STRUCTURE-FUNCTION STUDIES OF THE MAMMALIAN 2-OXOGLUTARATE DEHYDROGENASE COMPLEX

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by

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ABBREVIATIONS

In addition to the accepted abbreviations (Instructions to authors, Biochem.J.(1985) **225**, 1-26), the following have been adopted:

BCOADC	branched chain 2-oxoacid dehydrogenase complex
DAHP	3-deoxy-D-arabino-heptulosonate 7-phosphate
DTNB	[5,5'-dithiobis(2-nitrobenzoic acid)]
DTT	dithiothreitol
EDTA	ethylenediamine tetra acetic acid
FPLC	fast protein liquid chromatography
GdnHCl	guanidinium hydrochloride
Iodogen	1,3,4,6-tetrachloro-3α,6α-diphenyl glycouril
Leupeptin	acetyl-L-leucyl-L-leucyl-L-arginal
M_r	molecular mass (relative)
MOPS	(3-[N-morpholino]propane-sulphonic acid)
NEM	N-ethylmaleimide
OGDC	2-oxoglutarate dehydrogenase complex
PDC	pyruvate dehydrogenase complex
PEG	polyethylene glycol
PMSF	phenylmethylsulphonylfluoride
PVDF	polyvinylidene difluoride
RBS	rat blood serum
SDS	sodium dodecylsulphate
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylethylenediamine
TPP	thiamine pyrophosphate
Tween 20	polyoxyethenesorbitan monolaureate
TX-100	Triton X-100

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SUMMARY

It has been shown that N-terminal sequence homology exists between protein X from *S. cerevisiae* and the bovine heart OGDC E1 enzyme. The N-terminus of OGDC E1 exhibited lipoyl-like domain characteristics; however, it was found to be non-functional as judged by its inability to be reductively acetylated in the presence of radiolabelled substrate. By degrading the bovine heart OGDC E1 enzyme specifically using trypsin, a stable lower M_r species, termed E1^l was formed rapidly as a result of N -terminal cleavage of E1, generating a highly-immunogenic peptide with an approx. M_r value of 8,000. Tryptic degradation of OGDC also resulted in the removal of the lipoyl domain from E2 at longer time intervals, the E3 enzyme remaining intact under these conditions. On gel permeation chromatography of trypsin-treated complex where E1^l had been formed, the E3 enzyme was found to exhibit a reduced affinity for the complex; in addition E1^l also showed a marked tendency to dissociate from the E2 core assembly. Similarly, protease arg C treatment of OGDC causing extensive but specific cleavage of E1 only, also resulted in release of E3 from the core.

On treatment with trypsin, the overall activity of OGDC was found to decrease. This decline in activity was not caused by inactivation of E1 as the modified E1 enzyme retained approx. 100% activity. Loss of OGDC function is instead attributed to the reduced affinity of the E3 (and E1) enzymes for the core structure. There are indications that E1^l/E1 heterodimers of E1 may be able to sustain overall complex activity.

From the above experiments coupled to sequence alignment analysis of protein X from *S. cerevisiae*, it is proposed that a truncated, non-functional 'lipoyl-like' domain is situated at the N-terminus of the E1 enzyme of OGDC. Evidence is presented that this region is followed closely by an E3 binding domain, cleavage of which, results in E3 and E1 dissociation from the complex.

The exact mode of association which exists between E1 and E3 within OGDC has not, as yet, been clearly defined.

Dissociation of intact OGDC in conditions of high ionic strength has no significant effect on complex activity; however, after tryptic degradation of OGDC to varying extents to form differing amounts of the $E1^{l}$ species, the residual complex activity exhibits an increased salt sensitivity profile. It was also shown that treatment of the complex with 0.25M $MgCl_2$ representing high ionic strength, was sufficient to render the E3 enzyme susceptible to inhibition by N-ethylmaleimide but only in an NADH dependent manner. Similar partial protection of the redox-active disulphide cysteine pair on E3 in the intact complex was observed in the presence of the specific active-site inhibitor p-aminophenyldichloroarsine. These findings are consistent with the view that the active-site of E3 is shielded from the external environment in the intact complex.

In bovine heart PDC, it has been proposed that protein X performs a role in protecting the active site of E3. Since it is postulated that protein X in this complex and the N-terminal region of bovine heart OGDC E1 are both involved in binding E3, the possible role of the N-terminal region of OGDC E1 in the active site protection of E3 was investigated. The E3 enzyme of OGDC was found to be susceptible to inhibition by N-ethylmaleimide in an NADH dependent manner when the complex was treated with high salt concentrations; in contrast, protease treatment of the complex with trypsin had little effect on E3 activity in the presence of NADH and N-ethylmaleimide. Protease arg C treatment, however, allowed N-ethylmaleimide in the presence of NADH to effect an overall reduction in complex activity of approx. 50%. These results indicate that the large $E1^{l}$ species may still protect released E3 from modification by N-ethylmaleimide. However, no evidence was obtained for the presence of a stable $E1^{l}$/ E3 subcomplex.

The importance of the N-terminal region of E1 of OGDC in mediating the re-folding of the complex after denaturation of the enzyme with GdnHCl or in regulating its

sensitivity to Ca^{2+} was investigated. It was established that this region was not critical to the stability of the enzyme or its capacity to renature. The Ca^{2+} dependence of its activity also appeared to be unaltered. Such data are consistent with the view that the primary role of this region of the E1 enzyme is a structural one in promoting E3 and E1 interactions with the core of the complex.

CHAPTER ONE

INTRODUCTION

1.1 MULTIENZYME COMPLEXES

Compartmentation is one of the fundamental aspects in the organisation of living matter, as illustrated in for example, the existence of differentiated cells within a multicellular organism. Within eukaryotic cells, the intracellular space is further divided by the presence of organelles each possessing their own unique microenvironment. It is clear that the cell cannot be visualised as a simple 'bag' filled with enzymes and metabolites which are freely dissolved in solution. It has been calculated that the protein concentration in the matrix of the actively metabolising mitochondrion is approximately 560 mg/ml (Hackenbrock, 1968) which is thought to be similar to the protein concentration in enzyme crystals (Srere, 1982). In view of this, it is very unlikely that both enzymes and metabolites are able to move around freely in such a viscous environment.

Two levels of organisation are thought to exist for enzymes in the cell: the first is purely functional where the enzyme has evolved to have catalytic and kinetic properties which are compatible with other enzymes both in the same and other pathways; the second is structural-functional organisation involving the macromolecular association of enzymes in cases where precise topographical positioning of enzymes is critical, as in the membrane integrated mitochondrial respiratory chain complexes (De Pierre and Ernster, 1977). Evidence now suggests that the structural organisation of enzymes within cells is now more common than envisaged previously.

Many pathways or consecutive enzyme sequences in the cell may be present in multienzyme clusters comprising two groups, complexes and conjugates. Within multienzyme complexes each enzyme is represented by non-covalently associated polypeptide chains; this is in contrast to multienzyme conjugates where the different enzymic activities reside on a single polypeptide chain. Multienzyme complexes can be represented by tryptophan synthase (Miles, 1991) and the 2-oxoacid dehydrogenase complexes (Reed, 1974; Perham, 1975). Examples of conjugates are

the *arom* multienzyme conjugate from *Neurospora crassa* (Welch and Gaertner, 1980) and fatty acid synthase from eukaryotic sources (Lynen, 1972).

The *arom* multienzyme conjugate is common to micro-organisms but is absent in animals. It represents the primary route for the biosynthesis of polyaromatic compounds, including the aromatic amino acids, from non-aromatic sources. In *N. crassa* all five enzyme activities are present on a single polypeptide chain which is coded for by a single gene. *In vitro*, the *arom* multienzyme conjugate has been shown to effect a reduction in the transient time (Welch and Gaertner, 1975) which is defined as the speed at which a new steady state is attained in a particular sequence of reactions. It has been shown that transient times can be reduced if enzymes are in close proximity to each other (Easterby, 1989). In addition, co-ordinate effects involving all five of the component enzymatic activities were observed such that, on incubation of the conjugate with its first substrate, DAHP, the enzyme cluster exhibited a co-ordinated activation of enzyme activity (Welch and Gaertner, 1976).

In a stable multienzyme complex in which the components are permanently associated, the appropriate spatial arrangements of active sites relative to each other, ensures that the intermediates will bind with a high probability to the next enzyme instead of leaving the complex. This is most clearly seen with the tryptophan synthase multienzyme complex which catalyses the final two reactions in the biosynthesis of L-tryptophan. In *E. coli* the tryptophan synthase complex is composed of 2 non-identical dissociable subunits; the $\alpha_2\beta_2$ tetramer formed from α monomers and β_2 dimers, has a higher affinity for substrate as a result of mutual activation of the individual subunits. Indole produced in the active site of the α subunit becomes a substrate for the β subunit where it is converted to L-trytophan. In *Salmonella typhimurium* it has been shown that the active centres of the neighbouring α and β subunits are 25Å apart and are connected by a tunnel with a diameter equal to, or greater than the dimensions of indole (Hyde *et al.*, 1988). The tunnel probably provides a pathway for the internal diffusion of indole between the two active sites and prevents the escape of indole to the solvent. This ensures that indole does not reach a

diffusion-controlled equilibrium in the bulk phase of the cell. This can result in protection of metabolites and/or the cell from either degradative enzymes or toxic substances such as aldehydes which are known to be reactive towards protein side chains.

Substrate channelling can also decrease the transit time or the time it takes for a metabolite to diffuse towards the next enzyme in the pathway. Owing to the small distances within the cell, encounter times between metabolites and enzymes would be expected to be faster than the enzymic catalytic rate; however, it appears that the calculated difference between diffusion times and enzyme turnover rates is not as large as originally thought (Welch, 1977). In the absence of substrate channelling within the cell, a situation could arise where the volume occupied by total cellular metabolites would be so large that the solvent capacity of the cell would be rate limiting, thereby affecting transit times and cellular flux rates (Atkinson, 1969). Multienzyme complexes can also act to create a special environment around a pathway, the so called 'hot potato hypothesis' such that the cell acquires the means of maintaining a high flux of substrates with a moderate number of intermediates. It does so by protecting intermediates which may be insufficiently stable to survive the normal diffusion process in an aqueousenvironment(Perham, 1991).

It is now thought that higher levels of enzyme organisation may exist in the cell; for example, some evidence has been presented which indicates that glycolytic enzymes may form functional particles known as glycosomes (Gorringe and Moses, 1978). The term 'quinary structure' is often used when discussing interactions between different protein species for example, the enzymes involved in the citric acid cycle which are present in the mitochondrial matrix, are thought to exist as a putative complex or quinary metabolon associated with the inner surface of the inner mitochondrial membrane (Srere *et al.*, 1987). It is possible to prepare slightly damaged mitochondria with exposed citric acid cycle enzymes which remain stably bound to the membrane even at high ionic strength (Robinson and Srere, 1985). When mitochondria are fully disrupted using sonication, all of the matrix proteins with

Table 1.1 Specific reactions catalysed by the 2-oxoacid dehydrogenase complexes

Complex	$R-C(=O)COOH$	Substrate	Product
OGDC	$CO_2CH_2CH_2-R$	2-oxoglutarate	Succinyl CoA
PDC	CH_3-R	Pyruvate	Acetyl CoA
BCOADC	$(CH_3)_2CHCH_2-R$	2-oxoisocaproate	Isovaleryl CoA
	$CH_3CH_2CH_3CH-R$	2-oxo-3-methyl-valerate	2-methyl-butyryl CoA
	$(CH_3)_2CH-R$	2-oxo-iso-valerate	Isobutyryl CoA
		2-oxobutyrate	Propionyl CoA
		4-methyl thio 2-oxobutyrate	4-methylthio-propionyl CoA

the exception of succinate dehydrogenase are released; it was subsequently shown that these solubilised enzymes could be cross-linked preferentially back onto the inner membrane of the mitochondrion adjacent to each other. Moreover, the solubilised citric acid cycle enzymes were capable of being cross-linked to each other demonstrating the specificity of such interactions (Srere *et al.*, 1987). Within the cell the enzymes would function more efficiently at low apparent concentrations of substrate, since locally high concentrations could be maintained in the active site region of the enzyme.

1.2 THE 2-OXOACID DEHYDROGENASE COMPLEXES

The 2-oxoacid dehydrogenase multienzyme complexes are high M_r assemblies containing multiple copies of three different enzymes which, acting in concert, are responsible for catalysing the oxidative decarboxylation of 2-oxoacids in the cell. The three members of this multienzyme family have been purified from many diverse sources (Section 1.2.1) and include the pyruvate dehydrogenase multienzyme complex (PDC) which is specific for pyruvate and 2-oxobutyrate, the 2-oxoglutarate dehydrogenase complex (OGDC) whose substrate is 2-oxoglutarate and the branched chain 2-oxoacid dehydrogenase complex (BCOADC) which shows specificity for the branched chain amino acids in the cell derived from valine, leucine and isoleucine (Table 1.1). In addition, BCOADC is also capable of metabolising 2-oxobutyrate, an intermediate formed during the breakdown of threonine and methionine (Jones and Yeaman, 1986). 2-oxobutyrate can either be formed directly by the transamination of threonine or indirectly via a trans-sulphuration pathway during methionine oxidation. Another pathway exists for methionine metabolism in the cell whereby its transamination results in the formation of the substrate 4-methylthio-2-oxobutyrate (Livesey, 1984).

2-oxoglutarate is a branch point metabolite in cellular metabolism generated by carbohydrate oxidation and by glutamate dehydrogenase during the deamination of

amino acids. It is also produced by the transamination of glutamate as part of the malate/aspartate shuttle which functions to transfer reducing equivalents from the cytoplasm into the mitochondrial matrix. Succinyl CoA, the product of 2-oxoglutarate oxidation, can be withdrawn for energy-requiring biosynthetic pathways such as those involved in the production of porphyrins, leucine and methionine. The citric acid cycle represents the major catabolic energy generating function in the cell and since OGDC is the major regulatory enzyme in the latter stages of the cycle, the flux of metabolites through OGDC is coupled to the energy state of the mitochondrion.

The oxidative decarboxylation of pyruvate to acetyl CoA, catalysed by PDC, represents the committed step in glucose homeostasis in mammals as it is the first physiologically irreversible reaction in the oxidative pathway of glucose metabolism. Acetyl CoA formed by PDC can undergo one of two possible fates: (a) it can be completely oxidised to form CO_2 via the citric acid cycle or (b) it can perform a biosynthetic role in eukaryotic lipogenic tissues whereby, acetyl CoA entering the citric acid cycle via PDC can be utilised for fatty acid synthesis by condensing with oxaloacetate to form citrate, an intermediate in lipid synthesis.

BCOADC catalyses the committed step in the degradation of the branched chain amino acids in the cell. Inherited defects affecting the enzyme subunits of BCOADC may give rise to the symptoms of Maple Syrup Urine Disease which can be distinguished by amino- and ketoacidaemia (Herring *et al.*, 1991).

1.2.1 PURIFICATION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES FROM DIVERSE SOURCES

In order to investigate the properties and structure of these enzymes in greater detail, the 2-oxoacid dehydrogenase complexes have been isolated and purified to homogeneity from many sources, both prokaryotic and eukaryotic. For example, OGDC has been isolated from the following prokaryotic sources; *Acetobacter xylinum* (Kornfeld *et al.*, 1977), *Acinetobacter lwoffi* (Parker and Weitzman, 1973),

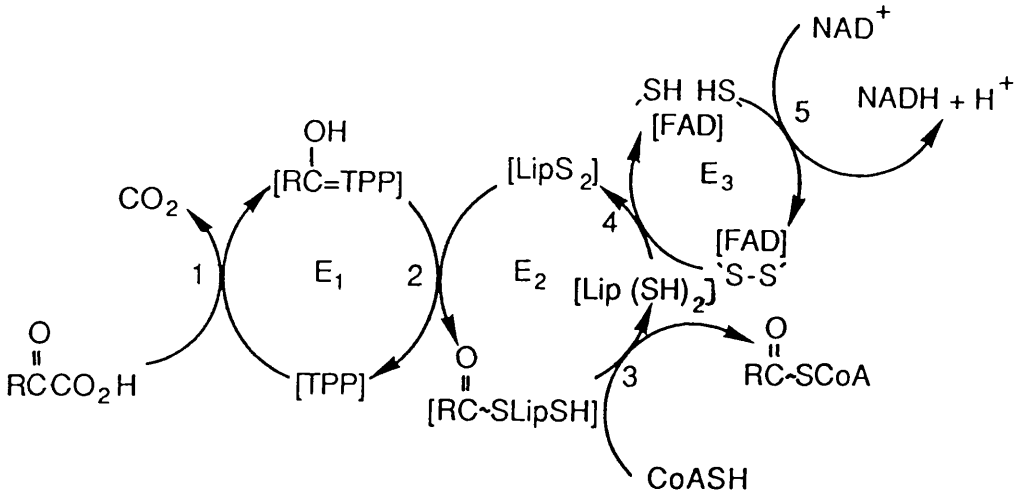
Dictyostelium discoideum (Heckert *et al.*, 1989), *Escherichia coli* (Reed and Mukherjee, 1969; Pettit *et al.*, 1973) and also from eukaryotic sources which include bovine kidney (Reed and Oliver, 1968), bovine heart (Stanley and Perham, 1980), pig heart (Massey, 1960; Hirashima *et al.*, 1967; Koike and Koike, 1976; McCormack and Denton, 1979), pigeon breast (Severin and Gomazkova, 1971) and cauliflower mitochondria (Poulsen and Wedding, 1970).

In a similar manner PDC has been purified from *Bacillus stearothermophilus* (Henderson and Perham, 1980), *Bacillus subtilis* (Hodgson *et al.*, 1983), *Escherichia coli* (Koike et al., 1960; Reed and Mukherjee, 1969; Danson et al., 1979), *Pseudomonas aeruginosa* (Jeyasselen *et al.*, 1980) and eukaryotic sources such as *Saccharomyces cerevisiae* (Kresze and Ronft, 1981), bovine heart (Stanley and Perham, 1980), bovine kidney (Linn *et al.*, 1972; Cate and Roche, 1979; Kresze and Steber, 1979) and pig liver (Roche and Cate, 1977).

Sources from which BCOADC has been purified include *Pseudomonas putida* (Sokatch *et al.*, 1981a), bovine kidney (Pettit *et al.*, 1978) and bovine liver (Danner *et al.*, 1979; Heffelfinger *et al.*, 1983), rabbit liver (Paxton and Harris, 1982) and rat kidney (Odessey, 1982).

1.2.2 REACTION CATALYSED BY THE 2-OXOACID DEHYDROGENASE COMPLEXES

Each of the multienzyme complexes is composed of multiple copies of three enzymes: a substrate specific 2-oxoacid dehydrogenase (E1) which requires TPP and Mg^{2+} for activity, a distinct dihydrolipoamide acyltransferase (E2) which contains a covalently attached lipoic acid group and dihydrolipoamide dehydrogenase (E3), a flavoprotein which is common to all of the complexes (Koike and Koike, 1976). In PDC and OGDC the E1 and E2 components are specific to their respective complexes whereas, E3 has been shown to be functionally interchangeable between both (Mukherjee *et al.*, 1965).



$$RCCO_2H + CoASH + NAD^+ \rightarrow RC\sim SCoA + CO_2 + NADH + H^+$$

Fig. 1.1 Reaction scheme for the oxidative decarboxylation of 2-oxoacids by the respective multienzyme complexes

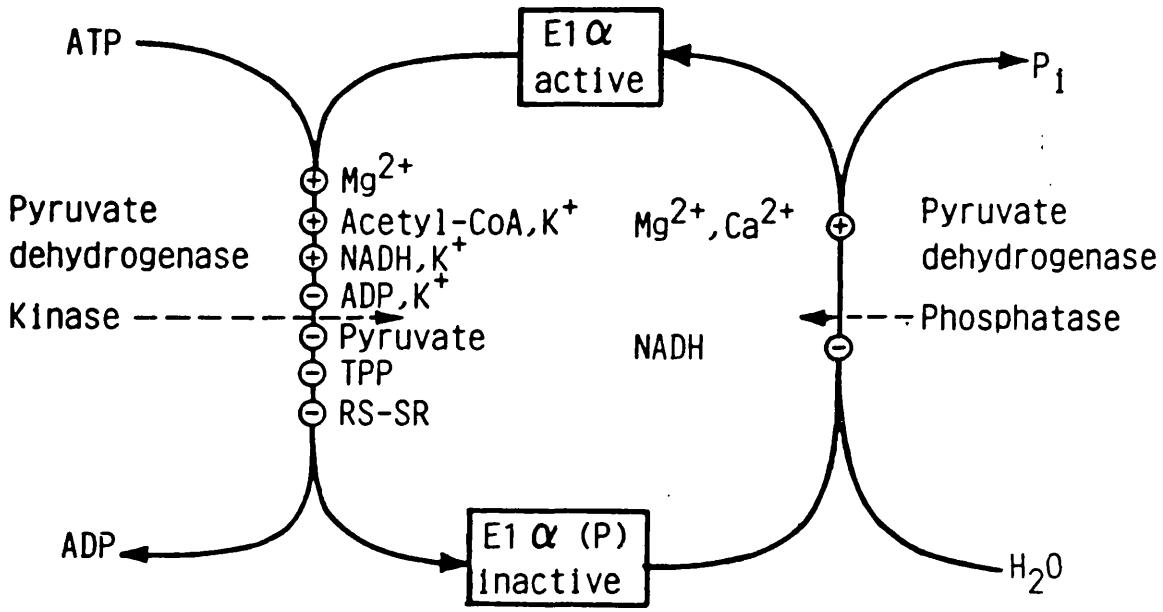
The three constituent enzymes of OGDC are 2-oxoglutarate dehydrogenase (E1, EC 1.2.4.2), dihydrolipoamide succinyltransferase (E2, EC 2.3.1.61) and dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4). For PDC the corresponding enzymes are pyruvate dehydrogenase (E1, EC 1.2.4.1), dihydrolipoamide acetyltransferase (E2, EC 2.3.1.12) and in common with OGDC, dihydrolipoamide dehydrogenase (E3, EC 1.8.1.4). The branched-chain 2-oxoacids in the cell are oxidatively decarboxylated by BCOADC which has comparable subunit structure to both PDC and OGDC (Randle *et al.*, 1987).

The overall reaction catalysed by the 2-oxoacid dehydrogenase complexes can be represented by:

$$RCO\text{-}COOH + CoASH + NAD^+ \rightarrow RCO\sim SCoA + CO_2 + NADH + H^+$$

The complexes catalyse the oxidative decarboxylation of the 2-oxoacids by a series of reactions which generate CO_2 and the corresponding acyl CoA as shown in Fig. 1.1. (Reed, 1974). The substrate-specific E1 enzyme catalyses the decarboxylation of the 2-oxoacid and is also responsible for the reductive acylation of the lipoic acid moiety which is covalently attached to E2 via a specific lysine residue (Bleile *et al.*, 1979). The lipoyl moieties present on the E2 enzyme comprise an interacting network that functions as an acyl group and electron pair relay system through thiol disulphide and acyl transfer reactions amongst all of the lipoyl groups. In PDC, inter- and intra-chain transfers of acetyl groups and electron pairs can occur between S-acetyldihydrolipoyl groups and oxidised lipoyl moieties. A similar situation exists for OGDC except that only interchain transfers can occur since the individual E2 subunits possesses only a single lipoyl group (Collins and Reed, 1977). E3, an FAD-linked enzyme, is responsible for the re-oxidation of the dihydrolipoyl moiety with NAD^+ as the ultimate electron acceptor.

Fig. 1.2 Schematic representation of the covalent modification of pyruvate dehydrogenase complex and its regulation by various metabolites (Reed *et al.*, 1980)



1.2.3 REGULATION OF 2-OXOACID DEHYDROGENASE COMPLEX ACTIVITY

It is important that the activity of the 2-oxoacid dehydrogenase complexes is closely regulated as they play both a central and critical role in cellular metabolism. Both OGDC and PDC are inhibited by an accumulation of product metabolites in the cell by a system of regulatory feedback inhibition, such that they are inhibited by succinyl CoA and acetyl CoA respectively (Smith *et al.*, 1974; Tsai *et al.*, 1973). The inhibitory effect that NADH has upon the activity of OGDC isolated from bovine kidney, can be reversed by either ADP or Ca^{2+} ions (Lawlis and Roche, 1981a). Adenine nucleotides such as ATP inhibit OGDC activity, with both ADP and inorganic phosphate capable of activating the complex by altering the affinity of the E1 enzyme for 2-oxoglutarate such that the K_m value is decreased (Lawlis and Roche, 1981b). Effects of the adenine nucleotides are also observed with OGDC purified from other sources such as bovine and porcine heart, suggesting that similar regulatory systems may be in operation in tissues throughout the body (McCormack and Denton, 1979).

The mammalian 2-oxoacid dehydrogenase complexes (excluding OGDC), are subject to covalent modification whereby phosphorylation of specific serine residues present on the E1α subunit of the enzyme complex causes inactivation of overall complex activity (Reed and Yeaman, 1987). In PDC isolated from bovine kidney and heart and porcine heart, phosphorylation has been shown to occur on three serine residues in the E1α subunit, the amino acid sequence surrounding the phosphorylation sites possessing a high degree of homology (Yeaman *et al.*, 1978; Sugden *et al.*, 1979). The kinase responsible is tightly bound to the E2 subunit in PDC and consists of an α subunit and a β subunit (Stepp et al., 1983). In contrast, the phosphatase responsible for de-phosphorylation and subsequent activation of the complex is much more loosely associated with the complex (Linn *et al.*, 1972). Fig. 1.2 shows the effectors responsible for the regulation of kinase and phosphatase activity in PDC whereacetyl CoA, NADH, ADP and pyruvate exert their effects directly upon the kinase. In PDC Ca^{2+} ions are required to bind the phosphatase, but not the kinase nor

the E1 enzyme to the core structure (Pettit *et al.*, 1972). This Ca^{2+} ion controlled association of the phosphatase with the E2 core provides a means for regulation of complex activity: Ca^{2+} ions act to stimulate PDC phosphatase by decreasing the K_m for the inactive phosphorylated PDC (Denton *et al.*, 1972); this is in contrast to the antagonistic effects exerted by Ca^{2+} ions on the kinase which is slightly inhibited at submicromolar concentrations of Ca^{2+} ions (Cooper *et al.*, 1974). In BCOADC, phosphorylation of the E1α subunit of the complex by the BCOADC kinase causes inactivation of the complex (Paxton and Harris, 1982), peptide mapping experiments indicating that threedistinct sites within the E1α subunit are phosphorylated (Cook *et al.*, 1983). The relative activity states of PDC and BCOADC are usually very different and independently regulated in any given tissue, owing to different factors regulating the respective kinases and phosphatases of these complexes (Harris *et al.*, 1986). The branched chain 2-oxoacids are known to inhibit the kinase (Jones and Yeaman, 1986); however, other substrates as well as the products of the reaction catalysed by the complex, do not seem to regulate the kinase in a manner similar to that observed with PDC (Paxton and Harris, 1984). These differences between the complexes probably reflect the different metabolic role that each complex performs in controlling their respective pathways.

Mitochondria from vertebrate sources possess pathways both for the uptake and efflux of Ca^{2+} ions; accumulation of Ca^{2+} ions in the matrix occurs via a uniport mechanism, the inner mitochondrial membrane possessing an independent efflux pathway which functions as a $Ca^{2+}/2Na^{+}$ antiporter in mitochondria from most tissues including heart, brain and brown adipose tissue. In liver, the same pathway functions as a $Ca^{2+}/2H^{+}$ antiporter (Nicholls and Ferguson, 1992). The Ca^{2+} ion concentration present in heart mitochondria is approx. two to three times that of the cytoplasmic concentration, and represents the concentration range in which OGDC and PDC activity are affected (Denton *et al.*, 1978). The main effect of Ca^{2+} ions upon OGDC activity is to decrease the K_m for 2-oxoglutarate (Lawlis and Roche, 1981a; McCormack and Denton, 1979), although micromolar concentrations of Ca^{2+} ions

may also reduce NADH inhibition by direct binding of these ions to the E1 subunit (Lawlis and Roche, 1981b). OGDC isolated from non-vertebrate sources does not exhibit sensitivity to calcium so, as a means of regulation, this could be considered to be an evolutionary development (McCormack and Denton, 1981). It was originally thought that OGDC from plant sources was not subject to regulation by calcium (Poulsen and Wedding, 1970; McCormack and Denton, 1979), although it has recently been demonstrated that OGDC isolated from cauliflower is capable of being activated in a calcium dependent manner raising doubts about earlier hypotheses (Karam and Bishop, 1989).

Alternative mechanisms exist for the regulation of OGDC in some bacterial species; OGDC production is repressed in anaerobically grown *E. coli* which form succinyl CoA from oxaloacetate rather than by oxidation of 2-oxoglutarate. OGDC production can be induced either by 2-oxoglutarate or glutamate but it is not formed detectably on either glucose or lactate until substantial metabolite accumulation has occurred (Amarasingham and Davies, 1965). In *B. subtilis* levels of OGDC production are also induced by either 2-oxoglutarate or glutamate at the end of exponential growth owing to the requirement for a functional citric acid cycle needed for sporulation of this species (Ohne, 1975).

1.3 MACROMOLECULAR ORGANISATION OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

The 2-oxoacid dehydrogenase complexes are large multimeric enzyme assemblies with M_r values ranging between 2.5-2.7 x 10^6 for OGDC and 7.0-9.0 x 10^6 for PDC (Linn *et al.*, 1972). The general architecture of the complex is that of a symmetrical core consisting of E2 polypeptides around which are arranged E1 and E3 subunits which bind non-covalently (Reed, 1974).

SDS/polyacrylamide gel analysis reveals that mammalian OGDC is composed of three individual polypeptide chains. The apparent M_r values of these chains in pig

heart are 113,000, 55,000 and 48,000 (Koike *et al.*, 1974) and in *E. coli* 94,000, 54,000 and 47,000 (Pettit *et al.*, 1973). Resolving the complexes into their constituent chains by subjecting them to gel filtration analysis under dissociating conditions allows activities to be assigned to the enzyme subunits (Tanaka *et al.*, 1972). This revealed that for pig heart OGDC, the chains of M_r 113,000, 55,000 and 48,000 corresponded to E1, E3 and E2 activities respectively (Koike *et al.*, 1974) and for *E. coli* OGDC the chains of 94,000, 54,000 and 47,000 represented the E1, E3 and E2 enzyme activities (Pettit *et al.*, 1973).

Similar treatments can be applied to PDC complexes such that *E. coli* PDC can be shown to have three polypeptide chains of M_r 100,000, 80,000 and 56,000 which correspond to the E1, E2 and E3 enzyme subunits (Perham and Thomas, 1971; Vogel and Henning, 1971). PDC from mammalian sources differs slightly in its structure as the E1 enzyme is composed of non-identical E1α and E1β subunits. Thus, PDC isolated from bovine heart is composed of five subunits with M_r 72,000, 55,000, 50,000, 42,000 and 37,000 which correspond to the E2, E3, protein X (Section 1.4), E1α and E1β polypeptide chains (Linn *et al.*, 1972).

Two distinct forms of the E2 core exist which are easily identifiable by electron microscopy - the cube and the pentagonal dodecahedron, where a morphological unit consisting of three E2 subunits appears to be important in the assembly of both types of polyhedral forms (Oliver and Reed, 1982). The cube form is associated with cores which consist of twenty four identical E2 subunits arranged with octahedral (432) symmetry. E2 cores with this symmetry can be found in PDC isolated from *E. coli* (Reed, 1974; Danson *et al.*, 1979), OGDC from *A. vinelandii* (Hanemaaijer *et al.*, 1989), *E. coli* (Danson et al., 1979) and mammalian sources (Reed, 1974). BCOADC from mammalian sources also exhibits octahedral symmetry (Griffin *et al.*, 1988). In contrast to the cube, the pentagonal dodecahedron is composed of sixty E2 subunits arranged with icosahedral (532) symmetry and E2 cores with this design are present in PDC from *B. subtilis* (Lowe *et al.*, 1983), *B. stearothermophilus* (Henderson and Perham, 1980), *S. cerevisiae* (Keha *et al.*, 1982) and mammalian

sources (Reed, 1974). The M_r values of the E2 polypeptides as calculated by SDS/polyacrylamide gel electrophoresis can be misleading as they may exhibit anomalous electrophoretic mobility due to the presence of elongated linker sequences which are responsible for the conformational flexibility of this polypeptide (Bleile *et al.*, 1979). Sedimentation equilibrium analysis in the presence of 6M guanidine hydrochloride gave an M_r value of 97,000 for E1 of pig heart OGDC as opposed to a value of 113,000 as determined by SDS/polyacrylamide gel electrophoresis (Tanaka *et al.*, 1972).

Multiple copies of the E1 and E3 enzymes bind tightly but non-covalently to the E2 core complex. Binding studies reveal that they do not interact with each other, but that each associates with E2 in a manner which does not prevent the E2 enzymes from associating with each other to form the large multimeric core of the complex (Tanaka *et al.*, 1972; Pettit *et al.*, 1973). *E. coli* OGDC is capable of binding six E1 dimers and eighteen E3 dimers although maximum activity was obtained when only six E3 dimers were bound to the E1/E2 subcomplex. This implies that binding of the E1 and E3 enzyme subunits occurs in an ordered manner to give a native subunit composition of 12 E1:24 E2:12 E3 (Pettit *et al.*, 1973). Titration with the transition state analogue inhibitor of E1, thiamine thiazolone pyrophosphate, confirmed the presence of twelve E1 active sites per complex (Angelides and Hammes, 1979). In a similar manner, OGDC from mammalian sources contains six E1 dimers and six E3 dimers associated with the 24-meric E2 core (Koike and Koike, 1982).

There are two structural types of PDC; type 1 which is found associated with Gram-negative bacteria and type 2 which is present in both mammalian mitochondria and Gram-positive bacteria. Type 1 is represented by PDC isolated from *E. coli* which has twelve E1 dimers and six E3 dimers which position themselves around the icosahedral core, the E1 enzyme binding along the twelve edges and the E3 dimers along the six faces of the cube (Reed *et al.*, 1975). In contrast, type 2 PDC isolated from bovine heart can associate with 20-30 E1 tetramers ($\alpha_2\beta_2$) and the six E3 dimers which position themselves along the thirty edges and the twelve faces of the

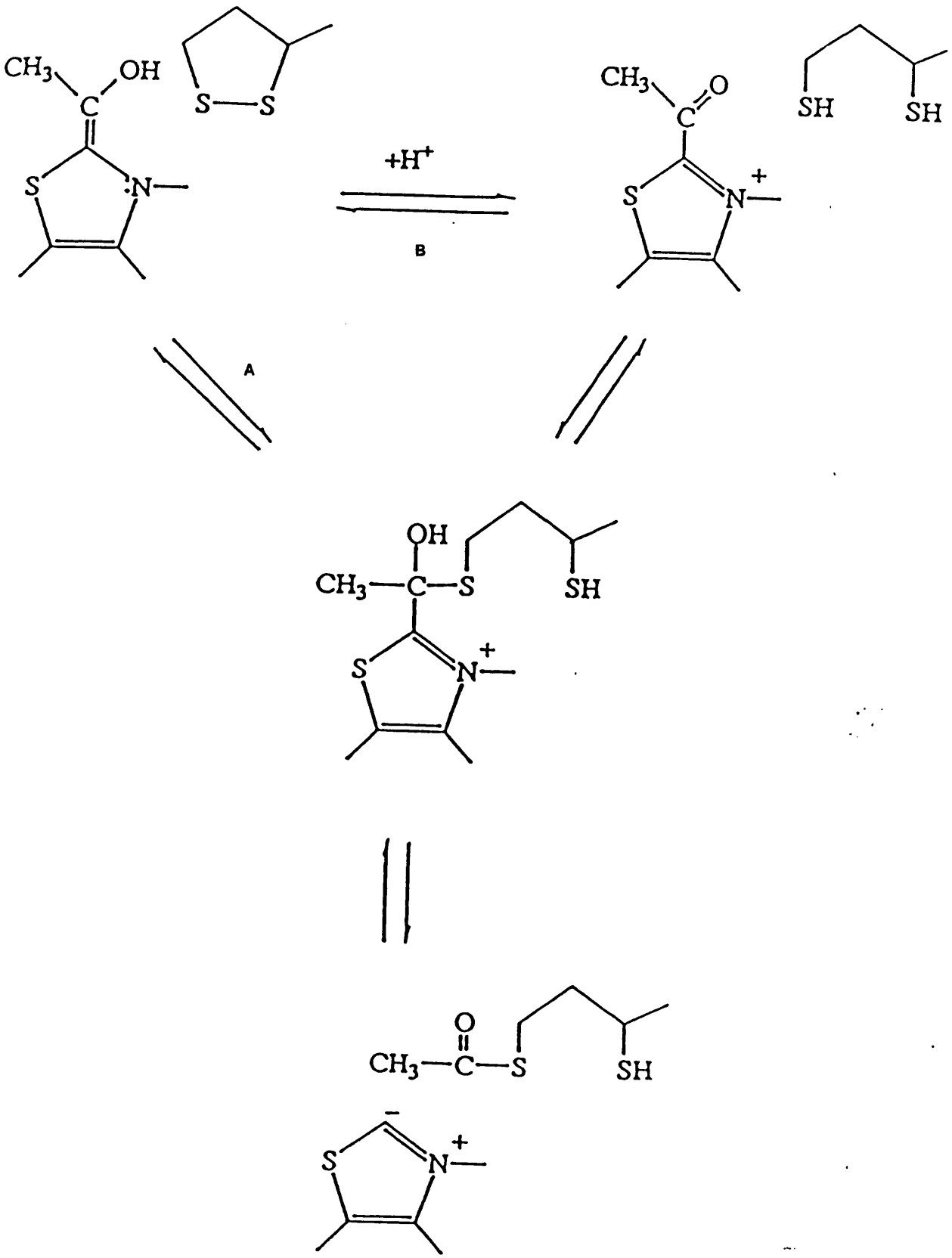
pentagonal dodecahedron E2 core (Barrera *et al.*, 1972). These structural features may be of evolutionary interest as *B. stearothermophilus* and *B. subtilis*, both Gram-positive bacteria, possess PDC with type 2 structures. This raises the possibility that the forerunner of mitochondria may have exhibited Gram-positive rather than Gram-negative characteristics (Keha *et al.*, 1982).

BCOADC is similar to PDC in that the E1 enzyme forms an $\alpha_2\beta_2$ heterodimer (Reed, 1974). Although it is generally assumed that this complex has six E3 dimers associated with the core, it is difficult to be precise about this since the E3 enzyme dissociates readily from the complex during its purification (Pettit *et al.*, 1978).

1.3.1 SUBSTRATE SPECIFICITY OF THE 2-OXOACID DEHYDROGENASE (E1) ENZYME

The 2-oxoacid dehydrogenase complexes all contain a substrate-specific dehydrogenase, designated E1, which catalyses the TPP-dependent decarboxylation of substrate in the rate-limiting step of the overall catalytic mechanism (Walsh, 1976; Bates *et al.*, 1977; Danson *et al.*, 1978; Cate *et al.*, 1980). In studies performed on pig heart OGDC, all of the protein bound TPP was found to be associated with the E1 component (Koike *et al.*, 1974). The TPP content of the enzyme was estimated at 1 mole per E1 dimer (M_r 216,000) as determined by enzymatic assay of TPP and chemical analysis of total thiamine present. Furthermore, E1 activity was found not to be stimulated by the addition of exogenous TPP suggesting that it remains associated with the enzyme during its isolation (Tanaka *et al.*, 1972). Comparison of the primary amino acid sequences in all TPP-utilising enzymes suggests the presence of a common structural motif which may represent a specific binding site for TPP on the E1 enzyme (Hawkins *et al.*, 1989). This area of homology begins with the highly conserved sequence -GDG- concluding with the highly conserved sequence -NN- . The region between these two sequences comprises approx. 30 residues which although less well conserved, exhibits several common features: the tenth residue is normally either a glutamic acid or aspartic acid residue which is followed later by a conserved alanine

Fig. 1.3 Mechanistic pathways for the reductive acetylation of lipoyl cofactors by 2-(1-hydroxyethylidene)-TPP at the active sites of pyruvate dehydrogenase



(Adapted From Frey *et al.*, 1989)

and proline residue respectively. The region immediately preceding the -NN- sequence also contains a cluster of 6-7 amino acids all of which contain hydrophobic side chains. The predicted secondary structure of this region shows similarities to a region in the active sites of a large number of dinucleotide-binding enzymes. In these enzymes the binding of the dinucleotide occurs in a typical β-turn-α–turn-β secondary structure, in which the conserved sequence pattern -G-X-G-X-X-G- forms a tight turn allowing a favourable interaction between the N-terminus of the α-helix and the pyrophosphate group of the dinucleotide. The -GDG- sequence present on the E1 enzyme may be involved with allowing a similar β turn-αβ structural motif so promoting TPP binding.

In addition to catalysing decarboxylation of the substrate, E1 is also responsible for the reductive acylation of the lipoyl groups present on E2 which results in the concomitant opening of the dithiolane ring generating a free thiol. Acylation occurs initially at the S^8-position of lipoic acid and not at the S^6-position although isomerisation can occur in aqueous solution (Yang and Frey, 1986). Complete acylation of the complexes is possible with a limited number of active E1 polypeptide chains indicating that the lipoyl domains in addition to interacting with each other, must also be able to interact with any given E1 active site (Perham *et al.*, 1981; Stepp *et al.*, 1981).

Early studies of the 2-oxoacid dehydrogenase complexes gave results that could be interpreted in terms of the involvement of acyl intermediates such as acetyl-TPP (Das *et al.*, 1961). Acetyl-TPP and succinyl-TPP were indirectly implicated as intermediates in reactions catalysed by these complexes (Steginsky and Frey, 1984). The product of the E1 reaction sequence for PDC, 2-(1-hydroxyethylidene)-TPP, remains tightly bound to E1 with the least well understood step of the reaction mechanism being the interaction of this intermediate and the lipoyl group on E2 to form TPP and S-acetyldihydrolipoamide (Frey *et al.*, 1989). Two mechanisms have been proposed for this reaction as shown in Fig. 1.3; the first, depicted as pathway A in the figure, involves the coupling of the electron and group transfer reactions such

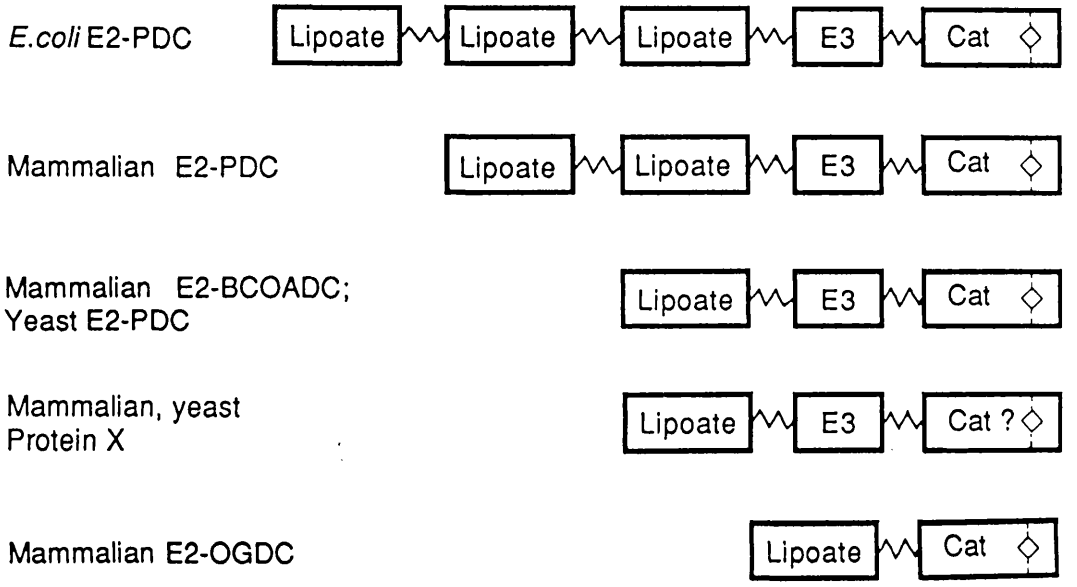

E.coli E2-PDC
Lipoate
Lipoate
Lipoate
E3
Cat
Mammalian E2-PDC
Lipoate
Lipoate
E3
Cat
Mammalian E2-BCOADC;
Yeast E2-PDC
Lipoate
E3
Cat
Mammalian, yeast
Protein X
Lipoate
E3
Cat ?
Mammalian E2-OGDC
Lipoate
Cat

Fig. 1.4 Schematic representation of the domain structure of the E2 acyltransferases and protein X component

~~ - extended flexible linker region

LIPOATE- lipoic acid containing domain, where all OGDC and BCOADC have been found to contain one lipoyl domain only, regardless of the source from which they are isolated.

E3- E3 and/or E1 binding domain

Cat- catalytic domain containing acyltransferase active site

that 2-(1-hydroxyethylidene)-TPP reacts with lipoamide to form the tetrahedral transition adduct in a single step (Breslow and McNelis, 1962; White and Ingraham, 1962). The second mechanism, shown as pathway B in Fig. 1.3, is thought to occur when the electron transfer and group transfer steps are distinct such that initiation of the reaction occurs by oxidation of 2-(1-hydroxyethylidene)-TPP to form acetyl-TPP and the reduction of lipoamide to produce dihydrolipoamide. This step is then followed by an acetyl group transfer to form TPP and S^8-acetyldihydrolipoamide (Daigo and Reed, 1962).

The main distinguishing feature between the two mechanisms is the compulsory involvment of acetyl-TPP in the second pathway where electron transfer and group transfer are distinct. It has recently been shown that acetyl-TPP is transiently formed in the overall reaction catalysed by PDC, although it cannot be determined whether this is a compulsory intermediate on the available evidence (Gruys et al., 1989). Thus at present, both pathways must be considered as being viable.

1.3.2 THE DIHYDROLIPOAMIDE ACYLTRANSFERASE (E2) ENZYMES

All dihydrolipoamide acyltransferase (E2) enzymes possess a unique multidomain structure, comprising an extended flexible outer domain in conjunction with a compact inner domain (Guest *et al.*, 1989; Perham and Packman, 1989; Reed and Hackert, 1990). A schematic representation of the domain structure of the E2 polypeptide chain is depicted in Fig. 1.4. The N-terminal region of the E2 polypeptide contains the extended, flexible lipoyl domains where the lipoic acid residues capable of being acylated are located. Each domain is approx. 80 amino acids in length (Dardel *et al.*, 1991), with the number of lipoyl domains present on a particular E2 polypeptide dependent upon the source material; for example, *E. coli* PDC possesses three homologous tandemly arrayed lipoyl domains in contrast to OGDC which only has one lipoyl domain, regardless of the source from which it is isolated. All of the domains present on the E2 polypeptide are linked to each other by conformationally

flexible linker domains, represented by the zig-zag lines in Fig. 1.4, which are an important feature with respect to active site coupling within the complex. Downstream of the lipoyl domain a small structurally distinct domain responsible for binding the E3 and/or the E1 enzymes to the core is present. Located at the C-terminus of the E2 polypeptide is the compact inner domain which, in addition to possessing the catalytic site of acyltransferase activity also contains the E2 binding sites, so allowing the E2 polypetides to bind to each other forming the large multimeric core of the complex. Each of the above features will be dealt with in turn in the remainder of this particular section.

1.3.2.1 LIPOYL DOMAINS

The lipoyl domains present at the N-terminus of the E2 polypeptide represent the site of acylation within the complex. As was shown in Fig. 1.4, the number of lipoyl domains present on a particular E2 polypeptide can vary between the different 2-oxoacid dehydrogenase multienzyme complexes. There appears to be no correlation between the number of lipoyl domains per E2 chain, the symmetry of the E2 core or the source of the E2 core (Allen and Perham, 1991). Estimation of the number of lipoic acid containing regions within the E2 polypeptide can be achieved in several ways however, these methods can give rise to erroneous results. For example, the number of lipoyl domains present on E2 of *E. coli* PDC was initially determined by measuring the content of radioactive lipoic acid in PDC isolated from *E. coli* cells that had been grown in the presence of DL-[^{35}S] lipoic acid (Eley *et al.*, 1972). Differing results were often obtained when using this method, as biosynthesis and incorporation of endogenous lipoic acid was still occurring at an unknown rate within the cell. If PDC is incubated with [2-^{14}C] pyruvate in the absence of CoA, radioactive acetyl groups subsequently become incorporated into the lipoyl domains of E2. Employing this method, it was initially estimated that *E. coli* PDC contained two lipoic acid residues per E2 chain (Danson *et al.*, 1981). Cloning of the E2 enzymes of the complexes from various sources has eliminated many of the ambiguities present in the

earlier type of experiments. Direct sequencing of the cloned enzyme allows for easy identification of the sequences associated with the presence of lipoyl domains. For example, it has since been shown that there are in fact three lipoyl domains present on the E2 enzyme of *E. coli* PDC, as it appears that earlier estimations suffered from systematic errors in estimating the extent of reductive acylation (Spencer and Guest, 1985). Crosslinking studies were performed with bovine heart PDC and OGDC treated with phenylene-o-bismaleimide in the presence of substrate; in the case of OGDC, a single cross-linked dimer was produced indicating the presence of only one lipoyl group per E2 subunit of OGDC. In contrast, a more complicated variety of cross-linked products indicating trimers with higher M_r aggregates were produced for PDC. This result was consistent with the acetylated lipoyl groups of different E2 and protein X subunits in PDC being able to interact in all possible combinations (Hodgson *et al.*, 1988).

The observed differences in the number of lipoic acid residues per E2 chain, raises the question of whether individual lipoyl domains perform different roles in the enzyme mechanism. Protein engineering experiments involving the deletion of either one or two of the homologous lipoyl domains from the *aceF* gene of *E. coli*, resulted in the truncated E2 chains being unimpaired in their catalytic activity proving that lipoyl domains are able to fold and function independently of each other (Guest *et al.*, 1985; Graham *et al.*, 1986). *E. coli* mutants were also constructed which consisted of different combinations of both functional and non-functional lipoyl domains; in each case where the mutant possessed at least one functional domain the enzyme activity was comparable to that of the wild type (Allen *et al.*, 1989). It is not clear why the E2 polypeptide subunit of *E. coli* PDC has three lipoyl domains, an apparent surplus of lipoyl groups, compared with two for the mammalian complex and one lipoyl domain for both mammalian OGDC and yeast PDC (Fig. 1.4). It is unlikely that all of the lipoyl domains present within these complexes would be fully acetylated at any time. A possible explanation for this apparent surplus of lipoyl groups could be that they are

Table 1.2 Primary amino acid sequence surrounding the lipoylated lysine residue on the E2 enzyme of the 2-oxoacid dehydrogenase complexes.

COMPLEX	SOURCE	PRIMARY AMINO ACID SEQUENCE
OGDC	Bovine	I E T D K* T S V Q V P S P A N G
	E.coli	I E T D K* V V L E V P A S A D G
PDC	Bovine,Rat	V/I E T D K* A T V/I G F E V Q E E G
BCOADC	E.coli	V E G D K* A S M E V P A/S P F/Q A G
	Bovine,Human	V Q S D K* A S V T I T S R Y D G

* Signifies the lysine residue to which the lipoic acid moiety is attached

(Adapted from Yeaman, 1989)

acting as reservoirs, storing acetyl groups until such times as they are required by the cell.

Lipoic acid co-factors are attached in amide linkage to the N^6-amino groups of specific lysine residues. The sequence surrounding the lipoate attachment site has been determined for both bacterial and mammalian sources as shown in Table 1.2 and appears to be highly conserved (Stephens *et al.*, 1983; Spencer *et al.*, 1984; Bradford *et al.*, 1987(a, b); Hummel *et al.*, 1988). Experiments have been performed with a subgene encoding a hybrid lipoyl domain such that, residues 1-33 and residues 238-289 corresponding to the first and third lipoyl domains respectively of wild type *E. coli* PDC E2 enzyme, were taken to represent the first 85 residues of a single lipoyl domain. Overexpression of this subgene in a wild type *E. coli* host, resulted in the formation of both lipoylated and unlipoylated domain products. (Miles and Guest, 1987). This hybrid lipoyl domain when expressed in an *E. coli* lipoic acid biosynthetic mutant in the absence of lipoic acid, resulted in the production of unmodified lipoyl domains in conjunction with octanoylated domains (Ali *et al.*, 1990). Similarly, Dardel *et al.* (1990) expressed a subgene encoding a lipoyl domain from *B. stearothermophilus* in *E. coli*, resulting in the formation of unlipoylated, lipoylated and octanoylated domains. From their results the authors assumed that the octanoyl modification of these domains was a direct result of lipoyl-deficiency, unsure of whether this modification represented a true intermediate on the lipoyl biosynthetic pathway. The results of both groups differed from those of Packman *et al.* (1991) who achieved full lipoylation of native PDC *E. coli* lipoyl domains in *E. coli*, with no apparent mis-modification. In view of the results obtained by Packman and co-workers, it would appear that octanoyl substitution in the lipoyl domains is caused by an abberation in the post-translational modification of the recombinant protein, the *E. coli* lipoylation system being unable to cope with the excessive amount of protein generated by the expression vectors.

The lipoyl groups present on the E2 polypeptide of the 2-oxoacid dehydrogenase complexes are attached to small, folded protein domains. It has been shown that free

lipoic acid or lipoamide are capable of acting as substrates for both the E2 and E3 enzymes of the *E. coli* PDC complex, yet reductive acetylation of these compounds by E1 could not be detected to any significant extent (Reed *et al.*, 1958; Reed, 1966). Lipoamide is a very poor substrate for the E1 enzyme of *E. coli* PDC with K_m values in excess of 2mM (Graham *et al.*, 1989). Lipoylated decapeptides with amino acid sequences identical to that surrounding the critical lipoylated lysine residues in *E. coli* PDC, were also found to have similarly high K_m values. In contrast, lipoyl domains proteolytically excised from either bovine heart PDC (Bleile *et al.*, 1981) or *E. coli* PDC (Packman *et al.*, 1984), were reductively acetylated by E1 in the presence of substrate and the necessary cofactors with a K_m value of approx. 26μM (Graham *et al.*, 1989). These results indicate that lipoyl domains must be folded in the correct conformation in order to be reductively acetylated by E1.

1.3.2.2 LINKER DOMAINS

A distinctive property of the E2 domains of the 2-oxoacid dehydrogenase complexes is the large degree of conformational flexibility that they exhibit. This phenomenon is especially important for the mechanism of active site coupling. The flexibility of the E2 chain is promoted by linker regions which act to connect the lipoyl domains to each other and to the subunit-binding/catalytic domains. The linker regions show similarities to each other between different species i.e. they are characteristically rich in alanine and proline residues although the relative amounts of the amino acids present may differ. In several cases hydrophobic and charged amino acids have also been shown to be present within the linker region (Perham and Packman, 1989).

Evidence for the flexibility of these regions has come from several sources: ^{1}H-n.m.r. spectroscopy reveals the presence of sharp resonances which show that the linker regions have a high degree of flexibility with respect to the bulk of the structure. *In vitro* deletions of the *aceEFlpd* operon in *E. coli* PDC to delete two of the three

lipoyl domains resulted in the disappearance of these sharp resonance patterns (Radford *et al.*, 1987). In later experiments, nested deletions were made in the long alanine-proline rich linker sequence; this represents the region which separates the lipoyl domain from the E3 binding domain in the genetically engineered one lipoyl domain PDC complex. The full length linker region was 32 residues in length and deletions were performed which reduced this to 20, 13, 7 and 1 residue(s) respectively. In all cases, these deletions were accompanied by a corresponding fall in the ^{1}H-n.m.r. intensities (Miles *et al.*, 1988). Progressive deletions in the alanine-proline linker region resulted in impairing the overall complex reaction without affecting the individual enzyme activities. Shortening the interdomain sequence from 32-20 residues left the system of active site coupling unimpaired; however, reducing the length of this region to 13 residues or less resulted in substantial losses in active site coupling. These effects were accompanied by corresponding reductions in acetylation of the lipoyl domains. The existence of a multiple random coupling mechanism was proposed for these complexes, where one lipoyl group services each E1 subunit and an extensive lipoyl-lipoyl interaction network exists for the exchange of electrons and acyl groups (Hackert *et al.*, 1983). Further analysis with synthetic peptides of 32 residues in length which were identical to, or related to the long alanine-proline rich region, supported the view that the linkers were exposed to solvent and had a substantial conformational flexibility within the enzyme complex. Furthermore, circular dichroism experiments showed that although disordered in structure, the linker regions did not represent random coils but from their predicted conformation appeared to cause a slight stiffening of the peptide structure.

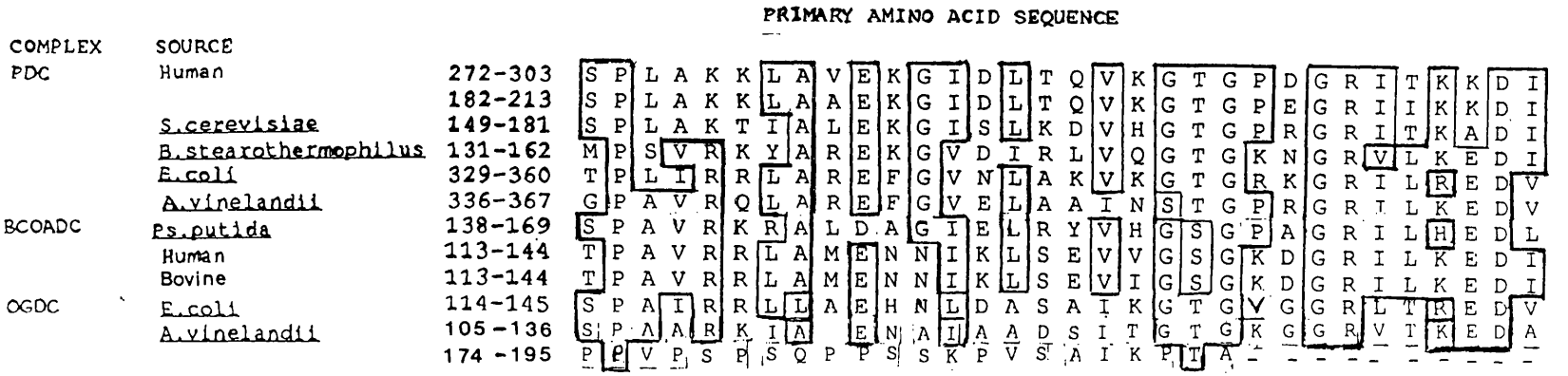
Limited proteolytic digestion studies have revealed the existence of the multi-domain structure of E2; it is possible to remove lipoyl domains preferentially from the core structure by cleaving within the proteolytically-sensitive linker domains. The E2 subunit of *E. coli* PDC (M_r 64,500) produces two fragments when digested with trypsin; one corresponding to the outer lipoyl bearing domain region (M_r 31,600), the other to the inner catalytic/subunit binding domain with (M_r 29,600) (Bleile *et al.*, 1979). In a

similar manner, *E. coli* OGDC digested with trypsin results in cleavage of the region between the lipoyl domain and the E3 binding site causing release of a single lipoyl domain (Packman and Perham, 1987). These cleaved lipoyl fragments, unlike the inner E2 core, proved difficult to visualise by electron microscopy owing to their physical flexibility (Bleile *et al.*, 1979).

The E2 polypeptides of the complexes provide a flexible lipoyl arm of approximately 1.4nm which allows the dithiolane ring to rotate and interact with both the E1 and E3 enzymes and other E2 subunits so promoting active site coupling (Koike *et al.*, 1963). The interaction between different lipoyl groups can be analysed using fluorescence spectra measurements where the formation of excimers arises due to the close proximity and interaction of neighbouring lipoic acids (Angelides and Hammes, 1979). When this method was applied to both complexes excimer formation was detected with PDC but not with OGDC, signifying that in PDC, inter- and intrachain transfer of acetyl groups and electron pairs can occur between s-acetyldihydrolipoamide groups and oxidised lipoamide groups. The situation for OGDC was different to that of PDCwhereonly interchain transfer could occur since OGDC possesses only a single lipoyl moiety per E2 chain (Collins and Reed, 1977).

Reporter groups such as maleimide groups carrying a nitroxide spin label, can be introduced into lipoic acid groups on the E2 polypeptides of these complexes and their movements detected using electron spin resonance (e.s.r.). Reporter groups were introduced into the E2 enzyme of *E. coli* PDC, the e.s.r. spectra showing that the nitroxide group had a high degree of mobility on the spin labelled enzyme. Calculation of rotational correlation values of the order 10^{-9} sec indicated that the reporter group seemed essentially free to rotate within the enzyme complex (Ambrose and Perham, 1976). Results of structural and kinetic studies carried out with *E. coli* PDC indicated that the distance between the active sites was 4.5nm with the span of the lipoyl-lysine arm being 3nm or less. This implied that the distances between the catalytic sites were too large to be linked by the rotation of a single lipoyl-lysine group during the catalytic cycle, and that two or more lipoic acid groups were used to

Table 1.3 Sequence motif of the putative E3 binding domain located on the acyltransferase enzymes of the 2-oxoacid dehydrogenase complexes from prokaryotic and eukaryotic sources



(Adapted from Nakano *et al.*, 1991)

transfer the intermediates between the catalytic sites of the complex (Shepherd and Hammes, 1977). This differs from *E. coli* OGDC where a normal catalytic cycle involves the rotation of a single lipoic acid between the catalytic sites although, both E1 and E3 can interact with two or more lipoic acid groups during catalysis (Angelides and Hammes, 1977). It is possible to remove the lipoyl domains from either OGDC or PDC with either trypsin or lipoamidase; trypsin selectively removes the lipoyl domains from E2 in contrast to lipoamidase which cleaves the lipoyl moiety itself from the domain structure (Stepp *et al.*, 1981). Release of lipoyl domains by trypsin and lipoyl moieties by lipoamidase proceeded at rates faster than the accompanying loss of overall activity. When *E. coli* PDC was treated with trypsin, approx. 50% of the total number of lipoyl domains were removed without any significant effect on overall activity, the catalytic cycle proceeding via the remaining lipoyl domains. Lipoamidase treatment of both *E. coli* OGDC and PDC, in which only the lipoyl moiety was removed from E2, resulted in a decrease of complex activity whereby the modified lipoyl domain was proposed to act as a dead-end inhibitor of either the E1, E2 or E3 active sites. This model may help to explain previous observations made with respect to active site coupling within the complexes.

1.3.2.3 THE E3 ENZYME BINDING DOMAIN

On the basis of sequence homology amongst enzymes of the 2-oxoacid dehydrogenase complexes, a stretch of 32 amino acids has been identified as representing an E3 binding site (Thekkumkara *et al.*, 1989). The sequence implicated as representing the E3 binding domain is shown in Table 1.3 with striking conservation of the amino acid sequence within this region being observed; this is not entirely unexpected since the E3 enzyme is a common component of all of these complexes (Hummel *et al.*, 1988; Lau *et al.*, 1988). Several amino acid residues within this region are strikingly conserved in all of the E2 sequences that were compared, with the exception of the OGDC E2 sequence from rat, and are shown

boxed in Table 1.3. Within the E3 binding domain, sequence homology as compared to the human PDC E2 sequence, is 91% for rat PDC E2 and 50% for *E. coli* PDC E2. Sequence homology for the E2 components of the OGDC and BCOADC compared with human PDC E2 is generally lower, in the region 40-50%. Recently, the amino acid sequence of the rat OGDC E2 subunit was deduced from the cDNA sequence of the cloned gene (Nakano *et al.*, 1991); close analysis of the entire gene sequence revealed the absence of a region representing the E3 binding domain. The same sequence also showed very little sequence homology to the majority of the E2 sequences shown in Table 1.3. The absence of an E3 binding domain for this enzyme suggests that an alternative site within the complex is involved in binding the E3 enzyme to the core structure. In view of the absence of an E3 binding domain within the E2 subunit, the possibility existes that it may be located at an alternative site in the complex perhaps on the E1 enzyme (see results).

Different complexes bind the E3 subunit in different ways. For example, electron microscopy techniques coupled with disassembly-reconstitution experiments performed with *E. coli* PDC, revealed that the E3 enzyme binds along the faces of the octahedral E2 core, whereas the E1 enzyme binds along the core edges (Reed *et al.*, 1975). In this type of complex the E3 enzyme binds to a separate site from E1. Therefore, on limited proteolysis, the E3 enzyme dissociates from the complex while the E1 enzyme remains associated. In *B. stearothermophilus,* the E3 binding domain is capable of also binding the E1 enzyme, such that limited proteolysis of the E2 enzyme causes both the E1 and E3 enzymes to dissociate from the complex leaving only a naked inner core (Packman *et al.*, 1988; Perham and Wilkie, 1980). The three dimensional structure of an E3 binding domain has been described recently by analysis of the solution structure of a synthetic peptide corresponding to the E3 binding domain of *E. coli* OGDC E2 (Robien *et al.*, 1992). When the sequences shown in Table 1.3, representing the E3 binding domains, were compared with data derived from the three-dimensional structure, several characteristic features emerged; it was shown that there are two helical regions which lie parallel to each other in conjunction with an irregular

and more disordered loop whose orientation relative to the main body of the domain cannot be determined. Proline, a structurally restrictive amino acid, which commonly occurs at the N-termini of helices, is absolutely conserved at a position near the start of the E3 binding domain, suggesting that this residue probably represents the N-terminal boundary of the domain.

1.3.2.4 ACTIVE SITE OF THE DIHYDROLIPOAMIDE ACYLTRANSFERASE ENZYME

Chloramphenicol acetyltransferase (CAT) (EC 2.3.1.38) catalyses the O-acetylation and subsequent inactivation of chloramphenicol in antibiotic resistant bacteria, it does so by using acetyl CoA as the acyl donor in the inactivation reaction. The amino acid residue histidine 195 in the sequence His-Xaa-Xaa-Xaa-Asp-Gly, located at the C-terminus of CAT, has been implicated in the catalytic mechanism (Kleanthous *et al.*, 1985). The C-terminal domain of the PDC E2 enzyme contains the lipoate acetyltransferase activity (Bleile *et al.*, 1979; Angier *et al.*, 1987); this region shows remote, but significant similarity with CAT, suggesting that the lipoate acyltransferases use a general base-catalysed mechanism analogous to that proposed for CAT (Guest, 1987). Alignment of the sequences of several lipoate acyltransferases revealed the existence of a highly conserved sequence motif Asp-His-Xaa-Xaa-Asp-Gly which closely resembled the CAT active site sequence (Russell and Guest, 1991).

That structural and functional relationships existed between the lipoate acyltransferases and CAT, was confirmed from the results of site-directed substitutions of the putative active site histidine and serine residues in both *E. coli* PDC E2 (Russell and Guest, 1990) and bovine BCOADC E2 (Griffin and Chuang, 1990); substitution of the putative active site histidine 602 for cysteine in *E. coli* PDC E2 and histidine 391 for asparagine in bovine BCOADC, resulted in losses of acetyltransferase activity in both cases. Further site-directed substitutions involving serine residues thought to be important in stabilising the transition-state of the enzyme,

also caused losses of acetyltransferase activity. These results differ from that seen with site-directed substitutions performed on the *S. cerevisiae* lipoate acetyltransferase; mutation of the putative active site histidine 427 to either alanine or asparagine, effected no loss of enzyme activity (Niu *et al.*, 1990). Further analysis of *E. coli* PDC which involved mutating the active site histidine to either cysteine, alanine or glycine, resulting in losses of acetyltransferase activity in all cases, confirmed the importance of this residue in the catalytic mechanism (Russell *et al.*, 1992). The result obtained for *S. cerevisiae* is puzzling since a high degree of sequence conservation exists between the E2 enzymes. It is unlikely that the role of histidine 427 in *S. cerevisiae* varies greatly from that of the active site histidine present in *E. coli*, unless some feature of the *S. cerevisiae* E2 enzyme allows for replacement of this residue without affecting the enzyme activity.

1.3.3 DIHYDROLIPOAMIDE DEHYDROGENASE (E3)

The dihydrolipoamide dehydrogenase enzyme (E3) is responsible for re-oxidising dihydrolipoamide groups which are bound to lysine residues present on the E2 enzymes of the 2-oxoacid dehydrogenase complexes. The E3 enzyme, present as a homodimer in the enzyme complex, catalyses the oxidation of lipoic acid groups in a NAD^+ dependent manner (Guest, 1978). Catalysis occurs via two half reactions involving the reduction of the oxidised enzyme followed by a re-oxidation step. The reaction sequence can be represented as follows:

$$Lip(SH)_2 + NAD^+ \longrightarrow LipS_2 + NADH + H^+$$

[where $Lip(SH)_2$ represents reduced lipoic acid and $LipS_2$ oxidised lipoic acid]
In mammalian complexes, E3 exhibits a low degree of immunogenicity, probably related to a high degree of primary sequence conservation which it has retained

throughout its evolution (De Marcucci *et al.*, 1985). This evolutionary stability of E3 may be explained in terms of it being a common component in all of the mammalian 2-oxoacid dehydrogenase complexes. Isolated E3 also has the property of being resistant to a wide range of harsh denaturing conditions including urea, high temperatures and various proteolytic enzymes (Williams, 1976).

In addition to its involvement with the 2-oxoacid dehydrogenase complexes, E3 is present as the L-protein of the glycine cleavage system (GCS), found in animal and plant mitochondria, which catalyses the reversible oxidation of glycine in the cell (Olson, 1989). E3 is also a member of the family of pyridine nucleotide-disulphide oxidoreductases which includes glutathione reductase, thioredoxin reductase and mercuric reductase (Williams, 1976; Curti *et al.*, 1991). Both structural and functional similarities exist amongst several enzymes in this family in particular, between dihydrolipoamide dehydrogenase and glutathione reductase, the latter responsible for catalysing the NADPH dependent reduction of glutathione disulphide in order to maintain a high ratio of reduced to oxidised glutathione in the cell. Although both these enzymes function physiologically in opposite directions by passing reducing equivalents to NAD^+ or from NADPH respectively, both contain a redox-active disulphide bridge which undergoes oxidation-reduction during catalysis causing them to cycle between oxidised and two electron reduced forms. Site directed substitution of the active site cysteines (Cys 44 and 49) for serine residues in the *lpd* gene of *E. coli*, led to a complete loss of E3 enzyme activity confirming the role of these cysteine residues in the catalytic mechanism (Williams *et al.*, 1989). It has also been shown that the thiol group nearer the N-terminus interacts with the dithiol substrate, in contrast to the C-terminal thiol which associates with the FAD cofactor (Williams *et al.*, 1982). In addition to the sequence conservation observed at the active site, considerable homology extends throughout the primary amino acid sequence of these molecules; for example, at the FAD and NAD(P) binding sites (Rice *et al.*, 1984). Reports of the three-dimensional crystal structures of the E3 enzyme from

Table 1.4 M_r values of dihydrolipoamide dehydrogenase enzyme isolated from eukaryotic and prokaryotic sources

Source	M_r (Da)	Complexes
Human liver	[a]50,216	OGDC; PDC; BCOADC; GCS?
	[a]49,702	
Bovine kidney	55,000	OGDC; PDC; BCOADC
Rat liver	56,000	
S. cerevisiae	56,000	OGDC; PDC
	[a]51,558	OGDC; PDC
E. coli	51,274	OGDC; PDC
Ps. putida	49,000	BCOADC
	56,000	OGDC; PDC; GCS
Ps. aeruginosa	50,000	BCOADC
	54,000	OGDC; PDC
A. vinelandii	56,000	PDC
B. subtilis	---	OGDC; PDC-BCOADC
B. stearothermophilus	54,000	PDC
H. halobium	[b]58,000	---

a-M_r value obtained from amino acid sequence deduced from the nucleotide sequence
b-lacks the 2-oxoacid dehydrogenase complexes

(Adapted from Carothers *et al.*, 1989).

S. cerevisiae (Takenaka *et al.*, 1988) and *A. vinelandii* (Schierbeek *et al.*, 1989), should allow for conserved sequence motifs to be matched to specific structural features of these enzymes.

1.3.3.1 SPECIES DIFFERENCES WITH RESPECT TO DIHYDROLIPOAMIDE DEHYDROGENASE

Although similar E3 enzymes exist for a wide range of both prokaryotic and eukaryotic species large variations are often observed in their M_r values (Table 1.4). The M_r of E3 from human sources is commonly determined from the amino acid sequences deduced from the nucleotide sequences of the cloned enzymes; using this approach a calculated M_r of approximately 50,000 is obtained as opposed to migration of these proteins as 55,000 species in SDS/polyacrylamide gel electrophoresis (Pons *et al.*, 1988). Full length cDNA clones obtained seem to indicate that E3, from human and *S. cerevisiae* sources, is encoded by a single nuclear gene and synthesised as a precursor with a cleavable leader sequence (Kim *et al.*, 1991; Browning *et al.*, 1988). In addition to the E3 enzyme, many mammalian enzymes including the E1 and E2 subunits from the 2-oxoacid dehydrogenase complexes, are synthesised as larger precursors (Hunter and Lindsay, 1986). These presequences, which are important for targetting enzyme subunits to their correct subcellular locations, vary in length from 15-70 amino acids and are located at the N-terminus. Although having no obvious sequence similarity with each other, they are usually rich in positively charged amino acids which form amphipathic α-helices on import of the protein through the plasma membrane (Glover and Lindsay, 1992).

E. coli contains the OGDC and PDC complexes but lacks BCOADC. The E1 and E2 enzymes of OGDC are encoded by the *sucAB* operon and the same enzymes in PDC are coded for by the *aceEF* operon. For both complexes, the E3 enzyme is produced by the *lpd* gene which is adjacent to the *aceEF* operon such that most of the E3 enzyme necessary for PDC activity is produced as a full length transcript from the

aceEFlpd genes, with an independently transcribed *lpd* transcript providing much of the E3 enzyme required for OGDC activity (Guest, 1978). The *Pseudomonas* species is unique in that it has been proven to have two genetically distinct E3 proteins (Sokatch *et al.*, 1981b; McCully *et al.*, 1986); *Ps. aeruginosa* produces two proteins of M_r 50,000 and 54,000, the former species associating specifically with BCOADC with the high M_r enzyme interacting with both the OGDC and PDC complexes. The proteins produced in *Ps. aeruginosa* are analogous to those produced in *Ps. putida.*

In several cases dihydrolipoamide dehydrogenase activity has been detected in species which are known to lack functional 2-oxoacid dehydrogenase complexes. For example, the halophilic archaebacteria which catalyse the oxidative decarboxylation of both 2-oxoglutarate and pyruvate using ferredoxin oxidoreductases (Kerscher and Oesterhelt, 1982; Kerscher *et al.*, 1982) have been shown to also contain dihydrolipoamide dehydrogenase activity (Danson *et al.*, 1984). *Halobacterium halobium* contains an enzyme which can be chemically modified and inactivated with the trivalent arsenical compound p-Aminophenyldichloroarsine, which is diagnostic for the presence of a catalytically essential disulphide bond as is found in the dihydrolipoamide dehydrogenases (Danson *et al.*, 1986). Despite the existence of similar active site residues in E3 isolated from *H. halobium* , *E. coli* and pig heart, amino acid sequence comparisons show that a very low level of homology exists between the mammalian and *H. halobium* E3 proteins, the latter containing higher mole fractions of acidic residues and reduced numbers of basic amino acid residues. These sequence differences probably reflect adaptations related to the harsh environmental conditions in which these halophilic organisms exist (Carothers *et al.*, 1989). E3 has been isolated from the bloodstream of the African parasite *Trypanosoma brucei* where it appears to be associated with the plasma membrane of the organism (Danson *et al.*, 1987). Complete subcellular fractionation of *T. brucei* confirmed that E3 associated exclusively with the membrane and was absent from other cellular fractions, although it remains to be established whether the attachment of E3 to the plasma membrane signifies a function specific to that location (Jackman *et*

al., 1990). *lpd* mutants of *E. coli* have been isolated which show low abundance residual E3 activity; it has been postulated that the E3 species present may be involved with protein dependent transport of sugars such as galactose and maltose in the cell (Richarme, 1989). These discoveries raise the question as to whether dihydrolipoamide dehydrogenases perform other distinct functions within the cell in addition to their established role in the 2-oxoacid dehydrogenase complexes.

1.4 PROTEIN X

The eukaryotic pyruvate dehydrogenase complex, in addition to its constituent enzymes E1, E2 and E3, also contains an additional tightly bound polypeptide of M_r 51,000. This extra band, commonly referred to as component or protein X, remained undetected for many years since it does not separate readily from the E3 subunit on SDS/phosphate gels run under denaturing conditions (Weber and Osborn, 1969). The eventual resolution of protein X from the E3 enzyme in mammalian PDC was achieved on SDS/polyacrylamide gels which were electrophoresed in Tris/glycine buffers (De Marcucci and Lindsay, 1985; Jilka *et al.*, 1986). Protein X, originally observed in PDC isolated from a variety of sources including bovine heart (Stanley and Perham, 1980), bovine kidney (Bleile *et al.*, 1981) and *S. cerevisiae* (Kresze and Ronft, 1981; Behal *et al.*, 1989), was thought to be the associated kinase activity or a degradation product of either the E2 or E3 enzymes (Bleile *et al.*, 1981); however, immunological, limited proteolysis and peptide mapping studies provided evidence that protein X was a distinct polypeptide of PDC (De Marcucci *et al.*, 1986). High titre antisera were also produced to both the E2 and protein X polypeptides, with each antiserum found to be monospecific in reacting only with its parent antigen. This lack of observed cross-reactivity between the antisera demonstrated that the two polypeptides were immunologically distinct (De Marcucci and Lindsay, 1985). In contrast to these findings, Jilka and co-workers (1986) prepared antisera to protein X which cross-reacted with both protein X and the E2 enzyme. These irregularities in the cross-

reactivity patterns exhibited between the antisera prepared by the two groups may reflect differences in the methods used to produce them, such as the immunisation regime used and each animal's individual immune response.

Immune mapping studies allow for the detection of proteolytic fragments derived from a single subunit in the presence of fragments from other subunits. The E2 enzyme and protein X polypeptides were isolated and purified from bovine heart PDC before digestion with elastase (De Marcucci and Lindsay, 1986). Cleavage of the E2 enzyme resulted in the production of two major proteolytic fragments, a fragment of M_r 45,000 which was highly immunogenic and a smaller M_r 29,000 fragment. At longer time intervals the M_r 45,000 peptide is degraded further while an increase in the M_r 29,000 band is observed. These fragments had been previously observed by Coomassie blue staining after elastase digestion of purified E2 enzyme, where the M_r 45,000 peptide corresponded to the lipoyl bearing domain and the M_r 29,000 peptide to the intersubunit binding domain of E2 (Kresze and Ronft, 1980). In contrast to the E2 enzyme, protein X produced two peptides with M_r values of 29,000 and 26,000 which were rapidly cleaved to form smaller peptides of M_r 15,000 or below. When the E2 enzyme and protein X polypeptides were labelled with [^{125}I]NaI for use in one and two dimensional peptide mapping studies, characteristic cleavage patterns were produced for each protein (De Marcucci et al., 1986; Jilka *et al.*, 1986). The differences observed in the peptide maps of E2 and protein X clearly demonstrated that protein X was a separate and distinct polypeptide species which did not originate from degradation of the larger E2 polypeptide. The cloning of both protein X and the E2 enzyme of PDC from *S. cerevisiae* (Section 1.5) has proved unequivocally that although these two species may be structurally similar, they are separate gene products which have probably evolved from a common ancestor.

1.4.1 STRUCTURAL PROPERTIES OF PROTEIN X

Protein X appears to be functionally active within the complex as incubating PDC with [2-^{14}C] pyruvate in the absence of CoASH results in the incorporation of radiolabel into both the E2 enzyme and protein X. Protein X retains 12-14% of radiolabel which suggests that there may be approximately eight to twelve molecules of the protein X polypeptide per E2 core (De Marcucci and Lindsay, 1985). Alternatively, both the E2 enzyme and protein X can be radiolabelled with [2,3-^{14}C]N-ethylmaleimide in a substrate dependent manner by incubating PDC either in the presence of pyruvate and absence of CoA or, by addition of NADH so generating the free thiol group (Hodgson *et al.*, 1986). The sites of acylation on the E2 enzyme have been identified as lipoic acid residues situated within domains present at the N-terminus; characterisation of the released radiolabelled product after hydrolysis in 6M HCl, confirmed that the acetylatable site on protein X was also a lipoic acid group (Hodgson *et al.*, 1986). Cross-linking studies with PDC were performed with phenylene-o-bismaleimide in the presence of acylating substrate to determine the number of lipoyl groups present on the protein X species; high M_r aggregates containing both the E2 enzyme and protein X were formed as a result of these cross-linking studies, with the formation of E2/E2, E2/X and X/X dimers in conjunction with trimers, proving that the lipoic acid groups of different E2 enzymes and protein X subunits were able to interact in all possible combinations. From the calculated M_r values of the cross-linked species, it was deduced that there were two lipoic acid groups per E2 subunit with a single lipoyl group present on protein X (Hodgson *et al.*, 1986). Proteolytic degradation of mammalian PDC with trypsin has also verified the presence of two lipoyl domains per E2 subunit; two major tryptic products are formed with M_r 45,000 and 26,000. The small fragment contains the acyltransferase active site in contrast to the larger peptide species which contains the lipoyl domains; further degradation of the large fragment to M_r 36,000 occurred as a

result of removal of the N-terminal lipoyl domain, with an estimated M_r 15,000 as deduced from SDS/polyacrylamide gel electrophoresis (Kresze and Ronft, 1980).

The genes which encode the E2 acetyltransferase and protein X species from *S. cerevisiae* have been cloned and sequenced (Niu *et al.*, 1988; Behal *et al.*, 1989). The N-terminal part of protein X (residues 1-195) appears to show some resemblance to E2, with particularly strong homology (approx. 50%) existing over the residues 1-84, the expected location of the lipoyl domain (Behal *et al.*, 1989). The sequence PALSPTM present on E2 which corresponds to the presence of a lipoyl domain further downstream, is also present in protein X although in a slightly modified form PAMSPTM, confirming that protein X possesses a lipoyl domain at its N-terminus. The remainder of the two proteins (from residues 195 onwards) are quite different to each other, especially at the C-terminus where protein X lacks the highly conserved acyltransferase active site sequence which is normally found in this region. The lack of the acyltransferase sequence motif from protein X might suggest that this subunit does not participate in acetyltransferase reactions within the complex; collagenase treatment of mammalian PDC results in removal of the lipoyl domains from E2, leaving those on protein X intact. After such treatment the complex retains approx. 10% of its original activity, suggesting that the lipoyl domain of protein X is capable of supporting the overall complex reaction (Rahmatullah *et al.*, 1990).

1.4.2 POSSIBLE FUNCTIONAL ROLE FOR PROTEIN X

Prior to the discovery of protein X, it had been shown that PDC kinase activity was capable of being stimulated by either pyruvate or acetyl CoA as a direct consequence of acetylation of lipoyl domains present on the E2 enzyme of the complex (Cate and Roche, 1978; 1979). The role of protein X as a modulator of PDC kinase activity was proposed, when it was shown that reduction and subsequent acetylation of the recently discovered protein X subunit by pyruvate or NADH and acetyl CoA resulted in increases in kinase activity associated with the complex (Rahmatullah and

Roche, 1987). In this way, the acetylation state of the complex was linked to changes in kinase activity; however, the sole involvement of protein X in kinase stimulation could not be proved conclusively, since the results did not eliminate the possibility that E2 itself may have been responsible for the observed kinase activation.

Protein X is known to associate very strongly with the E2 core structure of PDC as treating the complex under dissociating conditions and then subjecting the complex to gel filtration chromatography, causes release of E1 and E3 only from the E2/X core structure (Linn *et al.*, 1972; Kresze and Steber, 1979). Protease arg C degradation of isolated E2/X subcomplex prepared from intact PDC results in cleavage of the lipoyl domain from protein X, where the loss of overall PDC activity in subsequent reconstitution experiments paralleled the rate of cleavage of protein X (Gopalakrishnan *et al.*, 1989; Rahmatullah *et al.*, 1989). It was concluded from such analyses that the lipoyl domain of protein X was performing a major role in the overall reaction catalysed by PDC. In contrast to treatment with protease arg C which cleaves only the lipoyl domain from protein X, collagenase treatment of the complex selectively removes the lipoyl domains from the E2 subunits while leaving those on protein X intact (Rahmatullah *et al.*, 1990). After treatment of the complex with levels of collagenase sufficient to remove all of the E2 lipoyl domains, 10% of complex activity persisted suggesting that the lipoyl domains present on protein X were capable of supporting the overall complex reaction.

Protein X was also shown to be capable of interacting with the E3 enzyme; in microplate assays E3 was found to bind to the E2/X subunit fraction immobilised on the plate surface, the same group also showing that cleavage of protein X by protease arg C was reduced in the presence of exogenously added E3 (Gopalakrishnan *et al.*, 1989). Methods were developed which allowed for protein X to be removed from the isolated E2/X subcomplex, resulting in an E2 oligomer free from protein X (Powers-Greenwood *et al.*, 1989). The purified E2 oligomer was shown to be incapable of maintaining the overall reaction sequence in reconstitution experiments with E1 and E3, and exhibited a reduced affinity for binding the E3 enzyme. Neagle and Lindsay

(1991), while agreeing that protein X was involved in binding the E3 enzyme to the core, showed that proteolytic degradation of protein X using protease arg C causing removal of the lipoyl domain, had no effect on overall complex activity. The major difference between this and the previous group's experiments, was that intact bovine heart PDC was used in the later study as opposed to the isolated E2/X subcomplex. The decrease in complex activity observed by other groups during attempted reconstitution experiments was attributed to degradation of the E1α enzyme which also occurred as a result of protease arg C digestion. It may also be that observed differences are a reflection of the instability of the truncated protein 35kD species and its ability to maintain overall complex stability.

The existence of a small domain which shows distant but related homology to an E3 binding domain has been found on protein X (residues 144-180) of *S. cerevisiae* (Lawson *et al.*, 1991). When bovine heart protein X is cleaved using protease arg C to remove the lipoyl domain so producing an M_r 35,000 fragment, reduced binding of E3 is observed following release of the lipoyl domains. The release of E3 may reflect instability of the E3 binding region after lipoyl domain release; the putative E3 binding domain has an estimated M_r 5,000 and at longer incubation times, the M_r 35,000 species is further cleaved to form an M_r 30,000 species probably as a result of removal of this putative E3 binding domain (J. Neagle and S. Sanderson, unpublished results). Interestingly, collagenase treated E2/X subcomplex retains the ability to bind E3, where it is known that only the lipoyl domain and its associated linker domain are removed as a result of proteolytic treatment (Rahmatullah *et al.*, 1990). Lawson *et al.*, (1991), suceeded in isolating a mutant of *S. cerevisiae* in which the chromosomal copy of protein X had been disrupted. Although this deletion did not affect growth of the yeast under the conditions tested, extracts of mitochondria from this mutant were unable to catalyse the CoA/NAD^+ linked oxidation of pyruvate. Purification of PDC from the mutant showed that PDC contained the E2, E1α and E1β subunits only, lacking both E3 and protein X. In order to determine the particular region of protein X involved with binding E3, a series of deletion mutants were produced such that either

the lipoyl domain (residues 1-80) or the putative E3 subunit binding domain (residues 144-180) were deleted. The mutant in which the lipoyl domain had been removed showed little effect on overall activity; PDC purified from this mutant exhibited 60-70% of overall wild type activity, the complex also containing E3. It would appear that the lipoyl domain together with its covalently bound lipoyl moiety, is non-essential for protein X function in *S. cerevisiae*. The mutant in which the putative subunit domain had been deleted showed a total loss of overall PDC activity in conjunction with a loss of high affinity E3 binding, indicating that this domain performs a role in binding E3.

To date, no evidence has been presented for the existence of protein X in the 2-oxoacid dehydrogenase complexes except those found in mammalian PDC, although the possibility that a similar structure exists in the other complexes cannot be ruled out.

1.5 CLONING OF THE ENZYME SUBUNITS OF THE 2-OXOACID DEHYDROGENASE COMPLEXES

In recent years efforts have been directed towards the cloning and sequencing of the constituent enzyme subunits of the 2-oxoacid dehydrogenase complexes. This is desirable to allow for the comparative analysis of the nucleotide and deduced amino acid sequences, and to aid the identification of any common motifs or structural characteristics which may exist between the complexes isolated from both prokaryotic and eukaryotic sources.

The E1 enzyme of OGDC in *E. coli* is encoded by the *sucA* gene (Darlison *et al.*, 1984) and the E2 enzyme by *sucB* (Spencer *et al.*, 1984; Carlsson and Hederstedt, 1987). Both these genes are found arranged in an operon where the *lpd* gene coding for E3, common to both OGDC and PDC, is found not on the *sucAsucB* operon but as part of the *aceEaceF* operon encoding the E1 and E2 enzymes of PDC (Spencer and Guest, 1985). Organisation of the genes encoding the OGDC subunits in *B. subtilis* is similar to that of *E. coli*; the E1 (*odhA*) and E2 (*odhB*) genes constitute one operon

where the E3 gene is found on the operon which encodes the PDC subunits (Carlsson and Hederstedt, 1989). In addition to similarity in their gene organisation, OGDC from *E. coli* and *B. subtilis* are structurally very similar as indicated by complementation studies in which the E2 enzymes from both complexes were able to interact with each other forming active complexes both *in vivo* and *in vitro* (Carlsson and Hederstedt, 1987). The genes responsible for encoding OGDC in *A. vinelandii*, in contrast to those of *E. coli*, belong to one gene cluster; a common promoter in the 5' untranslated region of the gene together with a lack of promoter and terminator sequences for E2, suggests that all three genes are transcribed as a single mRNA transcript. The *lpd* gene possesses its own promoter allowing it to be expressed independently in order to synthesise the E3 necessary for PDC assembly.

Many of the subunits of these complexes have now been cloned from mammalian sources; the sequence of the E1 subunit of OGDC from *S. cerevisiae* (Repetto and Tzagoloff, 1989) and cDNA encoding E1 from a human foetal liver library (Koike et al., 1992) are now known. The human E1α subunit of PDC has also been cloned (Dahl *et al.*, 1987; De Meirlier *et al.*, 1988; Koike *et al.*, 1988; Ho *et al.*, 1989), where a high degree of sequence conservation exists between all of the cloned E1α sequences except in the case of Dahl *et al.* (1987), which shows considerable sequence variation when compared to the sequences of Koike *et al.* (1988) and De Meirlier *et al.* (1988). The E1α subunit of BCOADC has also been cloned from human liver (Zhang *et al.*, 1988) and bovine liver (Hu *et al.*, 1988). A common feature of many of these sequences is the presence of a leader sequence at the N-terminus, a feature responsible for the correct targetting and assembly of these large multimeric complexes.

The E2 enzymes of the three complexes have also been cloned from various sources. For example, PDC E2 has been cloned from *S. cerevisiae* (Niu *et al.*, 1988), *A. vinelandii* (Hanemaaijer *et al.*, 1988) and *B. stearothermophilus* (Borges *et al.*, 1990). The sequence obtained for human PDC E2 (Thekkumkara *et al.*, 1988) was identical to that for rat liver E2 (Gershwin *et al.*, 1987) in the region of the second lipoyl domain; however, the deduced amino acid sequence from rat liver did not

extend far enough into the N-terminal region of the protein to include the first lipoyl domain. The nucleotide sequence of the E2 enzyme from OGDC has also been deduced for rat heart (Nakano *et al.*, 1991), *E. coli* (Spencer *et al.*, 1984; Carlsson and Hederstedt, 1987), *B. subtilis* (Carlsson and Hederstedt, 1989) and *A. vinelandii* (Westphal and de Kok, 1990). BCOADC E2 from mammalian sources (Hummel *et al.*, 1988; Danner *et al.*, 1989) has a similar structural organisation to the PDC and OGDC E2 enzymes all of which are synthesised with a leader sequence. The presence of these leader sequences is essential for the correct targetting of these nuclear-encoded cytoplasmically synthesised proteins to the mitochondria (Glover and Lindsay, 1992). These similarities in the structure of the E2 proteins from the different complexes suggest that they may be evolutionary related. For example, the primary structure of the inner E2 core domain of bovine BCOADC is similar to PDC E2 isolated from both human and *E. coli* sources (Griffin *et al.*, 1988) and the E2 domain of OGDC (Lau *et al.*, 1988).

The gene encoding dihydrolipoamide dehydrogenase (E3) has been cloned from several sources including pig (Otulakowski and Robinson, 1987), human (Pons *et al.*, 1988), *S. cerevisiae* (Browning *et al.*, 1988), *E. coli* (Stephens *et al.*, 1983), *A. vinelandii* (de Kok *et al.*, 1987), *Ps. putida* (Burns *et al.*, 1989) and *Ps. fluorescens* (Benen *et al.*, 1989). Considerable homology has been demonstrated in the primary amino acid sequences of these proteins for example, human E3 shows 96% homology with pig heart E3 and 44% homology with *E. coli* E3. This high degree of sequence conservation is unsurprising since E3 is a common component in all of the 2-oxoacid dehydrogenase complexes, and as such has not shown large sequence variations throughout its evolution.

1.6 AIMS OF PROJECT

The members of the 2-oxoacid dehydrogenase complex family of enzymes are composed of three distinct enzymes E1, E2 and E3. In addition, PDC isolated from mammalian sources has been found to contain an extra polypeptide termed protein X,

implicated in assisting with binding E3 to the core. The absence of this particular subunit in OGDC and BCOADC is puzzling since both these complexes contain an identical E3 gene product species to that found in PDC.

The aim of this project therefore, was to investigate the possible existence of a protein X-like component in bovine heart OGDC and determine its location within the complex. Studies would then be made relating to its structural role within the complex, particularly its involvement in binding the E3 subunit. In addition, the role of any such component in the catalytic functioning of the complex would also be examined.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 CHEMICALS

Substrates and coenzymes for enzymatic assays, PMSF, benzamidine-HCl, MOPS, Tween-20, antifoam A concentrate, Coomassie brilliant blue type R250 and N-ethylmaleimide were obtained from Sigma Chemical Co., Poole, Dorset U.K. Pyronin Y dye was purchased from George T. Gurr Ltd., London. Polyethylene glycol 6000 grade and Triton X-100 were obtained from Fisons, Loughborough, England. Folin and Ciocalteu phenol reagent was from FSA laboratory supplies, Loughborough, England. Iodogen was purchased from Pierce Laboratories, Rockford, Illinois, U.S.A. DTT was from Boehringer Mannheim GmbH, Germany. Sodium salicylate was obtained from Aldrich Chemical Company, Ltd., Gillingham, U.K. Ultra pure acrylamide used for protein sequencing was from Fluka, Derbyshire, England. All other chemicals including reagents for polyacrylamide gel electrophoresis were of analaR grade from BDH Chemicals Ltd., Poole, U.K. or were of the highest grade available commercially.

2.1.2 RADIOCHEMICALS

[^{125}I]NaI, N-ethyl[2,3-^{14}C]maleimide and 2-oxo[5-^{14}C]glutaric acid were purchased from Amersham, Bucks., England. [2-^{14}C]pyruvic acid (sodium salt) was from Du Pont de Nemours GmbH, Germany.

2.1.3 ENZYMES AND PROTEINS

The following commercial preparations were obtained from Sigma Chemical Co., Poole, Dorset : protein A (*Staphylococcus aureus*, Cowan 1 strain), TPCK-treated trypsin, lipoamide dehydrogenase from pig heart and low molecular weight markers

for M_r determinations by tricine-SDS/polyacrylamide gel electrophoresis. Protease arg C was obtained from Boehringer Mannheim GmbH, Germany. Low molecular weight marker proteins for M_r determinations by SDS/polyacrylamide gel electrophoresis were bought from Pharmacia Ltd., Milton Keynes. Leupeptin was purchased from the Protein Research Foundation, Osaka, Japan.

2.1.4 ANIMALS

Bovine hearts were obtained from Paisley Abbatoir, Sandyford Road, Paisley and stored at 4°C until they reached the laboratory. On arrival the hearts were cut into small cubes, care taken to excise fatty deposits, and stored at -80°C until required. New Zealand White Rabbits for antisera production were purchased at 3 months old from MRC accredited sources.

2.1.5 MISCELLANEOUS

Hybond-C extra nitrocellulose was purchased from Amersham International, Bucks., U.K. Immobilon PVDF membrane for protein sequencing was bought from BioRad, Richmond, CA, U.S.A. 0.22μm nitrocellulose filters were from Millipore S.A., Molsheim, France. Rat serum, Freund's complete and incomplete adjuvant were obtained from Sigma Chemical Co., Poole, Dorset, U.K. Plast-X autoradiography cassettes were from Anthony Monk (England) Ltd., Sutton in Ashfield, U.K. FPLC columns and gel chromatography materials were from Pharmacia Ltd., Milton Keynes, England.

2.1.6 PHOTOGRAPHIC MATERIALS

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

was from Amersham, Bucks., England.

2.2 METHODS

2.2.1 CONCENTRATION OF PROTEIN SAMPLES

Protein samples were concentrated in one of several ways: freeze-drying involved freezing the sample in a dry ice/methanol bath prior to drying down under vacuum. Alternatively, samples for gel electrophoresis were concentrated by the addition of 4 volumes of acetone and then stored at -20° C overnight. Next day the sample was centrifuged at 11,600g, the acetone removed and the pellet resuspended in Laemmli sample buffer.

TCA precipitation was employed when it was necessary to remove salt or other reagents which might interfere with electrophoresis. TCA from a 100%(w/v) stock was added to a final concentration of 10%(w/v) and the sample kept at 4°C for 1-2 h. The pellets obtained after a short centrifugation at 11,600g for approx. 2 min were washed with ether to remove any remaining traces of TCA, air dried and resuspended in sample buffer.

2.2.2 MEASUREMENT OF PROTEIN CONCENTRATION

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

Stock solutions were prepared as follows:

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

Solution B - 4%(w/v) copper sulphate

Solution C- 1ml of solution A added to distilled water to a final volume of 100ml.

Solution D - Folin and Ciocalteu phenol reagent diluted 50:50 with distilled water.

A 1mg/ml stock of bovine serum albumin was used as the standard protein and a standard curve was constructed with protein amounts between 0-150μg. The final volume of the assay was made up to 1ml with distilled water before addition of 3ml of solution C. The tubes were then vortexed and allowed to stand at room temperature for 15 min. Colour development was effected by addition of 0.3ml of solution D and absorbance was measured at 660nm after 30 min.

2.2.3 CONDITIONS FOR RESOLUTION OF PROTEINS BY SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS

(a) CONDITIONS FOR POURING OF SLAB GELS

Protein samples were analysed using a discontinuous Tris-glycine system according to the method of Laemmli (1970). Gels were cast in slabs with the dimensions 16cm x 19.5cm x 0.15cm; however, in the case of gels which were to be processed for fluorography [Section 2.2.3 (e)] gels of thickness 0.8cm were poured. Gels were subjected to electrophoresis at a constant current of 40-60mA in 192mM glycine/0.1%(w/v) SDS/25mM Tris base, pH 8.3 using Bethesda Research Laboratory vertical gel electrophoresis apparatus. Electrophoresis was continued until the Pyronin Y tracker dye was approximately 0.25cm from the bottom of the gel. Stock solutions for the pouring of analytical gels were prepared as follows :

Acrylamide stock	29.2%(w/v) acrylamide
	0.8%(w/v) N,N^{l}-methylenebisacrylamide
Resolving gel buffer	0.75M Tris-HCl (pH 8.8)
	0.2%(w/v) SDS
Stacking gel buffer	0.17M Tris-HCl (pH 6.8)
	0.14%(w/v) SDS

Ammonium persulphate: 1g dissolved in 10ml of distilled water

Resolving gels of the required percentage of acrylamide were prepared from stock solutions according to the following table were all volumes shown are in ml.

	%(w/v) acrylamide		
	10%	12.5%	15%
Acrylamide stock	50.00	62.00	74.00
Resolving gel buffer	72.00	72.00	72.00
Ammonium persulphate	1.44	1.44	1.44
Distilled water added to a final volume of 144ml .			

These solutions were mixed, degassed and polymerisation initiated by the addition of 0.1%(v/v) TEMED before pouring into the casting apparatus. The resolving gel was overlaid with propan-2-ol to ensure an even surface on the top gel. Propan-2-ol was removed after the gel had set and the gel surface thoroughly rinsed with distilled water prior to pouring the stacking gel.

The 5% stacking gel was poured as follows :

Acrylamide stock	7.20
Stacking gel buffer	32.80
Ammonium persulphate	0.48
Distilled H_2O added to	48.00

(All volumes in table are in ml)

Polymerisation was initiated by the addition of 0.1%(v/v) TEMED and after setting, gel plates were wrapped in damp tissue and stored at 4°C .

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

Protein solutions were mixed with an equal volume of Laemmli sample buffer [2%(w/v) SDS/10%(w/v) sucrose/0.001%(w/v) Pyronin Y/62.5mM Tris-HCl, pH 6.8] containing 10mM DTT. Samples were boiled for 2-3 min prior to loading.

(c) STAINING OF GELS WITH COOMASSIE BLUE

After electrophoresis, gels were stained for protein in 0.04%(w/v) Coomassie brilliant blue R250/25%(v/v) methanol/10%(v/v) acetic acid for 1h at room temperature with gentle agitation.

(d) DESTAINING OF GELS

Excess stain was removed from gels by washing in 20%(v/v) methanol/10%(v/v) acetic acid. Destaining of gel took approximately 1-2h with the destaining solution being changed every 30 min during this period.

(e) FLUOROGRAPHY

Fluorography was performed according to the method of Chamberlain (1979) using sodium salicylate as fluor. Gels to be processed for fluorography were fixed overnight by incubation in 25%(v/v) propan-2-ol/10%(v/v) acetic acid. After fixing, gels were rinsed several times with distilled water over a 30 min period and then soaked in 1M sodium salicylate for 30 min. Gels were dried down under vacuum at 60°C and then exposed to Hyperfilm™ MP before being stored at -80°C .

(f) M_r DETERMINATION

The molecular weight of protein species was determined by comparing their mobility to those of a set of molecular weight standards which included phosphorylase b, M_r 97,000, albumin, M_r 67,000, ovalbumin, M_r 43,000, carbonic anhydrase, M_r 31,000, soya bean trypsin inhibitor, M_r 20,100 and lysozyme, M_r 14,000.

R_f value = $\frac{\text{distance migrated by protein}}{\text{distance travelled by dye front}}$

The calculated R_f value obtained for a individual polypeptide subunits is plotted against its log M_r to produce a calibration curve from which the subunit molecular weight of the unknown species can be determined by interpolation.

2.2.4 TRICINE-SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

(a) CONDITIONS FOR TRICINE-SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS OF PROTEINS

When used as the trailing ion, tricine allows for the resolution of small proteins at lower acrylamide concentrations than in the glycine SDS/polyacrylamide gel electrophoresis systems (Schägger and Von Jagow, 1987).

The stock solutions prepared for gel electrophoresis are shown in the table below:

Buffer	Tris(M)	Tricine(M)	%SDS(w/v)	pH
Anode buffer	0.2	---	---	8.90
Cathode buffer	0.1	0.1	0.1	8.25
Gel buffer	3.0	---	0.3	8.45

48%(w/v) acrylamide/1.5%(w/v) N,N^{l}-methylenebisacrylamide solution

Gels of dimension 16cm x 19.5cm x 0.15cm were poured which consisted of three distinct gel layers i.e. a small pore 16.5%(w/v) acrylamide resolving gel (14.5cm), 10%(w/v) acrylamide spacer gel (3cm) and 4%(w/v) acrylamide stacker gel (2cm).

Composition of gels shown in table: (all volumes in table are in ml)

	Stacking gel 4%(w/v)	Spacer gel 10%(w/v)	Resolving gel 16.5%(w/v)
Acrylamide/bisacrylamide mix	1.0	6.1	10.0
Gel buffer	3.1	10.0	10.0
Glycerol	---	---	4g
d. H_2O added to	12.5	30.0	30.0

Polymerisation of resolving and spacer gels were initiated by addition of 150µl of 10%(w/v) ammonium persulphate and 0.05%(v/v) TEMED. Stacking gel was polymerised by adding 100µl of 10%(w/v) ammonium persulphate and 0.08%(v/v) TEMED. During polymerisation the gel surface was kept even with an overlay of propan-2-ol; once the gel had set completely, this was removed and top of gel rinsed thoroughly with distilled water to ensure that all traces of propan-2-ol had been removed.

Gels were electrophoresed at room temperature, with cathode buffer in the upper reservoir and anode buffer in the lower reservoir, at a constant voltage of 30V for 1h or until the loaded protein had completely left the sample well. Once the sample had entered the spacer gel, the gel was electrophoresed at 80mA (constant voltage) which decreased to 50mA once the sample had entered the resolving gel.

(b) PREPARATION OF PROTEIN SAMPLES FOR TRICINE-SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS

Protein solutions were mixed with an equal volume of sample buffer, [4%(w/v)/12%(w/v)glycerol/2%(v/v)mercaptoethanol/0.01%(w/v)Pyronin Y /50mMTris-HCl, pH 6.8] and boiled for 2-3 min before being loaded into the sample wells.

(c) M_r DETERMINATION USING TRICINE-SDS/POLYACRYLAMIDE GEL ELECTROPHORESIS

The molecular weight for proteins run on this gel system was calculated using a set of marker peptides of known molecular weights, the majority of which were fragments of myoglobin: myoglobin residues 1-153, M_r 16,950; myoglobin (I+II) residues 1-131, M_r 14,400; myoglobin (I+III) residues 56-153, M_r 10,600; myoglobin I residues 56-131, M_r 8,160; myoglobin II residues 1-55, M_r 6,210;

glucagon, M_r 3,460 and myoglobin III residues 132-153, M_r 2,510. As described previously a calibration curve of R_f values versus log M_r was plotted from which the molecular weights of unknown peptides could be calculated.

2.2.5 PROTEIN SEQUENCING

(a) ELECTROPHORESIS CONDITIONS

This procedure was used in the microsequencing of proteins according to Speicher (1989) for either N-terminal or internal amino acid sequence determination.

Mini gels with a 5%(w/v) acrylamide stacker were cast with dimensions 7.3cm x 10.2cm x 0.75mm according to the method outlined for SDS/polyacrylamide gel electrophoresis in section 2.2.3(a), the only modification being that ultra pure acrylamide was used in the pouring of these gels. Prior to use the gel solutions were filtered through Millipore (0.22μm) nitrocellulose filters (type GS). Gels were run on a BioRad mini-protean II vertical slab gel apparatus according to the Method described in section 2.2.3(a) with the following modifications: pre-run electrophoresis - 0.05mM reduced glutathione was added to 70ml of electrophoresis buffer in the upper reservoir and the gel electrophoresed at 3mA constant current for 2h prior to loading of the sample. Sample electrophoresis - pre-run buffer in the upper reservoir was decanted and the reservoirs re-filled with 70ml of fresh buffer solution to which 0.1mM sodium thioglycolate was added. Gels were electrophoresed at 10mA constant current until the dye front reached the bottom of the gel.

(b) IMMUNOBLOTTING AND SEQUENCING OF RESOLVED PROTEINS

Resolved proteins were electrophoretically transferred onto Immobilon PVDF membrane as described in section 2.2.6(a). Modifications included reducing the transfer time to 1h at a constant current of 150mA and pre-wetting the membrane for

15 min in 100% methanol. After transfer the membrane was washed in distilled water for 5 min and then stained for 5 min in 0.5%(w/v) Coomassie brilliant blue R. 40%(v/v) methanol was used to destain the membrane (2 x 5 min wash), the membrane finally washed in distilled water. The protein band of interest was excised from the membrane using a scalpel and sequenced using an Applied Biosystems model 470A protein sequencer with online PTH amino acid analyses (model 120A), in conjunction with a modification of the manufacturer's Blott 4 programme.

2.2.6 IMMUNOBLOTTING TECHNIQUES

(a) IMMUNE BLOTTING (IMMUNE REPLICA ANALYSIS, WESTERN BLOTTING)

This protocol allows for the immunological detection of proteins following their electrophoretic transfer from polyacrylamide gels onto nitrocellulose paper (Towbin *et al.*, 1979; Batteiger *et al.*, 1982).

Proteins to be immunoblotted were resolved on 10%(w/v) polyacrylamide gels [Section 2.2.3(a)] and transferred electrophoretically onto Hybond-C nitrocellulose paper either at 40mA overnight or 400mA for 2.5 hours in a BioRad trans-blot cell in 192mM glycine/0.02%(w/v)SDS/20%(v/v) methanol/25mM Tris, pH 8.3. After transfer the nitrocellulose membrane was stained with Ponceau S solution to allow the visualisation of the transferred proteins, and then washed free of this stain with distilled water prior to immunoblotting. Excess binding sites were blocked by washing the nitrocellulose membrane for 1h in 0.15M NaCl/0.5%(v/v) Tween-20/20mM Tris, pH 7.2. The wash buffer was discarded and the blot was incubated with the desired antibody, normally at 1:100 dilution for 90 min in wash buffer supplemented with 5%(v/v) heat inactivated donkey serum. The membrane was then rinsed with several changes of wash buffer over a period of 1h to remove any unbound antibody.

At this stage, ^{125}I-labelled protein A (3 x 10^6 c.p.m.) was added to the nitrocellulose membrane with 50 ml of wash buffer for 1h, after which the blot was washed in a similar manner to that desribed in the previous step. After washing, the nitrocellulose immunoblot was allowed to dry in air and mounted in cassettes in close contact with X-Omat S film and exposed at -80°C for a period of 1-7 days.

(b) PREPARATION OF ANTISERA

Rabbits were injected initially with 0.25-1.0mg of desired antigen dissolved in 0.75ml of 0.9%(w/v) NaCl mixed with 0.75ml of complete Freund's adjuvant. Thorough mixing was achieved by passing the solution several times through a narrow gauge needle until a thick creamy emulsion was formed. This was injected subcutaneously at multiple sites in the neck, back and thighs of a New Zealand White Rabbit. A booster dose was then administered four weeks later consisting of 0.1-0.5mg antigen/0.75ml of 0.9%(w/v) NaCl/0.75ml Freund's incomplete adjuvant mixed to form an emulsion in a similar manner to that described above. Further booster doses were administered 2 weeks prior to bleeding. Rabbits were bled from an ear vein and the collected blood was reamed to ensure that fibrous clots formed away from the walls of the collecting tube permitting the serum to be easily removed. Serum was spun at 700g for 5 min to remove any remaining red blood cells and stored in 1ml aliquots at -80°C.

(c) PREPARATION OF IgG FRACTION FROM WHOLE ANTISERA

This method allows for the purification of immunoglobulin G (IgG) from whole antisera. IgG was normally prepared from antisera which gave unsatisfactory results in immunoblotting studies caused by its non-specific binding to the nitrocellulose membrane.

2ml of antiserum was stirred gently at 4°C while 1ml of saturated ammonium sulphate was added dropwise. This solution was stirred for a further 30 min. Precipitated IgG was recovered by centrifugation at 5000g for 10 min, the resultant pellet solubilised in 2ml of 0.9%(w/v) NaCl and the above step repeated twice more. After the third precipitation, the pellet was resuspended in 1ml of 0.9%(w/v) NaCl and dialysed overnight at 4°C in 170mM NaCl/3.4mM KCl/10mM Na_2HPO_4/80mM KH_2PO_4, pH 7.2. After dialysis the sample volume was decreased by covering the sample, still in its dialysis tubing, with polyethylene glycol until the desired volume was obtained. The IgG fraction of the antiserum was then stored at -80°C in 0.5ml aliquots.

2.2.7 PURIFICATION OF THE 2-OXOGLUTARATE DEHYDROGENASE COMPLEX FROM BOVINE HEART

The 2-oxoglutarate dehydrogenase complex was purified according to the method of Stanley and Perham (1980) involving the following modifications. All steps were performed at or near 4°C. Heart tissue (300g) was homogenised for 5 min in 2.7mM EDTA/0.1mM DTT/3%(v/v) Triton X-100/1mM PMSF/1mM benzamidine-HCl/silicone antifoam (0.5ml/l)/50mM MOPS, pH 7.0. The homogenate was then diluted with an equal volume of the same buffer and spun at 10,000g for 20 min. The pH of the resulting supernatant fraction was adjusted to pH 6.45 using 10%(v/v) acetic acid after which 0.15 volumes of 35%(w/v) polyethylene glycol was added rapidly. The solution was allowed to stir on ice for 30 min before the precipitated material was centrifuged at 18,000g for 15 min. The pellets were resuspended by homogenisation with a loose-fitting teflon-glass homogeniser in 200 ml of 2.7 mM EDTA/0.1 mM DTT/1%(v/v) Triton X-100/ 0.15μM leupeptin/1mM PMSF/1mM benzamidine-HCl/50mM MOPS, pH 6.8. The homogenised material was filtered through muslin in order to remove fat particles and adjusted to give final concentrations of 13mM $MgCl_2$ and 50mM sodium phosphate, pH 6.3, during which the pH of the solution was not

allowed to fall below 6.8. The pH was then lowered to 6.45 using 10%(v/v) acetic acid, 0.12 volumes of 35%(w/v)poly ethyleneglycol was added and the solution stirred for 30 min on ice. The precipitated material was spun at 25,000g for 10 min and the pellets resuspended as before in 80ml of 2.7mM EDTA/0.1mM DTT/1%(v/v) Triton X-100 and stored overnight at 4 °C in the presence of 1mM PMSF/1mM benzamidine-HCl/0.5%(v/v) rat serum. The latter was included in the buffer to prevent loss of 2-oxoglutarate dehydrogenase complex activity from occurring overnight (Wieland, 1975). If omitted, losses of up to 50% OGDC activity were found to occur during overnight storage.

Next day, the suspension of partially purified enzyme was clarified by centrifugation at 35,000g for 1h. PDC and OGDC were separated from each other by selective precipitation: the addition of 0.04 volumes of 35%(w/v) polyethylene glycol was sufficient to precipitate approx. 90% of the OGDC activity while leaving 95% of PDC activity in the supernatant fraction. The precipitated material was then pelleted at 25,000g for 10 min and the resulting OGDC pellet solubilised in 2.7mM EDTA/1% (v/v)Triton X-100/50mM MOPS, pH 6.8.

(a) FURTHER PURIFICATION OF THE MULTIENZYME COMPLEX BY GEL FILTRATION ON SEPHAROSE CL-2B COLUMNS

Partially purified OGDC could be purified further to near homogeneity by using gel filtration chromatography. A Sepharose CL-2B column (100cm x 3cm) was pre-equilibrated at 4°C with 2.7mM EDTA/1%(v/v)Triton X-100/50mM NaCl/50mM potassium phosphate buffer, pH 7.0. Approx. 100mg of OGDC was loaded onto the column in a final volume of 4ml and the column was eluted at 4°C using the same buffer as before at a flow rate of 24ml/h during which 12ml fractions were collected. A_{280} of the collected samples was monitored continuously with alternate fractions being assayed for both OGDC and PDC activity. The tubes containing peak OGDC activity in the absence of contaminating PDC, were pooled and spun at 130,000g for

2.5h to pellet the complex. The OGDC was resuspended in approx. 1ml of 2.7mM EDTA/0.1mM DTT/1%(v/v) Triton X-100/50mM MOPS, pH 6.8. Purified complex was stored at either 4°C for short term (1-3 weeks) or for longer periods at -20°C in 50% glycerol.

2.2.8 ENZYME ASSAYS

(a) 2-OXOACID DEHYDROGENASE COMPLEXES

The overall activities of the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase complexes were assayed according to the method of Brown and Perham (1976). Assays were performed at 30°C in a final volume of 0.67ml of 50 mM potassium phosphate buffer, pH 7.6, containing 3 mM NAD^+/1mM $MgCl_2$/0.2 mM TPP/2.6 mM cysteine-HCl/0.13 mM CoA/2 mM sodium 2-oxoglutarate (or 2mM sodium pyruvate when assaying for PDC activity). The assay was initiated by addition of enzyme and the reaction followed by monitoring the formation of NADH spectrophotometrically at 340nm (Danson *et al.*, 1978). One unit of activity (katal) is defined as the amount of enzyme required to convert one mole of substrate per second at 30°C under the conditions of the assay.

(b) 2-OXOGLUTARATE DEHYDROGENASE (E1) ACTIVITY

Assaying of 2-oxoglutarate dehydrogenase (E1) activity was performed according to the method of Khailova *et al.* (1976). Assays were carried out at 30°C by following the rate of change of absorbance at 600nm corresponding to the reduction of DCPIP (2,6-dichlorophenol-indophenol) in 0.67ml of 50mM potassium phosphate buffer, pH 7.6, containing 3mM NAD^+/1mM $MgCl_2$/0.2mM TPP/2mM 2-oxoglutarate/14μg DCPIP.

(c) DIHYDROLIPOAMIDE DEHYDROGENASE (E3) ACTIVITY

Assays were carried out at 30°C in a final volume of 0.67ml of 50 mM potassium phosphate buffer, pH 7.6, containing 3mM NAD^+/1mM $MgCl_2$/0.2mM TPP/20µg of reduced lipoic acid (Section 2.2.9) according to the method of Jackman *et al.* (1990). The reaction was initiated by addition of enzyme and activity followed by monitoring NADH formation at 340nm.

2.2.9 PREPARATION OF REDUCED LIPOIC ACID

Reduced lipoic acid was prepared according to the method described by Kochi and Kikuchi (1976) with the following modifications: 240mg of DL-6,8-thiotic acid amide (oxidised form) was solubilised in 4.8ml of 1M potassium phosphate buffer, pH 8.0 which was diluted 50:50 with ethanol. 9.6 ml of 5%(w/v) sodium borohydride was then added and incubated for 10 min at 4°C, after which the solution was neutralised by the slow addition of 4.8ml of 3M HCl. Reduced dihydrolipoamide was extracted with toluene and recovered by evaporation of the toluene under a stream of nitrogen, the recovered material being solubilised in ethanol. Estimation of the number of reduced sulphydryl groups formed by borohydride reduction was estimated by the use of DTNB according to the method of Ellman (1959).

2.2.10 RADIOLABELLING OF PROTEINS

(a) RADIOLABELLING OF PROTEINS USING IODOGEN

This method of radiolabelling proteins (Fracker and Speck, 1978) was used for the iodination of protein A for use in immunoblotting studies [Section 2.2.6 (a)]. Iodogen, a mild solid phase oxidising agent, was solubilised in 1ml of chloroform and the solvent evaporated by drying under an gentle air current so that a thin layer of the

Iodogen covered the base of the reaction vessel. Protein A (1mg) was dissolved in 0.5ml of 1.5M NaCl/0.1M Tris-HCl, pH 7.2 and added to the Iodogen. 500μCi of [^{125}I]NaI was added to the Iodogen-protein A mix and incubated for 15 min at room temperature with occasional mixing. The reaction mixture was then applied to a 10ml Sephadex G25 column which had been equilibrated previously with 50ml of 0.15M NaCl/0.1M Tris-HCl, pH 7.2. 1ml fractions were collected and the radioactivity in each sample counted to determine the fractions containing the iodinated protein A; these fractions were pooled and then dispensed into Eppendorf tubes to give 1.5-2.5μCi aliquots for use in immunoblotting studies.

(b) MODIFICATION OF PROTEINS USING [2,3-^{14}C]N-ETHYLMALEIMIDE

Proteins were labelled in this manner to allow for their use as marker proteins on gels which were processed for fluorography [Section 2.2.3(e)]. 100μg of protein was diluted with 100μl of Laemmli sample buffer (omitting DTT) and boiled for approximately 2-3 min to denature the protein. 1μCi of [2,3-^{14}C]N-ethylmaleimide was added and protein modification allowed to proced for 1h at room temperature. Four volumes of acetone were then added to the radiolabelled protein which was allowed to precipitate overnight at -20°C. Sample was spun at 11,600g for 5 min, the acetone removed and the sample allowed to dry. The protein pellet was resuspended in 100μl of sample buffer which included 10mM DTT and stored at -20°C.

2.2.11 ELUTION OF ENZYME SUBUNITS FROM SDS/POLYACRYLAMIDE GELS

This method permits the elution of protein bands from polyacrylamide gels (Tolan *et al.*, 1980). Complex (3mg) was electrophoresed on 10%(w/v) polyacrylamide preparative gels with only a single large sample well. This allows the enzyme subunits to migrate as one single band across the entire length of the gel. Once the protein subunits had been resolved, the gels were stained and destained as described

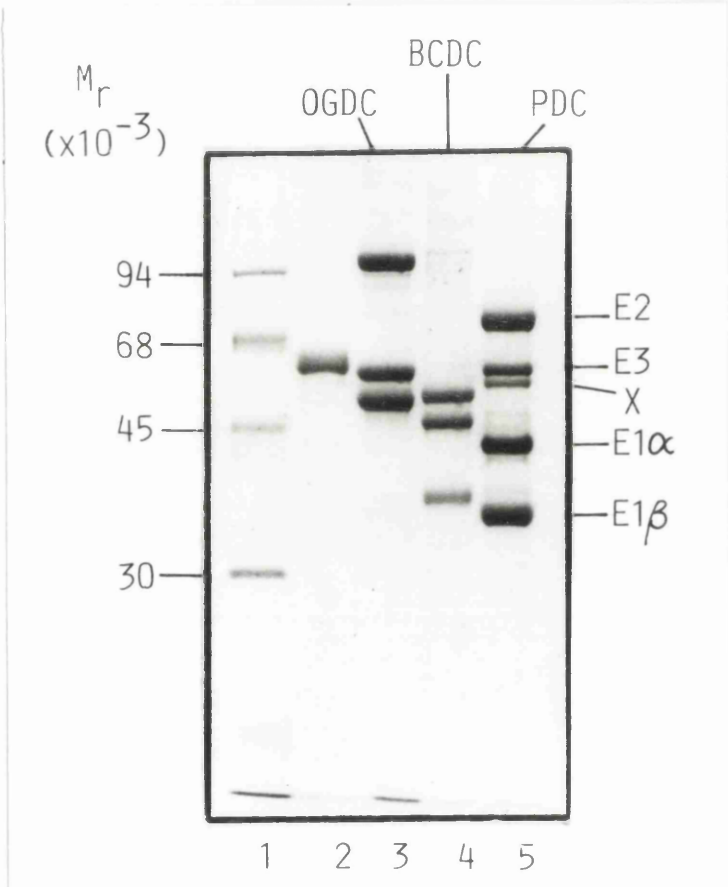
previously [Sections 2.2.3(c) and (d)]. The protein bands of interest were then carefully excised using a scalpel and rinsed with distilled water for 30 min after which the gel pieces were passed through 2ml disposable syringes to ensure fragmentation. The gel fragments were frozen in a dry ice/methanol bath and dried under vacuum overnight in the presence of sodium hydroxide pellets which helped to remove any acid still remaining from the destaining solution. The dried gel pieces were swollen in approx. 2-3ml of freshly prepared extraction buffer (50mM triethanolamine/1%(w/v)SDS/1mM DTT, pH 8.0) which was filtered through Millipore 0.22μm nitrocellulose filters (type GS) prior to use. The resulting suspension was heated for 5 min at 70°C and the protein extracted from the gel slurry by continuous stirring at room temperature for 16h. Gel fragments were removed by centrifugation at 1000g for 5 min after which the supernatant fraction, containing the extracted protein was removed. The remaining gel slurry was re-extracted and the supernatant obtained was pooled with the supernatent fraction collected from the first extraction. The pooled fractions were concentrated by freeze-drying, the dried samples re-dissolved in 1ml of distilled water. Protein was recovered by acetone precipitation (Section 2.2.2) and the pellets re-dissolved by boiling in 500μl 1%(w/v)SDS and stored at -20°C.

CHAPTER THREE

SIMILARITIES BETWEEN THE FAMILY OF E2 ACETYLTRANSFERASES AND THE 2-OXOGLUTARATE DEHYDROGENASE (E1) ENZYME OF BOVINE HEART OGDC

Fig. 3.1 Subunit profiles of the mammalian 2-oxoacid dehydrogenase complexes as examined by SDS/polyacrylamide gel electrophoresis

Samples of the three purified complexes were subjected to electrophoresis on a 10%(w/v) SDS/polyacrylamide gel and stained with Coomassie blue. Samples applied to each lane were as follows: lane 1, low M_r standards (10μg); lane 2, purified pig heart E3 (5μg); lane 3, bovine heart OGDC (15μg); lane 4, bovine kidney BCOADC (15μg) and lane 5, bovine heart PDC (15μg).


M_r
(x10^{-3})
OGDC
BCDC
PDC
94
68
45
30
E2
E3
X
E1α
E1β
1
2
3
4
5

3.1 INTRODUCTION

Each of the three main members of the family of 2-oxoacid dehydrogenase multienzyme complexes are known to be composed of three constituent enzymes E1, E2 and E3 which, acting in concert, are responsible for the decarboxylation of 2-oxoacids in the mitochondrial matrix. The complexes share similar structural features with the E2 enzymes binding together to form large oligomeric core structures around which the E1 and E3 enzymes arrange themselves (Reed, 1974). In addition to these three enzymes, PDC from eukaryotic sources such as bovine heart and *S. cerevisiae* has been shown to contain an extra polypeptide, termed component or protein X (De Marcucci and Lindsay 1985; Behal *et al.*, 1989). The absence of a comparable protein in both mammalian OGDC and BCOADC is surprising in view of similarities between the multienzyme complexes which all possess an identical E3 enzyme, particularly in view of the postulated role of protein X in assisting with the binding of E3 to the core structure (Neagle and Lindsay, 1991).

Evidence is presented in this chapter which suggests that OGDC may also contain protein X-like sequences; however, whereas in PDC these are present as a separate polypeptide species, in the OGDC, protein X like sequences are located at the N-terminus of the 2-oxoglutarate dehydrogenase (E1) enzyme.

3.2 RESULTS

3.2.1 ELECTROPHORETIC ANALYSIS OF THE 2-OXOACID DEHYDROGENASE MULTIENZYME COMPLEXES

The subunit structure of the 2-oxoacid dehydrogenase multienzyme complexes OGDC, PDC and BCOADC can be visualised by resolving the complexes using SDS/polyacrylamide gel electrophoresis as shown in Fig. 3.1. OGDC is composed of 3 polypeptide subunits of apparent M_r 96,000, 55,000 and 48,000 corresponding to

the E1, E3 and E2 enzymes respectively. PDC also contains the same 3 enzymes although with differing M_r values such that E2 has a M_r 70,000, E3, 55,000 and the E1 enzyme which is split forming a tetramer composed of E1α, M_r 41,000 and E1β, M_r 36,000. In addition to these enzymes PDC also contains a novel polypeptide termed protein X with M_r 51,000. The M_r values of the enzymes in BCOADC are E2, 52,000, E1α, 46,000 and E1β, 37,000 although from its gel profile, it is seen to lack the E3 enzyme. E3 is loosely associated with the BCOADC and is often lost on purification of the complex. The apparent loss of the E3 enzyme from this complex reflects the purification protocol used since methods do exist which allow for BCOADC to be purified containing endogenous E3 (Danner *et al.*, 1979; Heffelfinger *et al.*, 1983). In cases where the E3 enzyme is absent purified E3, usually from pig heart, must be added back to allow for measurement of the overall complex activity.

The M_r values of the integral polypeptides of these complexes as calculated from SDS/polyacrylamide gel electrophoresis are different to those deduced from the corresponding nucleotide sequence of these enzymes. Throughout the course of this thesis any references to the M_r values of the enzyme subunits of these complexes will be quoted as those determined from SDS/polyacrylamide gel electrophoresis unless otherwise indicated. Electrophoretic migration of the lipoyl bearing E2 chains in particular, is highly anomalous in the presence of SDS giving aberrantly high M_r values; this effect is attributed to lengthy alanine and proline rich sequences occurring within the regions which link the lipoyl domains together in the E2 domain structure (Guest *et al.*, 1985; Graham *et al.*, 1986).

3.2.2 N-TERMINAL SEQUENCE ANALYSIS OF THE E2 SUBUNITS OF BOVINE HEART OGDC AND PDC

The amino acid sequences of most of the constituent enzymes of the 2-oxoacid dehydrogenase complexes are now known either from cloning studies or by direct protein sequencing, usually from the N-terminus. The E1 enzyme of OGDC has been

Table 3.1 Comparison of the N-terminal amino acid sequences of the acyltransferases (E2) of the mammalian 2-oxoacid dehydrogenase complexes

N-terminal amino acid sequences were determined as outlined in the Methods with regions of sequence identity boxed as shown. An asterisk beside a sequence indicates that it was determined at Glasgow as opposed to being deduced from the published nucleotide sequence.

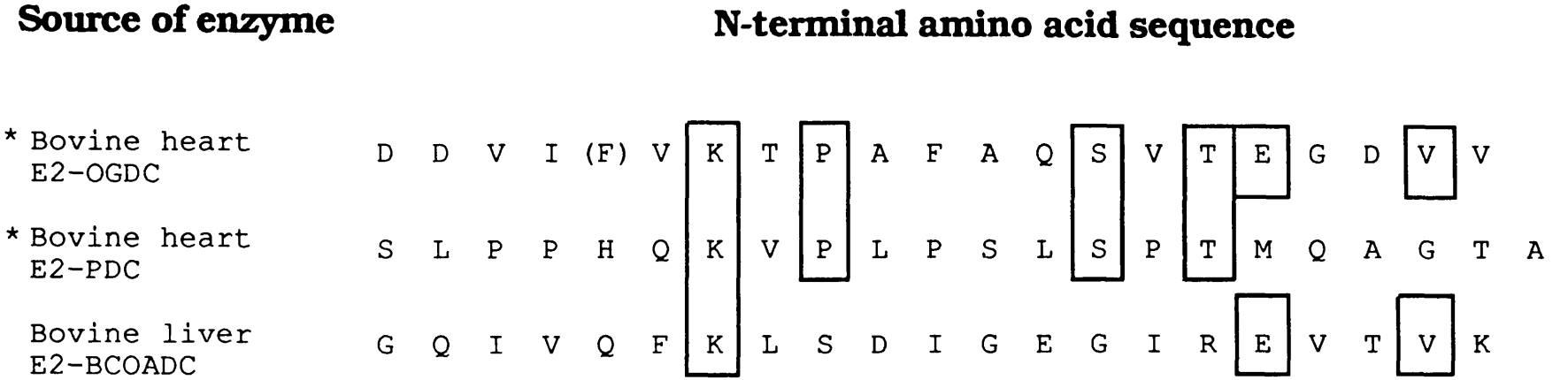
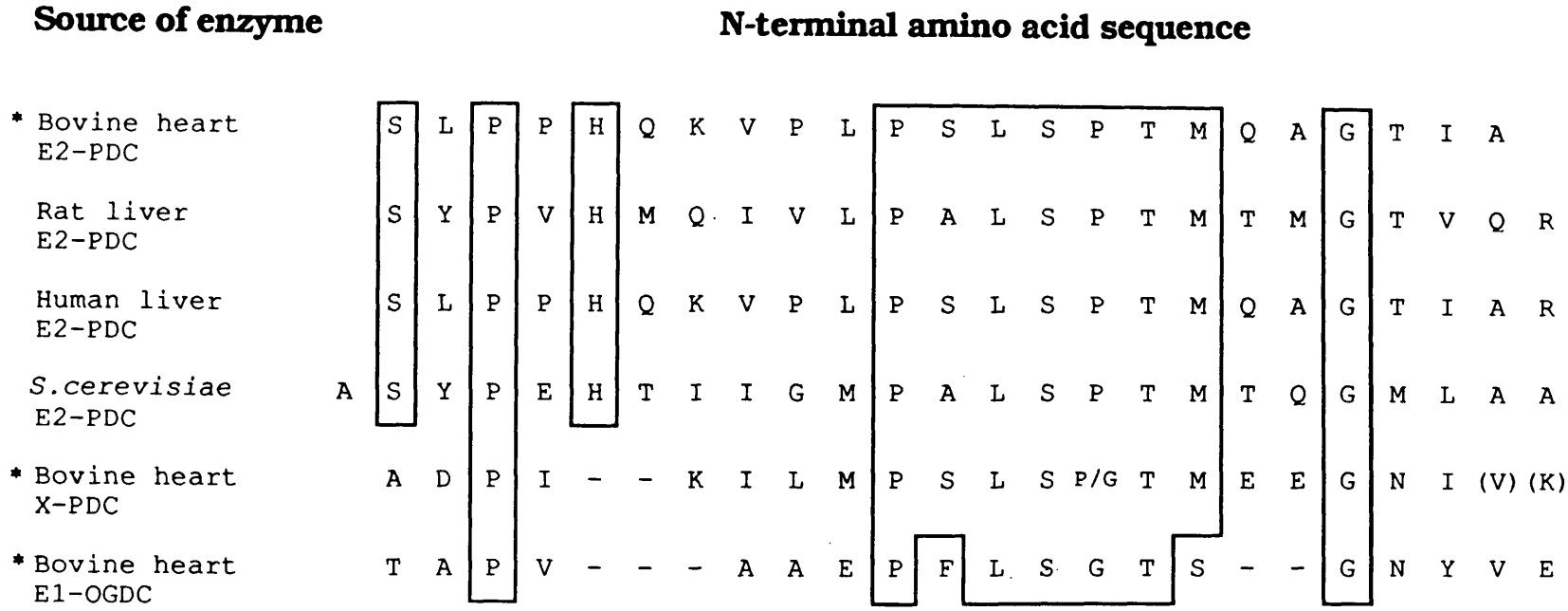

Source of enzyme
N-terminal amino acid sequence
* Bovine heart E2-OGDC
D D V I (F) V K T P A F A Q S V T E G D V V
* Bovine heart E2-PDC
S L P P H Q K V P L P S L S P T M Q A G T A
Bovine liver E2-BCOADC
G Q I V Q F K L S D I G E G I R E V T V K

Table 3.2 Comparative analysis of the N-terminal amino acid sequence of eukaryotic acetyltransferases (E2) with protein X and the 2-oxoglutarate dehydrogenase (E1) enzyme of bovine heart OGDC

N-terminal amino acid sequencing of proteins was performed as described in the Methods. Regions of sequence identity are boxed as shown were hyphens represent spacing to allow for the alignment of sequences: amino acid residues in parenthesis have only been identified tentatively. An asterisk beside a sequence indicates that it was determined at Glasgow in contrast to being deduced from the nucleotide sequence.


Source of enzyme
N-terminal amino acid sequence
* Bovine heart E2-PDC S L P P H Q K V P L P S L S P T M Q A G T I A
Rat liver E2-PDC S Y P V H M Q I V L P A L S P T M T M G T V Q R
Human liver E2-PDC S L P P H Q K V P L P S L S P T M Q A G T I A R
S.cerevisiae E2-PDC 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
* Bovine heart X-PDC A D P I - - K I L M P S L S P/G T M E E G N I (V) (K)
* Bovine heart E1-OGDC T A P V - - - A A E P F L S G T S - - G N Y V E

sequenced from various sources including *E. coli* (Darlison *et al.*, 1984), *B. subtilis* (Carlsson and Hederstedt, 1989), *S. cerevisiae* (Repetto and Tzagoloff, 1989) and human foetal liver (Koike *et al.*, 1992). An initial aim of this project was to sequence the E1 enzyme of OGDC from a mammalian source, since at the outset of this project, this had not yet been achieved. It was decided to sequence the E1 enzyme from bovine heart, such that the N-terminal sequence would be obtained and used in conjunction with internal amino acid sequence to produce oligonucleotide primers for use with the polymerase chain reaction (PCR). By using PCR in conjunction with these oligonucleotides, a probe would be produced for use in screening a λgt10 cDNA library to isolate a clone for the E1 enzyme of bovine heart OGDC.

Initial alignment of the primary structures of many proteins can be performed either by visual inspection or, by the use of specialised computer programmes which are capable of scanning for regions of sequence identity between particular protein sequences. In addition to sequence identity, conservative amino acid changes can also be taken into account which may act to increase the percentage similarity between sequences. Table 3.1 shows the N-terminal sequences of the acyltransferase (E2) enzymes as isolated from bovine heart and bovine liver. The overall observed similarity between the sequences is very low although a few residues do appear at common positions in each of the sequences; all of the acyltransferases contain a conserved lysine at position 7, whereas OGDC and PDC have additional sequence identity with proline at position 9, serine at position 14 and threonine at position 16. OGDC also exhibits sequence similarity to BCOADC with glutamic acid at position 17 and a valine at position 20. In all cases numbering is from the N-terminus towards the C-terminus. Although sequence identity is low, a number of conservative substitutions have also occurred between the sequences.

In contrast to the acyltransferases, comparison of corresponding sequences of the acetyltransferases (PDC E2) isolated from various sources including bovine heart, rat liver, human liver and *S. cerevisiae*, reveals the presence of regions of distinctive homology at the N-terminus as shown in Table 3.2. Features of this region include a

conserved N-terminal serine, a proline at position $^{3}/_{4}$ (position 4 with respect to the yeast sequence), a glycine at position $^{20}/_{21}$ (position 21 with respect to yeast) and the most distinctive feature, the sequence motif PSLS $^{P}/_{G}$TM. All of these sequence features are characteristic of the E2 lipoyl bearing domains of the acetyltransferase enzymes. Protein X shows a similar pattern of amino acid residues to those of the acetyltransferases in the N-terminal region except that it lacks the conserved histidine located at position $^{5}/_{6}$. It also possesses the sequence motif PSLS$^{P}/_{G}$TM in common with the acetyltransferases except for the substitution $^{P}/_{G}$ in position 5.

Table 3.2 also shows the N-terminal protein sequence obtained for the 2-oxoglutarate dehydrogenase (E1) enzyme of bovine heart OGDC. Observation of this sequence showed that significant similarity existed between OGDC E1, the E2 acetyltransferases and protein X. Comparison of the E1 sequence of bovine heart OGDC with those of the acetyltransferases revealed the presence of invariant proline and glycine residues at positions $^{3}/_{4}$ and $^{20}/_{21}$ respectively, although in common with protein X it lacked the N-terminal conserved serine and histidine residues which were present in the acetyltransferases. The 2-oxoglutarate dehydrogenase enzyme also contained a variation on the characteristic sequence PSLS$^{P}/_{G}$TM namely, PFLSGTS. Comparing limited N-terminal sequence for the E1 enzyme of OGDC with those of the E2 acetyltransferases, revealed the existence of sequence identity in the region of 30-40%; the highest homology between these sequences was observed with the bovine heart and rat liver E2 sequences, each having 37% sequence identity (7 identical residues) to the bovine heart E1 enzyme. This figure decreased to 32% (6 identical residues) when the sequence for E1 of OGDC was compared to both the human liver and *S. cerevisiae* E2 acetyltransferase sequences. The sequence which showed the greatest similarity to the N-terminus of the E1 OGDC sequence was protein X from *S. cerevisiae*; these sequences had 42% sequence identity (8 identical residues) to each other, although this figure increased to 47% depending on whether the valine residue at position 23 of the protein X sequence was taken into account. In all cases, the percentage identity was calculated by expressing the number of conserved identical

Fig. 3.2 Existence of cross-reactivity between protein X and the E1 enzyme of the mammalian 2-oxoglutarate dehydrogenase complex.

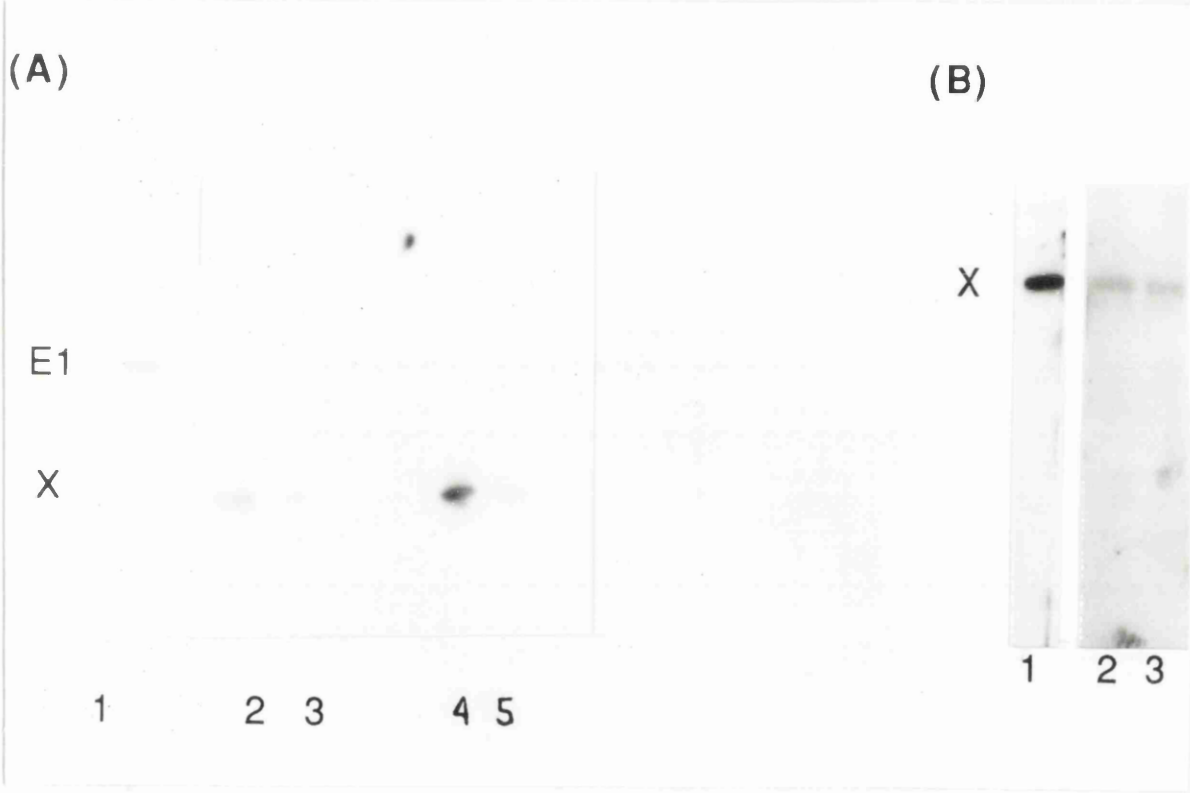
Proteins were resolved on 10%(w/v) SDS/polyacrylamide gels, transferred onto nitrocellulose membranes and challenged with antisera as described in the Methods.

Panel A: Lane 1, OGDC (2μg); lane 2, PDC (2μg); lane 3, PDC (1μg); lane 4, PDC (2μg); lane 5, PDC (1μg). Lanes 1-3 were challenged with the IgG fraction of antisera which had been raised to protein X (1:50 dilution). Lanes 4 and 5 were challenged with anti-(intact OGDC) serum (1:100 dilution).

Lanes 1,4 and 5 were exposed to film for approx. 10 days at -80°C; this was in contrast to lanes 2 and 3 which were exposed to film for 3 days only.

Panel B: Purified protein X was obtained by resolving bovine heart PDC on SDS/polyacrylamide preparative gels and eluting the polypeptide as outlined in the Methods.

Lane 1, PDC (1μg); lane 2, protein X (12μg); lane 3, protein X (8μg). Lane 1 was incubated with the IgG fraction of anti-X sera (1:50 dilution), lanes 2 and 3 were incubated with anti-(intact OGDC) serum (1:100 dilution).


(A)
E1
X
1
2 3
4 5
(B)
X
1
2 3

residues as a percentage of the total number of the E1 amino acid residues shown in Table 3.2.

In addition to considering sequence identity, conservative amino acid changes between the sequences were taken into account as described by Rice *et al.* (1984). Taking such changes into account did not significantly increase the percentage sequence similarity between the E1 enzyme of OGDC and the E2 acetyltransferase sequences; the only conservative amino acid change between the E1 enzyme of OGDC and protein X sequences, occurring where a valine residue in the E1 sequence was replaced by an isoleucine residue in the protein X sequence at position 4. This N-terminal sequence similarity exhibited by the E1 enzyme of bovine heart OGDC to the E2 acetyltransferases and protein X was entirely unexpected, and suggested that protein X-like sequences may exist on the E1 subunit of OGDC.

3.2.3 IMMUNOLOGICAL CROSS REACTIVITY BETWEEN PROTEIN X AND THE E1 ENZYME OF OGDC

Since protein X of PDC and the E1 enzyme of OGDC exhibit considerable sequence similarity at their N-termini, it is possible that further regions of homology may extend into their sequences. Any significant degree of similarity may be displayed as immunological cross-reactivity between the two enzymes.

Fig. 3.2 shows the results of cross-reactivity experiments in which bovine heart OGDC and PDC were resolved on SDS/polyacrylamide gels and then immunoblotted with antisera raised either to PDC, OGDC or protein X of PDC. Panel A shows the result of challenging OGDC with antisera specific for protein X of PDC (lane 1); a band corresponding to protein X of PDC was observed in this track as verified by comparison with control lanes 2 and 3, in which PDC was loaded and challenged with anti-X sera. This result is explained by the fact that purified OGDC is often contaminated with residual amounts of PDC, representing 1-5% of total protein in most preparations. In addition to cross reacting with the contaminating protein X of

PDC, a weak but specific cross reaction between the anti-X serum and the E1 enzyme of OGDC was observed in lane 1. The identity of this band was confirmed by comparison with a control lane in which intact OGDC was challenged with anti-(E1 OGDC) subunit specific serum (not shown). Lanes 4 and 5 in Fig. 3.2 show that on challenging PDC with antisera raised to intact OGDC, a weak cross-reacting band corresponding to protein X was identified. That this cross-reaction was not due to contamination of PDC with OGDC, was shown by the absence of any corresponding reaction with the E1 and E2 enzymes of OGDC; the cross-reacting species is also unlikely to be E3, as this particular subunit is known to have a low titre when challenged with earlier bleeds of anti-(intact OGDC) serum as in this case (De Marcucci *et al.*, 1985).

The possibility remained, however, that protein X was not the true identity of the cross-reacting species seen in lanes 4 and 5 when PDC was challenged with anti-(intact OGDC)serum. In order to incubate transferred protein on the same immunoblot with different antisera, it is necessary to cut the nitrocellulose into strips; this necessitates that the individual strips be re-aligned before exposure to the photographic film. Care was taken in the cutting and re-alignment of the nitrocellulose strips; however, a small mis-alignment of the nitrocellulose strips could result in mis-identification of the protein X (M_r 51,000) and E3 (M_r 55,000) protein species. This ambiguity was removed by resolving PDC on SDS/polyacrylamide preparative gels and selectively eluting the protein X band as described in the Methods (Section 2.2.11). In panel B of Fig. 3.2, lane 1 represents the control track in which PDC was challenged with antisera raised to protein X, thus demonstrating the monospecific nature of the anti-X sera with respect to the other subunits of this complex; lanes 2 and 3 shows gel purified protein X immunoblotted with anti-(intact OGDC) serum. A corresponding cross-reaction was observed to occur between protein X and the anti-(intact OGDC) serum. Immunological data demonstrate therefore, that a significant, albeit weak, cross reactivity can be observed between the E1 enzyme of bovine heart OGDC and protein X of mammalian PDC. It would appear that epitopes present on

Fig. 3.3 SDS/polyacrylamide gel analysis of tryptic digested bovine heart OGDC

Bovine heart OGDC was diluted with 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6 and treated with 0.01% (w/w) trypsin at 30°C. At the times indicated, aliquots (15μg) were removed into Laemmli sample buffer and resolved by electrophoresis on a 10%(w/v) SDS/polyacrylamide gel.

Lane M - low M_r marker proteins (10μg).

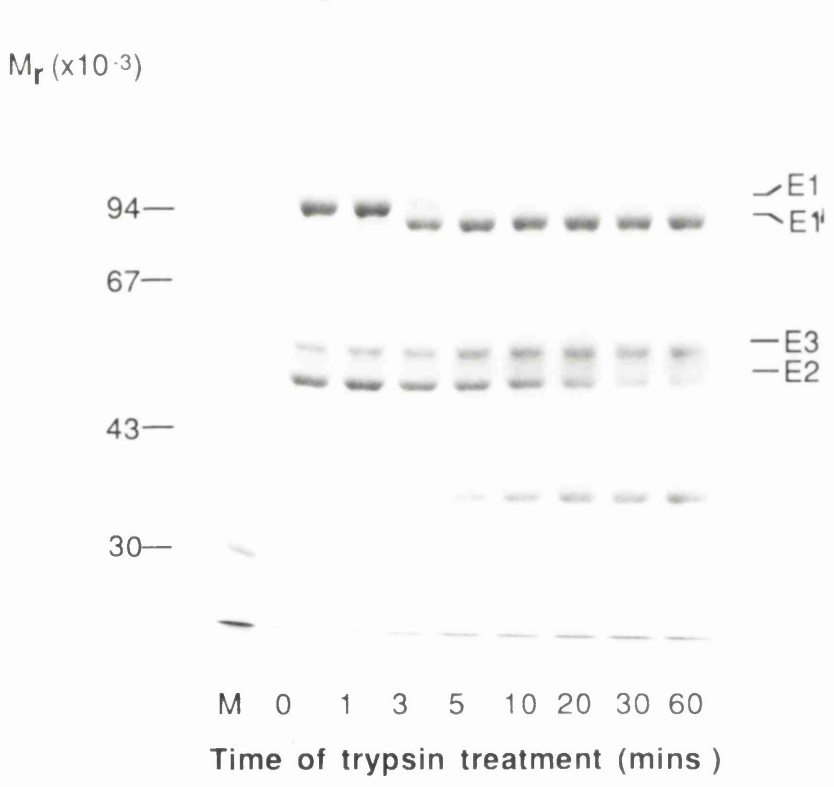
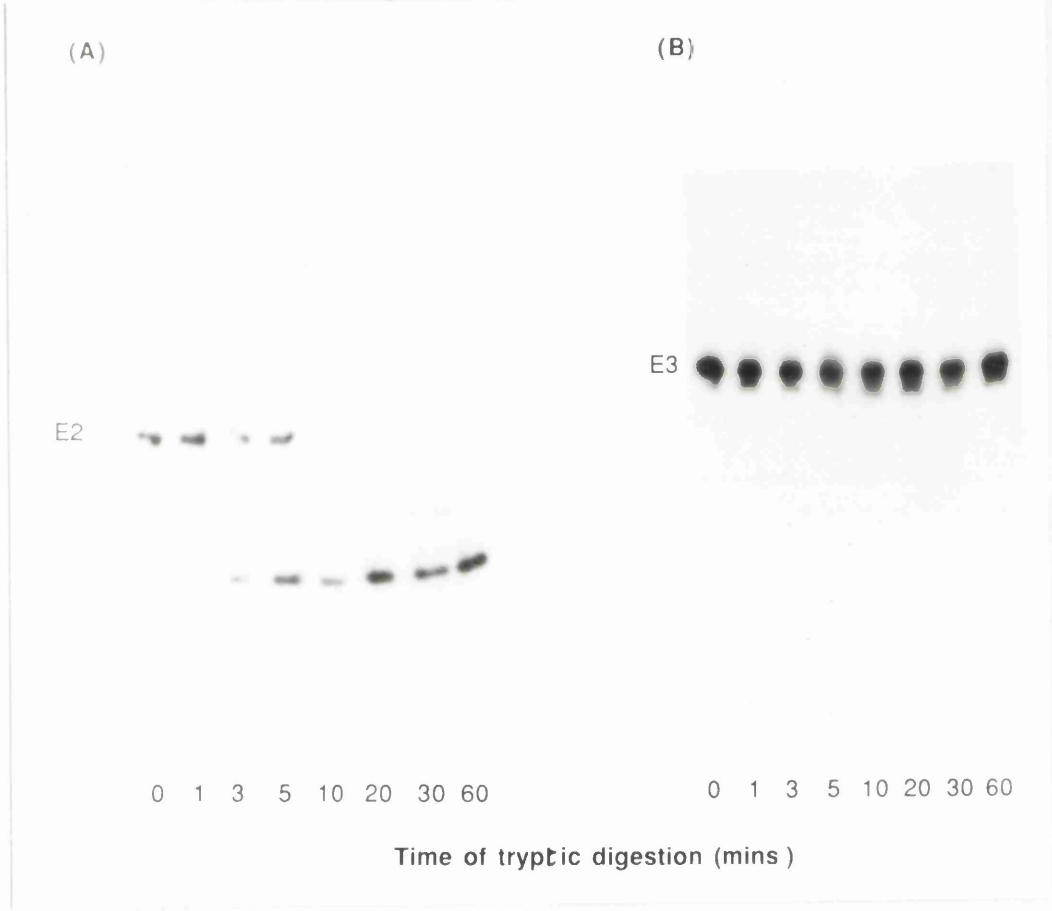

M_r (x10^{-3})
94
67
43
30
E1
E1'
E3
E2
M 0 1 3 5 10 20 30 60
Time of trypsin treatment (mins)

Fig. 3.4 Immunological detection of peptides derived from the dihydrolipoamide succinyltransferase (E2) and the dihydrolipoamide dehydrogenase (E3) enzymes after proteolysis of bovine heart OGDC with trypsin

Bovine heart OGDC was incubated at 30°C in 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6. 0.01%(w/w) trypsin was added and at the indicated time points aliquots (15μg) were removed into Laemmli sample buffer and boiled for 2-3 min. Samples (1μg) were resolved on 10%(w/v) SDS/polyacrylamide gels and immunoblotted as described in the Methods.

Panel A: products of OGDC tryptic digestion probed with anti-(E2 OGDC) serum (1:100 dilution).

Panel B: OGDC treated with trypsin challenged with anti-E3 serum (1:100 dilution).


(A)
(B)
E3
E2
0 1 3 5 10 20 30 60
0 1 3 5 10 20 30 60
Time of tryptic digestion (mins)

protein X are recognised by anti-(intact OGDC) serum however, in later experiments protein X was found not to cross react wth anti-(E1 OGDC) serum (not shown). These differences in cross-reactivity as exhibited by anti-(intact OGDC) and anti-(E1 OGDC) sera, may be related to differences in their titre which in some cases can vary 10-100 fold as determined by enzyme linked immunoabsorption assay (ELISA).

3.2.4 LIMITED TRYPTIC PROTEOLYSIS OF BOVINE HEART OGDC

OGDC from bovine heart has a distinctive and reproducible profile of digestion when incubated with trypsin. As illustrated in Fig. 3.3, it is apparent that E1 is the most proteolytically sensitive enzyme of the complex, being degraded within 3 min to form a stable, lower M_r species (approx. M_r 100,000) which is designated E1^l. In contrast to E1, the E2 enzyme of OGDC is not degraded to any appreciable extent until later in the time course when two peptides with M_r values 35,000 and 15,000 are formed. The M_r 15,000 species is not readily resolved on 10%(w/v) SDS/polyacrylamide gels (Fig. 3.3) as it tends to co-migrate with the dye front under these conditions. This M_r 15,000 peptide corresponds to the N-terminal lipoyl domain on E2, cleaved as a result of tryptic digestion. In cases where SDS/polyacrylamide gels containing higher percentages of acrylamide were run in an attempt to visualise this band, it proved difficult to detect, tending to stain very poorly with Coomassie blue. In contrast to the E1 and E2 enzymes of the complex, the E3 enzyme was extremely resistant to proteolytic degradation, remaining virtually intact under the conditions used in these experiments.

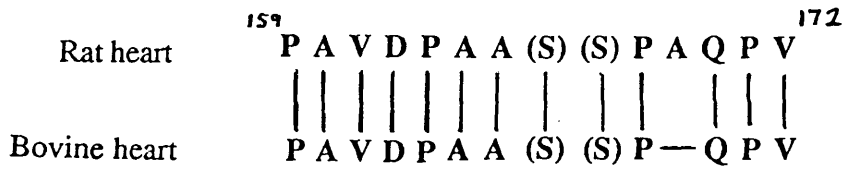
The origin of the M_r 35,000 band resulting from tryptic digestion was assessed by immunoblotting digested OGDC with monospecific antisera raised to the individual subunits of the enzyme complex (Fig. 3.4). Panel A shows the immunoblot obtained when trypsin treated OGDC was probed with anti-dihydrolipoamide succinyltransferase (E2) specific antiserum. The lower M_r species in panel A corresponds to a M_r 35,000 peptide which is formed as a direct result of digestion of

Table 3.3 Amino acid sequence of the M_r 35,000 peptide resulting from tryptic digestion of the E2 enyme of bovine heart OGDC

OGDC was diluted in 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6, and treated with 0.01%(w/w) trypsin for 60 min. Samples (5μg) were resolved on mini-protein sequencing gels and blotted onto Immobilon PVDF membrane as described in the Methods. The M_r 35,000 species of interest was excised from the membrane and sequenced on an Applied Biosystems 477A protein sequencer, with on-line 120A phenylthiohydantoin derivatives analyser using the manufacturer's BLOTT 4 programme. Amino acid residues in parenthesis have only been identified provisionally and those residue positions which are represented by a dash were unable to be conclusively determined.

The bovine heart E2 protein sequence obtained was aligned with sequence from the linker region of the rat heart E2 enzyme (Nakano *et al.*, 1991).

OGDC E2


159
172
Rat heart
P A V D P A A (S) (S) P A Q P V
Bovine heart
P A V D P A A (S) (S) P — Q P V

the E2 enzyme; it cross-reacts with the anti-(E2 OGDC) serum and its appearance closely corresponds to the disappearance of the E2 enzyme; as before, under these conditions the lower M_r 15,000 species appeared not to be resolved. The lipoyl bearing domains of these proteins are generally considered to be highly antigenic however, analysis by Lau *et al.* (1988) of the antigenicity associated with BCOADC E2 demonstrated that it is not the lipoyl domains which are immunogenic, but the highly flexible hinge regions linking the lipoyl domains to the catalytic core which are responsible for the antigenicity exhibited by these enzymes. It is possible therefore, that the M_r 15,000 peptide representing the cleaved lipoyl domain may have been resolved, but does not show a high level of immunogenicity owing to the absence of the linker domain region. In the same experiment, OGDC incubated with trypsin was immunoblotted with anti-E3 serum (Fig. 3.4 panel B); no immunologically-detectable proteolytic fragments were observed as a result of trypsin treatment, indicating that the E3 enzyme is not degraded to any significant extent under these conditions.

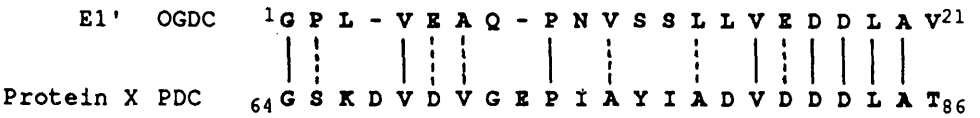
N-terminal amino acid sequence of the M_r 35,000 peptide species formed on tryptic treatment of OGDC was obtained and is shown in Table 3.3. Although only eighteen amino acid residues in length, it is apparent that this stretch of sequence is rich in both alanine and proline residues. This type of sequence is characteristic of the linker regions found within the E2 domain structure, an important feature necessary for the conformational flexibility associated with the catalytic activity of the complex. Analysis of the amino acid sequence as deduced from the recently cloned rat OGDC E2 enzyme (Nakano *et al.*, 1991) reveals the presence of a high proportion of alanine and proline residues in the region which links the lipoyl domain to the inner catalytic domain. Thus, tryptic digestion of the E2 enzyme of bovine heart OGDC results in two distinct species being formed; an M_r 15,000 peptide corresponding to the lipoyl domain which is cleaved from the N-terminus of the enzyme, and an M_r 35,000 species whichcontains both the flexible linker domain and the acyltransferase active site region.

Table 3.4 Comparison of the N-terminal sequence of the $E1^{l}$ species from bovine heart OGDC with protein X of *S. cerevisiae*

Bovine heart OGDC was diluted in 0.2mM TPP/2mM $MgCl_2$/3mM NAD^+/50mM potassium phosphate buffer pH 7.6, and digested with 0.05%(w/w) trypsin for 30 min. Samples (5μg) were resolved on a 10%(w/v) SDS/polyacrylamide sequencing gel and were subsequently blotted onto Immobilon PVDF membrane. The band corresponding to the lower M_r E^{l} species resulting from tryptic digestion of E1 was cut from the membrane, its N-terminal sequence obtained as described in the Methods. The amino acid sequence of the $E1^{l}$ species of OGDC, was compared to that of protein X from *S. cerevisiae* as deduced from the published nucleotide sequence.

Bold lines between residues are indicative of sequence identity in contrast to dashed lines which represent conservative amino acid changes.

(A)


E1' OGDC 1G P L - V E A Q - P N V S S L L V E D D L A V21
Protein X PDC 64G S K D V D V G E P I A Y I A D V D D D L A T86

(B)


E1' OGDC 1G P L V E A Q P N V S S L L V E D D L A V21
Protein X PDC 144V S L L L A E N N I S K Q K A L K E I A P164

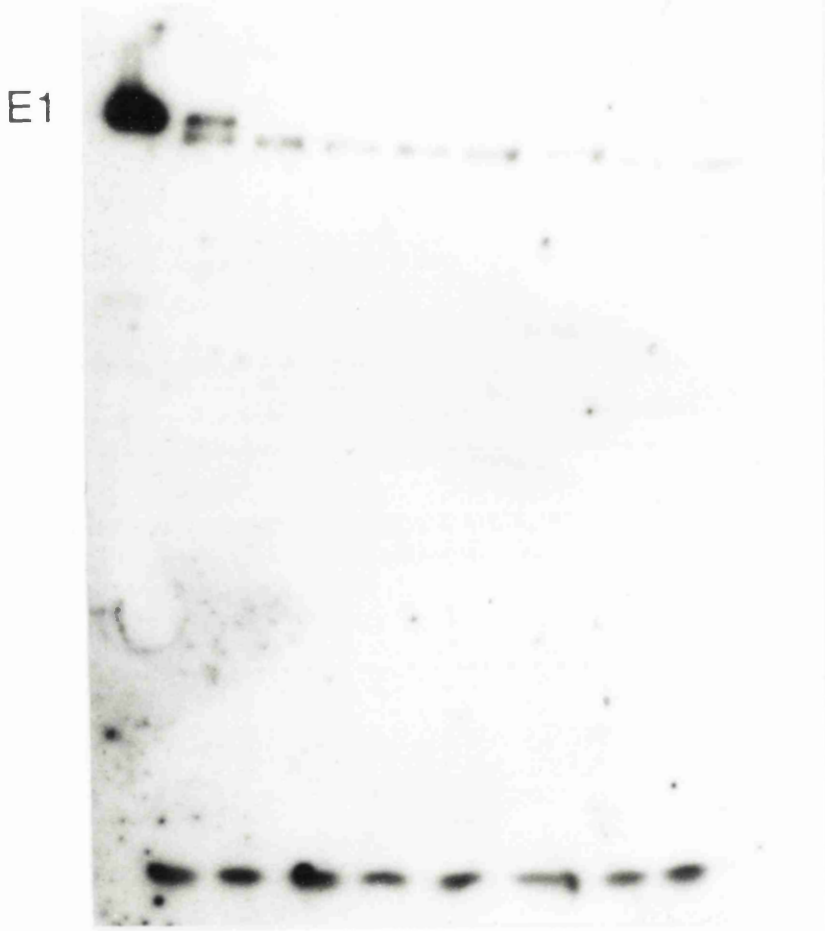
3.2.5 IMMUNOLOGICAL AND SEQUENCE ANALYSIS OF THE PEPTIDES RESULTING FROM CLEAVAGE OF THE E1 ENZYME OF MAMMALIAN OGDC

Digestion of OGDC with trypsin revealed that the E2 enzyme of the complex undergoes digestion in a manner similar to that of the acetyltransferases and protein X, with cleavage occurring within the flexible linker domain so causing removal of the lipoyl domain located at the N-terminus. Trypsin treatment of E1 resulted in its rapid cleavage to form the so called $E1^{l}$ species. It was necessary to know if E1 was being cleaved in a manner similar to that of protein X and the E2 acyltransferases, so as to determine if the peptide cleaved from E1 was formed as a result of N-terminal cleavage, analogous to removal of the lipoyl domain region. Thus, OGDC was degraded with trypsin to produce the stable $E1^{l}$ species which was then sequenced from its N-terminus. The results obtained are shown in Table 3.4.

It is evident from the results that when the N-terminal sequence of the $E1^{l}$ fragment is compared to the sequence obtained previously for E1 of OGDC, the sequences are quite distinct. This signifies that the $E1^{l}$ species is formed as a result of removal of the protein X-like sequence from the N-terminus of the E1 enzyme. In addition to undergoing N-terminal cleavage in a manner similar to protein X, the $E1^{l}$ sequence shows some degree of similarity to protein X from *S. cerevisiae*. The predicted amino acid sequence of protein X was deduced from the nucleotide sequence of the cloned gene (Behal *et al.*, 1989). When the similarity between E1 of OGDC and protein X is considered, the region of protein X to which $E1^{l}$ maps is dependent on the method used to match them. Table 3.4 shows the outcome of matching OGDC $E1^{l}$ to protein X of *S. cerevisiae* by eye (panel A) or by using a computer search programme such as SWISSPROT (panel B); both methods result in different regions of protein X being aligned with the $E1^{l}$ sequence. These searches were performed prior to publication of the entire amino acid sequence for the human OGDC E1 enzyme as deduced by Koike *et al.* (1992). For alignment by eye (panel A), $E1^{l}$ of OGDC was matched to residues 64-86 of the protein X sequence. This contrasts with the

Fig. 3.5 Immune blotting analysis of the extent of 2-oxoglutarate dehydrogenase (E1) enzyme degradation during digestion of OGDC

OGDC diluted with 3mM NAD^+/ 2mM $MgCl_2$/ 0.2mM TPP/ 50mM potassium phosphate buffer pH 7.6, was treated with 0.1%(w/w) trypsin at 30°C for the times shown. Samples (1μg) were resolved on a 12.5%(w/v) SDS/polyacrylamide gel after which proteins were transferred electrophoretically onto nitrocellulose paper for incubation with anti-(E1 OGDC) serum (1:100 dilution). Bound antibodies were detected by autoradiography after incubation with [^{125}I]-labelled protein A as described in the Methods.


E1
0 1 5 10 20 30 40 50 60
Time of tryptic digestion (mins)

computer search alignment (panel B) which recognised sequence similarity between residues 144-164. Both matches are closely comparable in the number of identical and conserved residues which they have between them; However, the match between $E1^{l}$ and residues 144-164 of the protein X sequence may be slightly more favourable as, unlike the visual match, no gaps were introduced into the $E1^{l}$ sequence to aid alignment. The significance of this result will be dealt with in greater detail in the final discussion chapter. However, the above results confirm that sequence similarity between protein X and the $E1^{l}$ species is not confined solely to the N-terminus.

Tryptic digestion of OGDC results in release of the N-terminal region of the E1 enzyme in a manner similar to the cleavage of lipoyl domains from either the acetyltransferases or protein X of PDC. As shown in Fig. 3.5, immunoblotting of tryptic digested OGDC with anti-(E1 OGDC) serum results in a very strong and specific cross-reaction. On digestion of E1 to form $E1^{l}$, the antigenicity of $E1^{l}$ is greatly reduced with respect to the undigested E1 control. The major immune response is now exhibited by the small peptide formed as a result of N-terminal cleavage of the E1 enzyme, which runs near the dye front. This small peptide elicits a high proportion of the total immune response against the original E1 subunit, even though it represents less than 10% of the overall sequence when compared to the entire length of the E1 enzyme. The high immunogenicity displayed by this peptide is very similar to that observed with lipoyl domain regions and their linker regions which dominate the antigenic response to the native undegraded E2 enzyme (Kresze *et al.*, 1980).

Lipoyl domains commonly show M_r values of approx. 15,000 when resolved by SDS/polyacrylamide gel electrophoresis. An accurate size determination of the N-terminal cleaved peptide was necessary, to allow a comparison to be made between it and the lipoyl domain on the basis of their M_r values. OGDC was digested using trypsin for a sufficient time to ensure that the E1 enzyme was fully converted to $E1^{l}$ and that this small N-terminal peptide was formed. The digested sample was then resolved on both 12.5%(w/v) and 15%(w/v) SDS/polyacrylamide gels (not shown) in

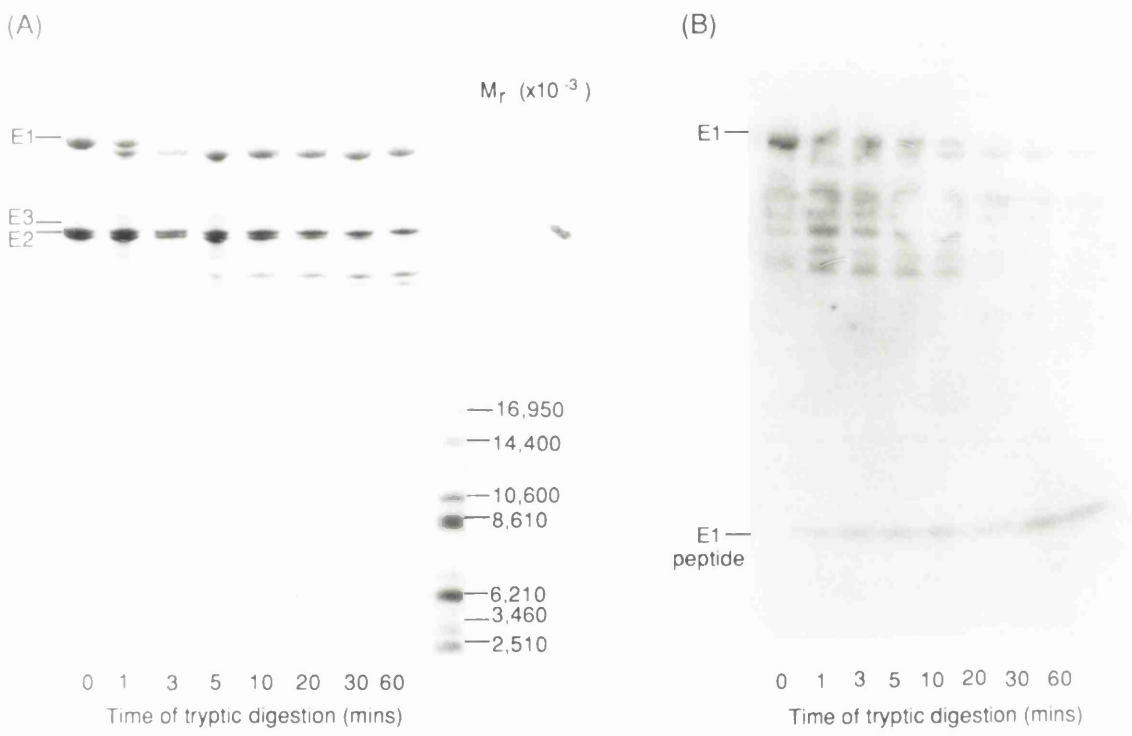
Fig. 3.6 Determination of M_r of peptide species cleaved from the N-terminus of the 2-oxoglutarate (E1) enzyme during tryptic digestion

OGDC from bovine heart was diluted with 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6, and digested with 0.1%(w/w) trypsin for times indicated. Samples were resolved on tricine-SDS/polyacrylamide gels (Section 2.2.4) after which the slab gel was cut in half: one half was stained with Coomassie blue (Panel A), the other half immunoblotted with subunit specific antisera (Panel B).

Panel A: samples of tryptic digested OGDC (20μg per track) were stained with Coomassie blue to allow visualisation of protein.

Lane M - marker proteins for M_r range determinations between 16,950 - 2,510 (10μg)

Panel B: resolved protein (5μg per lane), was transferred electrophoretically onto nitrocellulose membrane and blotted with anti-(E1 OGDC) serum (1:100 dilution).


(A)
M_r (x10^{-3})
E1
E3
E2
16,950
14,400
10,600
8,610
6,210
3,460
2,510
0 1 3 5 10 20 30 60
Time of tryptic digestion (mins)
(B)
E1
E1 peptide
0 1 3 5 10 20 30 60
Time of tryptic digestion (mins)

an attempt to resolve the peptide and calculate its M_r by comparison with standard marker proteins on the same gel. Difficulties were experienced when the peptide was resolved and the gels treated with either Coomassie blue dye or silver stain, since there was negligible staining of this low M_r peptide.

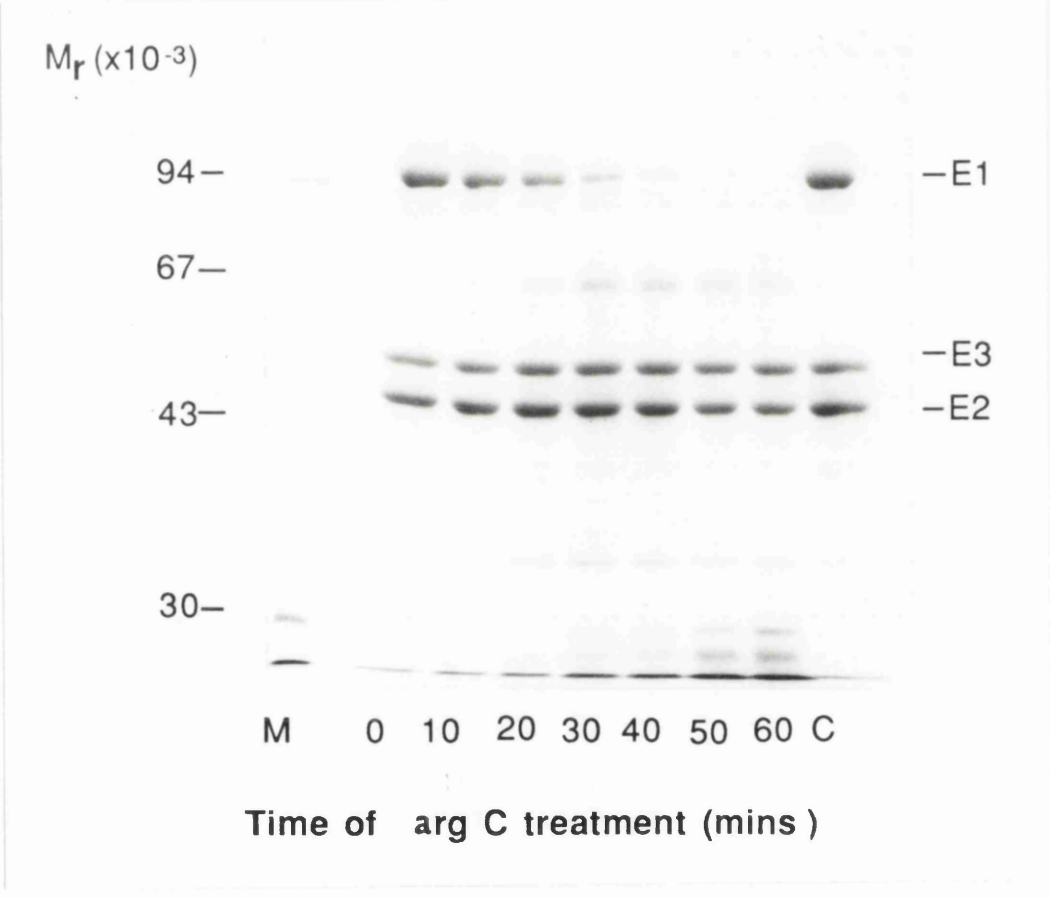
An alternative strategy was developed whereby tryptic digested OGDC was resolved on tricine-SDS/polyacrylamide gels (Section 2.2.4), the protein transferred onto nitrocellulose membrane and challenged with anti-(E1 OGDC) serum. The results of such an experiment are shown in Fig. 3.6. When tricine is used as the trailing ion, this allows for the resolution of small peptides at lower acrylamide concentrations than on glycine SDS/polyacrylamide gel electrophoresis (Schägger and Von Jagow, 1987). Panel A shows one half of the gel stained with Coomassie blue, panel B the other half blotted with anti-(E1 OGDC). From the Coomassie blue stained gel the small N-terminal cleavage product is not apparent; however, its presence can be detected on the immunoblot probed with anti-(E1 OGDC) serum. Although the anti-(E1 OGDC) antisera is specific for the small N-terminal peptide which appears to run near the bottom of the resolving gel, a large amount of cross-reactivity with this antiserum is seen in the top portion of the gel within the the region of the spacer gel. The tricine-SDS/polyacrylamide gel system is capable of separating complex mixtures of proteins to a high degree of resolution. It is possible that a small amount of non-specific E1 enzyme degradation has occurred during storage of the enzyme as the same non-specific cross-reaction with anti-(E1 OGDC) serum is also observed in the undigested control sample. On their resolution, the E1 degradation products were detected by immunoblotting, an extremely sensitive method capable of detecting amounts of protein as low as 0.1μg. The level of the anti-(E1 OGDC) cross-reaction within the spacer gel is seen to decrease during the time course of tryptic digestion, this is probably as a result of the original degradation products being cleaved to form smaller peptides which are no longer recognised by the antiserum. Direct comparison with the low M_r marker proteins on the Coomassie blue stained gel gave an approx. M_r 8,000 to the small N-terminal peptide. The calculated M_r of this peptide species is

Fig. 3.7 Treatment of bovine heart OGDC with protease arg C.

OGDC diluted in 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6, was digested with 9%(w/w) protease arg C for 1h at 30°C. The protease was added in 3 aliquots of 3%(w/w) at times 0 min, 20 min and 40 min respectively. Samples (15μg) were removed at times shown with the samples at 20 min and 40 min being taken before addition of the protease. Digested protein was then resolved on a 10%(w/v) SDS/polyacrylamide gel and stained with Coomassie blue.

Track M - M_r marker proteins (10μg).

Track C - Control OGDC i.e untreated OGDC incubated at 30°C for the duration of the experiment.


M_r (x10^{-3})
94
67
43
30
E1
E3
E2
M
0
10
20
30
40
50
60
C
Time of arg C treatment (mins)

lower than that expected for a lipoyl domain, although it may be postulated that this species represents a modified form of the domain. No evidence exists for the presence of a linker region associated with this peptide which exhibits a large immunogenic response when chalenged with anti-(E1 OGDC) serum.

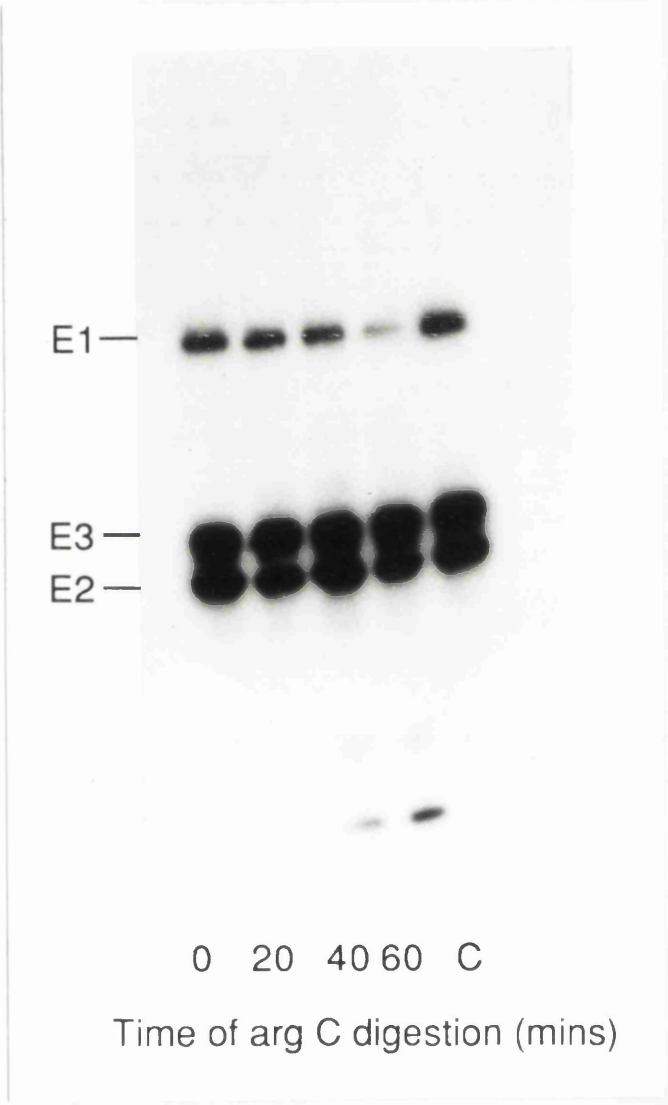
3.2.6 TREATMENT OF BOVINE HEART OGDC WITH PROTEASE ARG C

Bovine heart OGDC was also digested with the more specific protease arg C to determine if E1 showed a similar digestion profile with this protease as compared with trypsin. From the results shown in Fig. 3.7 it can be seen that although the E1 enzyme was degraded extensively resulting in the production of a variety of low M_r peptides, there was no detectable cleavage of either the E2 or E3 enzyme subunits. Extensive degradation of the E1 enzyme was not accompanied by formation of the stable $E1^l$ species as was observed with tryptic digestion. It seems unusual that a more specific proteolytic enzyme such as protease arg C which cleaves arg-arg bonds, is capable of totally digesting the E1 enzyme while trypsin, which cleaves peptide bonds at the C-terminal side of both arginine and lysine residues, causes a more specific cleavage of the E1 enzyme. It has been reported in the literature that protease arg C, in addition to cleaving arg-arg bonds, is also capable of cleaving lys-lys bonds (Chartier *et al.*, 1989) and lys-arg bonds (Bousfield and Ward, 1988). It would appear from this result that protease arg C may be capable of cleaving the E1 enzyme at several different sites at similar rates while lower levels of trypsin causes a rapid and very specific cleavage at a highly susceptible site. The possibility may also exist that proteolysis of E1 is promoted by a minor contaminating activity present in commercial preparations of protease arg C; this is unlikely as all batches of protease tested were found to cleave protein X of bovine heart PDC at two specific sites only, to produce a M_r 35,000 peptide, corresponding to the inner domain fragment which was further degraded to a M_r 30,000 species and a M_r 15,000 lipoyl domain peptide (J. Neagle and S. Sanderson, unpublished results).

Fig. 3.8 Immune replica analysis of mammalian OGDC with monospecific subunit antisera after digestion of complex with protease arg C.

OGDC was diluted in 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6. 9%(w/w) protease arg C was added in 3 equal aliquots at times 0 min, 20 min and 40 min respectively, samples (15μg) were removed prior to the addition of protease. Samples (1μg) were resolved on a 10%(w/v) SDS/polyacrylamide gel and after transferring onto nitrocellulose, the bound protein was incubated with anti-(E1 OGDC) serum (1:100 dilution). After the immunoblot had been exposed to photographic film for several days at -80°C, the nitrocellulose was removed from the film cassette and incubated in wash buffer [Section 2.2.6(a)] for 1h to remove the bound [^{125}I]-labelled protein A. Nitrocellulose was then re-probed with anti-(E2 OGDC) serum (1:100 dilution). This process was repeated once more when the same blot was re-challenged with anti-E3 serum (1:100) dilution.

Track C - Control OGDC (1μg) incubated at 30°C for the duration of the experiment.


E1
E3
E2
0 20 40 60 C
Time of arg C digestion (mins)

Immunoblotting of protease arg C digested OGDC (Fig. 3.8) further confirms that the E1 enzyme is extensively degraded in contrast to the E2 and E3 enzymes which are unaffected. The low M_r cross-reacting peptides seen at later time intervals of digestion were produced as a direct result of E1 enzyme degradation. This result differs from trypsin which cleaves the E2 enzyme forming the two peptide species described previously. This would suggest that, either the cleavage site present in the flexible linker domain of the E2 enzyme is specific for trypsin only or, that the same site is not susceptible to digestion by protease arg C.

3.3 DISCUSSION

In contrast to the individual acyltransferase (E2) enzymes of the family of 2-oxoacid dehydrogenase complexes which show little homology to each other in their N-terminal regions, the acetyltransferase enzymes of PDC exhibit sequence similarities at their N-termini which are characteristic of lipoyl bearing domains, in particular, the conserved amino acid sequence motif $P^S/_A$LSPTM which occurs approx. 30 amino acids before the lipoylated lysine residue in human E2. A major new piece of evidence is presented in this chapter relating to the discovery of an E2 acetyltransferase lipoyl like domain on the 2-oxoglutarate (E1) enzyme of mammalian OGDC, the region of sequence similarity located at the N-terminus; E1 was also shown to possess sequence similarity to protein X of bovine heart PDC in the region of their N-termini.

When considering if two sequences show significant similarity to each other, the number of identical or conservatively substituted amino acids which the two sequences have in common are taken into account. On analysis, the percentage identity existing between any two sequences which is considered to be the so called 'twilight zone' or lower limit of significant identity, is approx. 20% especially if gaps are inserted into the sequence to allow for optimisation of alignment. Matching of the N-terminal regions of both the OGDC E1 and protein X species gave an average percentage

identity in the range 42-47%, although gaps were introduced to allow for matching of the sequences. This value, which is significant and clearly above the lower limit of percentage identity, confirms that real sequence similarity does exist between these two proteins at their N-termini. Similarly, the percentage sequence similarity between OGDC E1 and the E2 acetyltransferases in the range 30-40%, demonstrates that significant sequence similarity also exists between these proteins at their N-terminal regions.

Correlations have been observed between the degree of immunological cross-reactivity and the degree of sequence homology existing between sequences, with values of approx. 60% sequence identity being necessary before an immunological cross-reaction is observed. This assumption is based on experiments performed by Prager and Wilson (1971a, b) who tried to examine in a quantitative manner, the relationship between immunological cross-reactivity in a family of lysosomal proteins with respect to their sequence homology. The immunological techniques used in these determinations such as immunodiffusion, precipitin analysis and micro-complement fixation studies, are known to be insensitive and inaccurate, so that the results obtained in studies of this type may not be entirely correct.

From the observed results E1 of OGDC was found to be capable of cross-reacting with antiserum raised to protein X. In addition, protein X exhibited cross-reactivity with anti-(intact OGDC). These results disagree with the assumptions of Prager and Wilson since E1 of OGDC and protein X do not possess 60% sequence similarity to each other, as can be verified by the computer matching of their sequences (not shown). It is perhaps too simplistic to relate the extent of cross-reactivity to sequence similarity except in cases were a high degree of sequence conservation exists. In the case of the dihydrolipoamide dehydrogenase (E3) enzyme which is a common component of the 2-oxoacid dehydrogenase complexes, the low level immunogenicity observed with this protein is attributed to the high degree of conservation of its primary structure and hence its tertiary structure during evolution (De Marcucci *et al.*, 1985). Cross-reactivity may not be solely dependent on sequence similarity but may

also be related to the conformation which the protein adopts therefore, although polypeptides may exhibit relatively low levels of sequence similarity to each other, they may assume similar three-dimensional structural epitopes allowing for cross-reactivity. Thus, for protein X and E1 of OGDC, sequence similarity and structural characteristics common to both of these polypeptides must be present on account of the observed cross-reactivity.

Cases have been reported were antisera raised to protein X has recognised the E2 enzyme (Jilka *et al.*, 1986), although it is possible to produce monospecific antiserum to protein X (De Marcucci and Lindsay, 1985). These conflicting results probably reflect differences in antisera production, since the epitopes which are responsible for eliciting antibodies in an individual animal depends on various host factors such as the immunoglobulin gene repertoire, the method of antigen presentation and sometimes even the regime used to immunise the animal.

Bovine heart OGDC has been shown to undergo a very specific pattern of degradation when subjected to limited proteolysis with trypsin. The E1 enzyme which appears to be the most proteolytically sensitive subunit of the complex, is cleaved by trypsin to form a lower M_r species which is termed E1^l. Protein sequence analysis showed that cleavage of the E1 enzyme was occurring at the N-terminal region, the result of which was the production of a peptide with an estimated M_r of approx. 8,000 as estimated by tricine-SDS/polyacrylamide gel electrophoresis. In addition, this small peptide was shown to be responsible for a large proportion of the E1 immunoresponse as shown by immunoblotting studies. The E2 enzyme of OGDC was cleaved by trypsin to produce two fragments of M_r 35,000 and 15,000; the smaller fragment representing the lipoyl domain cleaved from the N-terminus of the enzyme, the larger fragment possessing the catalytic acyltransferase active site. The nature of the polypeptides formed on E2 degradation were further confirmed by the use of anti-(E2 OGDC) serum in immunoblotting studies of the tryptic degraded OGDC. In contrast to E1 and E2, the E3 enzyme remained intact during tryptic degradation of OGDC as determined by immunoblotting analysis.

A different proteolytic degradation pattern was observed when bovine heart OGDC was treated with protease arg C; normally specific for arg-arg bonds only, this protease was shown both by SDS/polyacrylamide gel analysis and immunoblotting studies, to extensively degrade E1 producing a complex pattern of low M_r peptides while leaving the E2 and E3 enzymes intact. The extensive degradation of E1 with protease arg C in contrast to the lower M_r E1' species formed upon tryptic treatment, may reflect additional cleavage sites for protease arg C within the E1 enzyme; in some cases protease arg C has shown itself to be capable of cleaving arg-lys bonds (Bousfield and Ward, 1988) and lys-lys bonds (Chartier *et al.*, 1989).

In contrast to bovine heart OGDC which has three distinct polypeptides as shown by SDS/polyacrylamide gel electrophoresis, OGDC purified from bovine kidney, resolves into four bands (Linn, 1974). Three of these bands represent the known enzyme activities of E1, E2 and E3 together with an additional polypeptide of M_r 90,000. The origin of this polypeptide is unknown but it is thought to be formed as a result of proteolytic degradation of the E1 enzyme (M_r 96,000), by an enzyme termed inactivase. This enzyme is thought to be a thiol sensitive protease which although found in bovine kidney is also present in rat liver mitochondria. The specificity of inactivase has not been well characterised but in addition to cleaving E1, it also degrades E2 in the complex in a manner reminiscent of tryptic degradation. It would appear that inactivase may be cleaving the complex in a manner similar to the tryptic digestion of bovine heart OGDC.

E1 of bovine kidney OGDC can also be degraded specifically with papain (Kresze *et al.*, 1981) where the E1 species of M_r 96,000 is cleaved to form a species with an estimated M_r 94,000; the protease also cleaves at a susceptible site within the linker region of E2 to form two species of M_r 29,5000 and 27,000. A major difference existing between the tryptic and papain digestion profiles is that papain digests E2 at a much faster rate than E1; this situation is contrary to trypsin where E1 is proteolytically more sensitive than E2. With both proteases, E3 has been shown to be

resistant to proteolysis, remaining intact under conditions where both E1 and E2 are subject to degradation.

CHAPTER FOUR

ROLE OF THE E1 ENZYME OF THE 2-OXOGLUTARATE DEHYDROGENASE MULTIENZYME COMPLEX IN BINDING OF THE E3 ENZYME TO THE CORE COMPLEX

4.1 INTRODUCTION

In the previous chapter, it was established that the N-terminal region of E1 of bovine heart OGDC, exhibited a significant degree of sequence similarity to the N-termini of both the eukaryotic E2 acetyltransferase and protein X components of PDC. On account of this, the possibility that this region of E1 shared either functional or structural characteristics with the acetyltransferases and/or protein X was investigated. The conserved sequence $P^{S}/_{A}LSPTM$ which is found located at the N-terminus of all eukaryotic acetyltransferases, is indicative of the presence of a lipoyl domain; in human E2, this motif occurs approx. 30 amino before the lysine residue which is lipoylated. Since a modified form of this sequence is also found at the N-terminus of E1 of OGDC, it may indicate the presence of a modified lipoyl domain which, however, may still be functional, and therefore subject to acylation in a manner similar to that of the E2 acetyltransferases.

Protein X, which also shares sequence similarity with E1 of OGDC, is known to be involved in promoting an association between the E3 enzyme and the core assembly of PDC. The exact nature of this interaction is not as yet clearly defined; however removal of protein X from PDC, in particular the putative E3 binding domain, either by genetic or biochemical means causes the E3 enzyme to dissociate from the complex. Interestingly, OGDC and BCOADC, which both contain an identical E3 enzyme to that found in PDC, have not been found to possess a polypeptide analogous to protein X.

The aim of the work presented in this chapter was to determine if the sequence present at the N-terminus of E1 of OGDC was representative of either a functionally active lipoyl domain or, if it was implicated in assisting with the binding of E3 in this complex. Data presented in this chapter were obtained prior to the publication of the complete amino acid sequence for the OGDC E1 enzyme from a mammalian source. This has now been achieved by Koike *et al.* (1992) who recently cloned the human foetal liver E1 enzyme for OGDC.

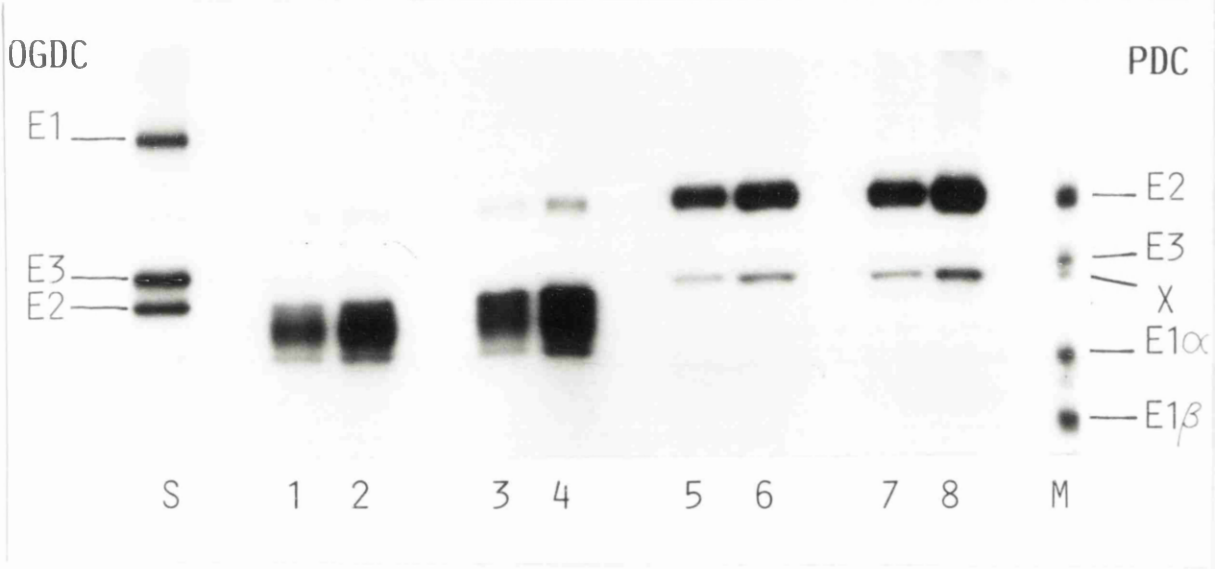
Fig. 4.1 Analysis of acetylated proteins after incubation of native OGDC and PDC with [2-^{14}C]pyruvate.

Bovine heart OGDC and PDC were diluted with 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6, to a final concentration of 1mg/ml. OGDC (150μg) and PDC (150μg) were both radiolabelled individually with 2μCi of [2-^{14}C] pyruvate for 10 min at room temperature. Laemmli sample buffer including 10mM DTT (see below) was added to terminate the reaction. Samples were incubated at 37°C for 5 min before resolution by 10%(w/v) SDS/polyacrylamide gel electrophoresis. Gels were subjected to fluorography as described in the Methods.

Lanes 1 and 3, OGDC (20μg); lanes 2 and 4, OGDC (40μg); lanes 5 and 7, PDC (20μg); lanes 6 and 8, PDC (40μg). Samples 1, 2, 5, and 6 were prepared in the presence of 10mM DTT, samples 3, 4, 7 and 8 prepared in the absence of DTT.

Lane S - radiolabelled OGDC marker protein (10μg)

Lane M - radiolabelled PDC marker protein (10μg)


OGDC
PDC
E1
E3
E2
E2
E3
X
E1α
E1β
S
1
2
3
4
5
6
7
8
M

4.2 RESULTS

4.2.1 MODIFICATION OF OGDC AND PDC WITH RADIOLABELLED SUBSTRATE

Both protein X and the E2 acetyltransferases of PDC contain covalently attached lipoyl groups which are capable of being acetylated by substrate in the absence of CoASH (De Marcucci *et al.*, 1986). The N-terminal region of E1 from bovine heart OGDC contains a variation on the sequence motif $P^{S}/_{A}LSPTM$, which precedes the lipoyl domains present on the E2 eukaryotic acetyltransferases. Owing to the unexpected presence of this sequence on E1 of OGDC, the experiments performed in this section sought to determine if this sequence represented a functionally active lipoyl domain on E1 which was capable of being modified by acylating substrate.

The result of incubating the complexes with [2-^{14}C]pyruvate, which was readily available in the laboratory, and subsequent analysis of the products by SDS/polyacrylamide gel electrophoresis and fluorography is shown in Fig. 4.1. DTT was omitted from the sample buffer in some tracks in an attempt to minimise the release of radiolabelled substrate from the enzyme subunits, since it has been shown that retention of radioactivity in these polypeptides is dependent on the method of sample preparation (De Marcucci *et al.*, 1986). Minimal losses of radioactivity could be achieved by solubilising the sample at 37°C in the absence of thiol reagents. Treatment of PDC with radiolabelled [2-^{14}C]pyruvate resulted in incorporation of label into both the E2 and protein X subunits as expected, owing to the formation of the S-acetyldihydrolipoamide intermediate. In contrast, incubation of OGDC with [2-^{14}C]pyruvate caused modification of the E2 subunit only; no incorporation of radiolabel into E1 was detected suggesting that it does not contain a functional lipoyl group.

Although E1 of OGDC did not become acetylated in the presence of [2-^{14}C]pyruvate, it is still possible that a lipoyl group may have been present. The putative lipoyl-like domain present on E1 may not incorporate label unless treated with

Fig. 4.2 Modification of native OGDC and PDC with 2-oxo[5-^{14}C]glutaric acid and [2-^{14}C]pyruvate

Bovine heart OGDC and PDC were diluted with 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6, to a final concentration of 1mg/ml. OGDC and PDC were labelled with either 2-oxo[5-^{14}C]glutaric acid (1μCi) or [2-^{14}C]pyruvate (1μCi) for 10 min at room temperature. Laemmli sample buffer containing no DTT was added to stop the labelling reaction. Samples were warmed at 37°C for 5 min and resolved by 10%(w/v) SDS/polyacrylamide gel electrophoresis. Gels were processed for fluorography as outlined in the Methods.

Lanes 1 and 2, OGDC (20μg), lanes 5 and 6, PDC (20μg); these samples were labelled with 2-oxo[5-^{14}C]glutaric acid. Lanes 3 and 4, OGDC (20μg), lanes 7 and 8, PDC (20μg); these samples were labelled with [2-^{14}C]pyruvate.

Lane M - radiolabelled OGDC marker protein (10μg)

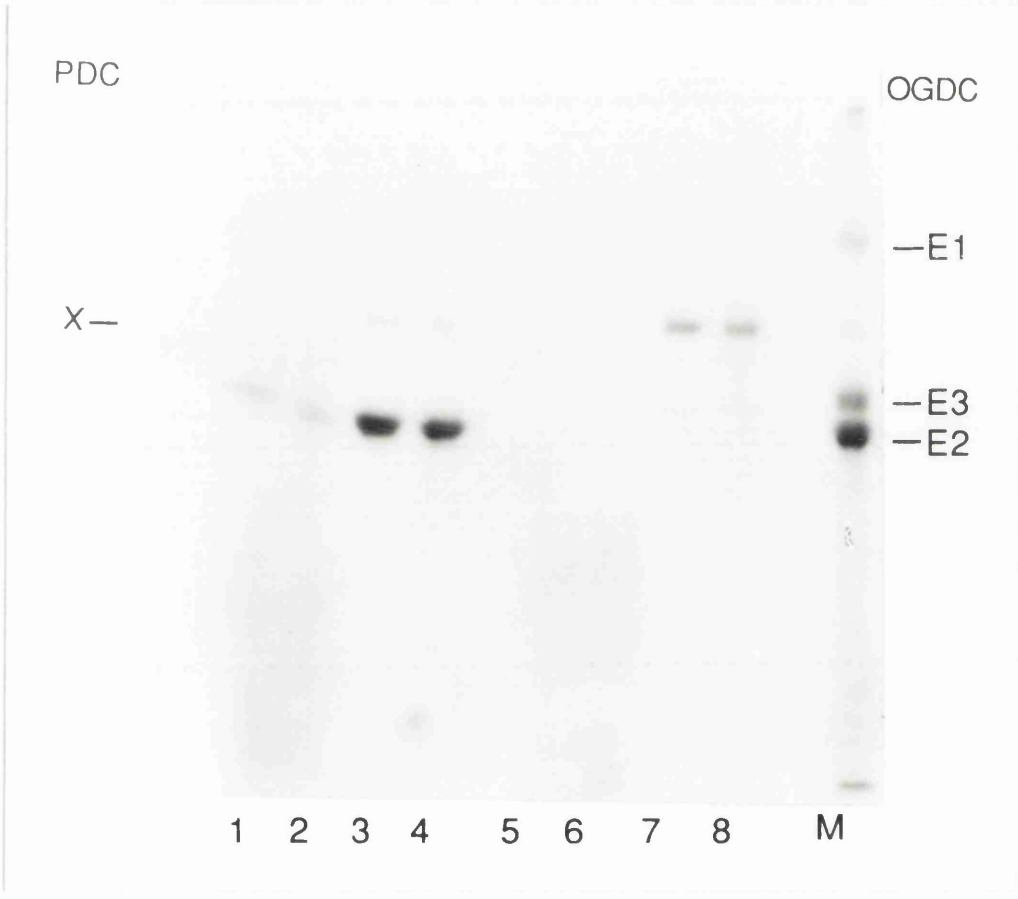
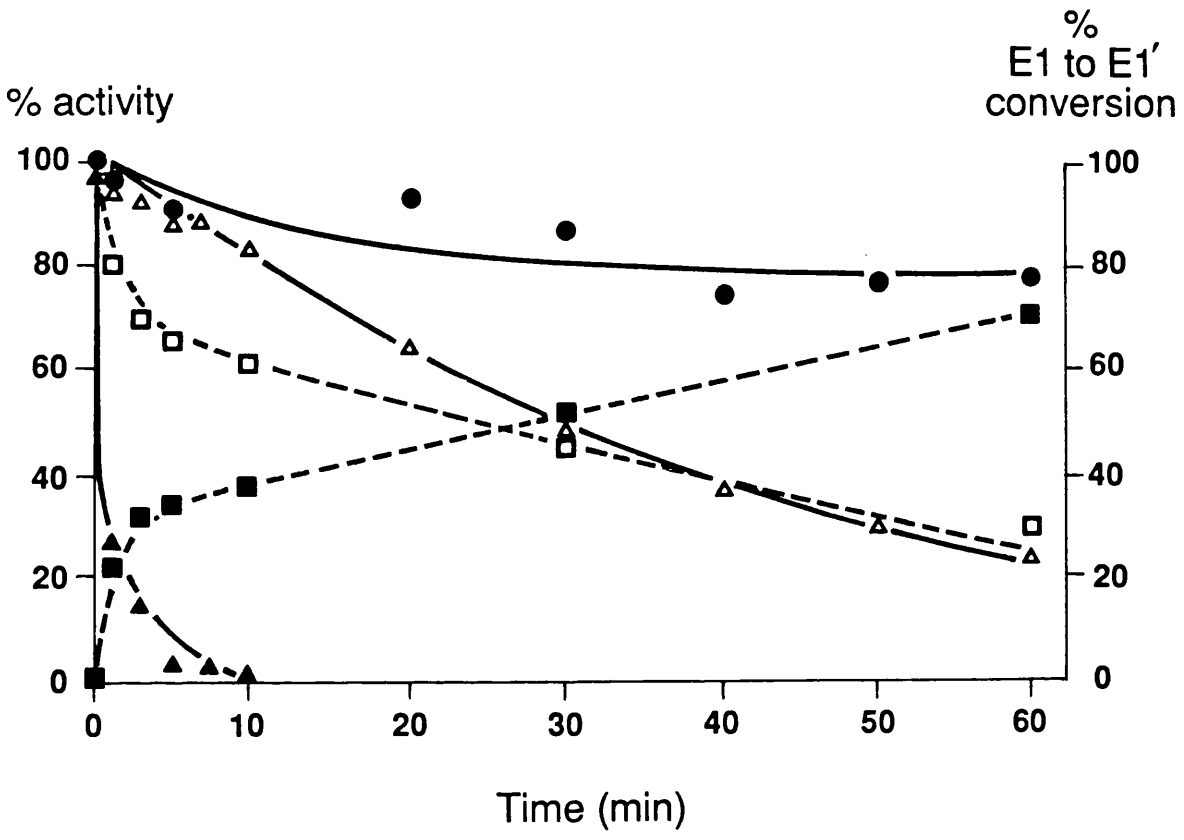

PDC
OGDC
—E1
X—
—E3
—E2
1 2 3 4 5 6 7 8 M

Fig. 4.3 Tryptic digestion of bovine heart OGDC; effect on 2-oxoglutarate dehydrogenase (E1) activity and OGDC activity.

OGDC diluted in 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6, was incubated with either 0.01%(w/w) trypsin (△—△) or 0.1%(w/w) trypsin (▲—▲) at 30°C. At times indicated samples were withdrawn and digestion stopped by addition of soya bean trypsin inhibitor, after which samples were assayed for overall OGDC activity. Samples digested with 0.1%(w/w) trypsin were also assayed for E1 activity (●——●).

Samples from the digestions were also resolved on 10%(w/v) SDS/polyacrylamide gels and stained with Coomassie blue (see Fig. 4.4). The areas of the bands coresponding to the amount of E1 remaining and $E1^l$ formed as a result of tryptic digestion was determined by densitometric scanning as outlined in the Methods. Variations in the levels of E1 (□- - -□) and $E1^l$ (■- - -■) are expressed with respect to the combined values of E1 and $E1^l$ at each time point.


% activity
% E1 to E1′ conversion
100
80
60
40
20
0
0
10
20
30
40
50
60
Time (min)

its natural substrate, 2-oxoglutarate. The results of such an experiment are shown in Fig. 4.2. On incubation with 2-oxo[5-^{14}C]glutaric acid, succinyl groups were incorporated into the E2 lipoyl domain but no labelling of the E1 subunit was detected. Surprisingly, only weak labelling of the E2 subunits was apparent. This may reflect the instability of the 8-succinyl dihydrolipoamide intermediate. This result provides strong evidence that E1 of OGDC lacks a functionally active lipoyl domain. Interestingly, neither the E2 or protein X subunits of PDC were modified when treated with 2-oxo[5-^{14}C]glutaric acid.

Since incorporation of [^{14}C]acetyl groups from [2-^{14}C]pyruvate into the E2 subunits of OGDC was observed, enzyme activity measurements were performed in which the activity of OGDC was monitored when pyruvate was used as substrate; E1 of OGDC is capable of using pyruvate as substrate such that acetyl groups are incorporated into the E2 enzyme; However, no measurable enzyme activity was observed when pyruvate was used as substrate. It would appear that incorporation of acetyl groups into E2 of OGDC is a result of the complex turning over very slowly, which under normal conditions functions at a rate of 1800 sec^{-1}.

4.2.2 EFFECT OF TRYPTIC DIGESTION ON OGDC ACTIVITY

Trypsin treatment of OGDC results in cleavage of the N-terminal region of E1. The effect of tryptic digestion of OGDC on complex activity was examined to determine the importance of this cleaved region in maintaining overall complex activity.

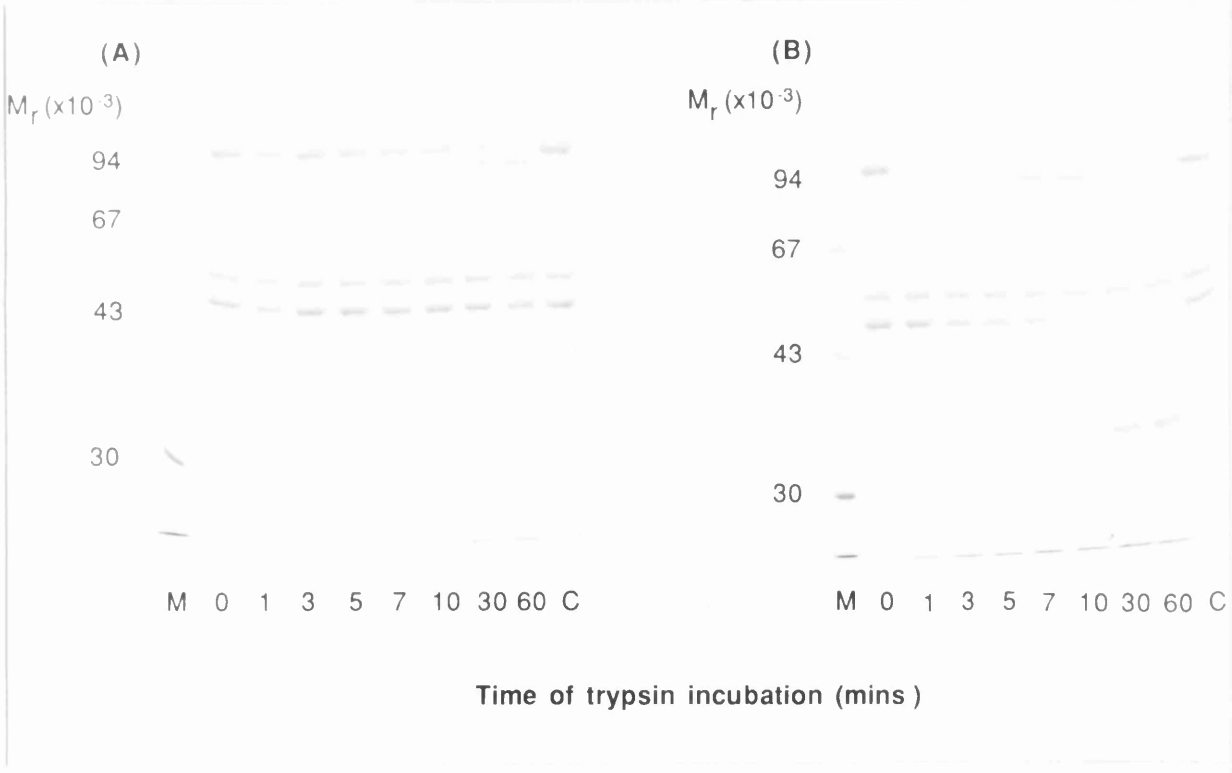
Fig. 4.3 shows the observed results when OGDC was digested with trypsin and assayed for residual complex activity. Digestion with 0.1%(w/w) trypsin resulted in a rapid loss of complex activity such that total inhibition occurred within 10 min. More gradual, controlled losses of complex activity were achieved by using lower concentrations of trypsin. Treatment with 0.01%(w/w) trypsin ensured that 50% of OGDC activity was still present after 30 min.

Fig. 4.4 Time course of tryptic digestion of bovine heart OGDC

OGDC was digested with 0.01%(w/w) trypsin (panel A) or 0.1%(w/w) trypsin (panel B) as described in Fig. 4.3. Samples (20μg) from each time point were taken and resolved by 10%(w/w) SDS/polyacrylamide gel electrophoresis. Gels were stained with Coomassie blue.

Lane M - low M_r marker proteins (10μg)

Lane C - OGDC (20μg) incubated at 30°C for duration of experiment in the absence of trypsin.

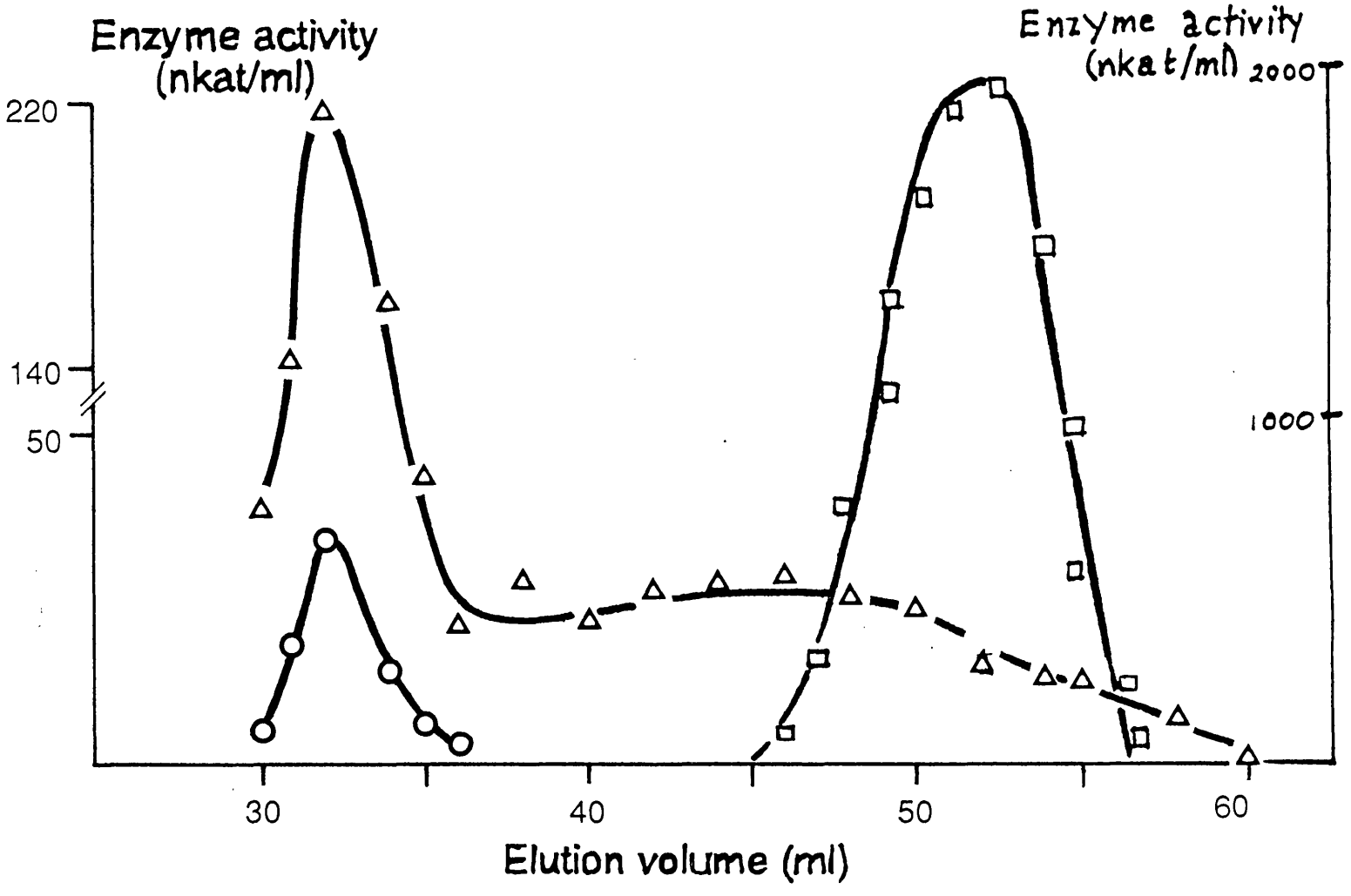

(A)
M_r (x10^{-3})
94
67
43
30
M 0 1 3 5 7 10 30 60 C
(B)
M_r (x10^{-3})
94
67
43
30
M 0 1 3 5 7 10 30 60 C
Time of trypsin incubation (mins)

The possibility that loss of overall OGDC function could be attributed to degradation of the E1 enzyme and concomitant loss of function was investigated initially since this is the rate-limiting enzyme within the complex. To test this hypothesis OGDC was digested with 0.1%(w/w) trypsin as previously shown and E1 activity monitored instead of overall complex activity. This level of trypsin, which was sufficient to cause complete loss of OGDC activity within 10 min , caused little or no decrease in E1 activity with approx. 80-100% of total E1 activity remaining after 1h of digestion. This result implied that loss of overall OGDC activity as a consequence of tryptic digestion could not be attributed to digestion of the E1 enzyme, with the resultant $E1^{l}$ fragment retaining full enzymatic activity under conditions in which OGDC activity was totally inhibited.

Samples of tryptic digested OGDC were removed at various time intervals during the above experiment and resolved by SDS/polyacrylamide gel electrophoresis. Fig. 4.4 shows the Coomassie blue stained gels where panels A and B represent OGDC digested with 0.01%(w/w) and 0.1%(w/w) trypsin respectively. From visual inspection of these gels there appears to be no detectable proteolysis of either E2 or E3, although at longer time intervals degradation of E2 is apparent. The major effect of tryptic cleavage is on the E1 enzyme, the extent of E1 proteolysis appearing to proceed in conjunction with the loss of OGDC activity. In order to quantify the rate of E1 to $E1^{l}$ conversion the Coomassie blue stained gels were subjected to densitometric scanning, the results shown in Fig. 4.3. The rate of E1 degradation appears to proceed in parallel with the loss of OGDC activity; however, in the early stages of the digestion, this conversion proceeds at a rate faster than the loss of overall complex activity. The combined areas under the E1 and $E1^{l}$ peaks representing a particular time point remained constant throughout the time course indicating that under these conditions, the $E1^{l}$ species is a stable intermediate formed by the specific degradation of E1. From these results it would seem that the E1 to $E1^{l}$ conversion effected by trypsin occurs approx. in parallel with the loss of overall OGDC activity, as determined by enzyme activity measurements and gel scanning analysis. However,

Fig. 4.5 Gel filtration of bovine heart OGDC and pig heart dihydrolipoamide dehydrogenase (E3)

Gel filtration was performed using a Superose 12 gel filtration column (HR 16/50, bed volume 100ml) attached to a Pharmacia FPLC system. The column was equilibrated and eluted with 10mM NaCl/50mM potassium phosphate buffer pH 7.6, at a flow rate of 1ml/min. Native bovine heart OGDC (2mg) was loaded onto the column and collected fractions were assayed for both overall OGDC activity (o——o) and E3 activity associated with the complex (Δ——Δ). In a separate experiment E3 from pig heart (2mg) was eluted from the column as described above, with the collected fractions being assayed for E3 activity (□——□).


Enzyme activity
(nkat/ml)
220
140
50
Enzyme activity
(nkat/ml)
2000
1000
30
40
50
60
Elution volume (ml)

loss of OGDC function cannot be attributed to the inhibition of E1, since the large stable $E1^{l}$ fragment retains approx. 80-100% of its original enzymatic activity.

4.2.3 CONDITIONS FOR RESOLVING DISSOCIATED E3 ENZYME FROM INTACT OGDC

It would appear from results obtained so far that the segment of polypeptide at the N-terminus of E1 of OGDC, which possesses primary amino acid sequence similarity to both the eukaryotic acetyltransferases and protein X from PDC, does not represent a functional lipoyl domain. The specific removal of this region by proteolytic degradation has a profound effect on complex activity resulting in a complete loss of OGDC activity while E1 remains fully active. This phenomenon may be explained by the involvement of the cleaved N-terminal region in a structural role whereby, it binds the E3 enzyme to the core assembly in a situation similar to that which exists in PDC for protein X. It was necessary to examine if dissociation of E3 from OGDC occurred as a direct result of the E1 to $E1^{l}$ conversion. In the following experiments OGDC was treated with either trypsin or protease arg C, both of these proteases known to degrade E1 specifically. In the event of E3 dissociation from the complex as a result of E1 degradation, it would be resolved from the enzyme core using gel permeation chromatography.

Initial efforts were concerned with finding a suitable gel filtration column which would allow for good resolution between intact OGDC and free E3. This was necessary to distinguish between E3 associated with the core from that which was released as a result of specific proteolytic degradation of E1 at its N-terminus. Optimal resolution between OGDC and free E3 was obtained on a Superose 12 gel filtration column (bed volume 100ml), which was used in conjunction with a Pharmacia FPLC system. Fig. 4.5 shows a typical elution profile obtained with bovine heart OGDC and free E3 isolated from pig heart . OGDC (M_r 3.25×10^{6}) eluted in a sharp peak with an elution volume of 32ml which was equivalent to the calculated void volume of the column, the activity profile confirming that the vast majority of E3 remained in

Fig. 4.6 Coomassie blue gel profile of tryptic digested OGDC subjected to gel filtration

Lanes 1 and 2, undigested OGDC (15μg); lanes 3 and 4, OGDC digested with 0.01%(w/w) trypsin for 3 min (15μg); lanes 5 and 6, OGDC digested with 0.02%(w/w) trypsin for 6 min (15μg).

Lane M - low M_r proteins (10μg)

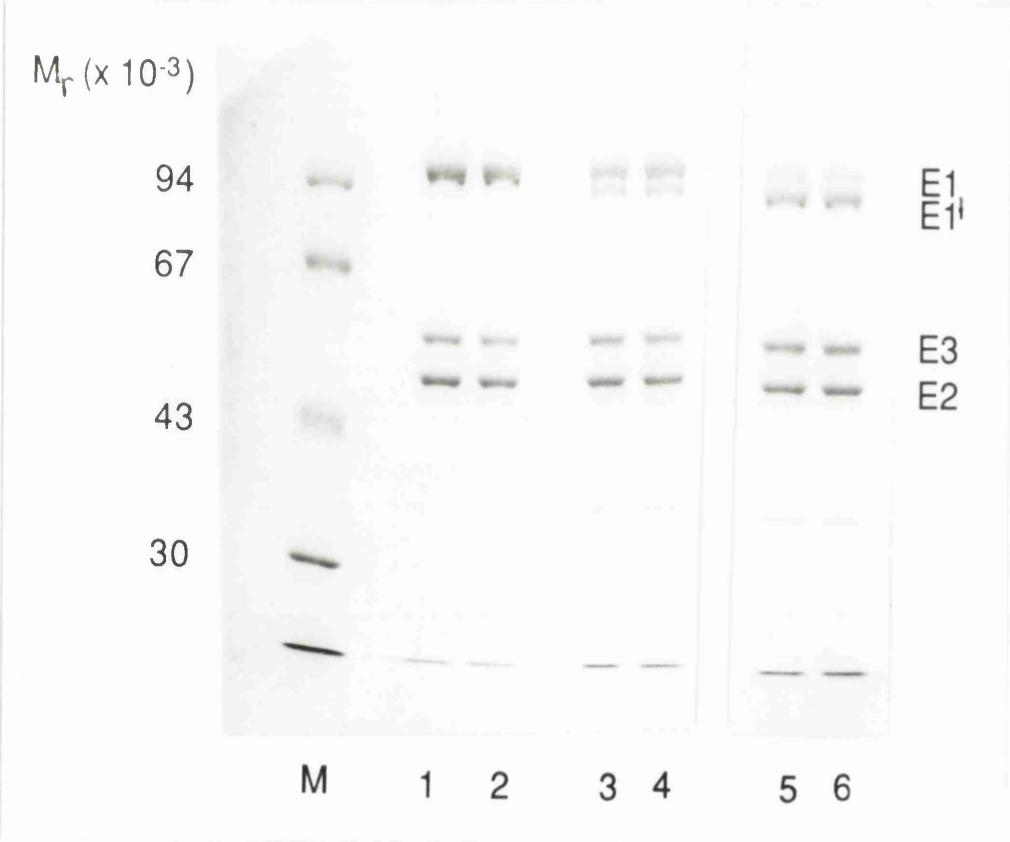
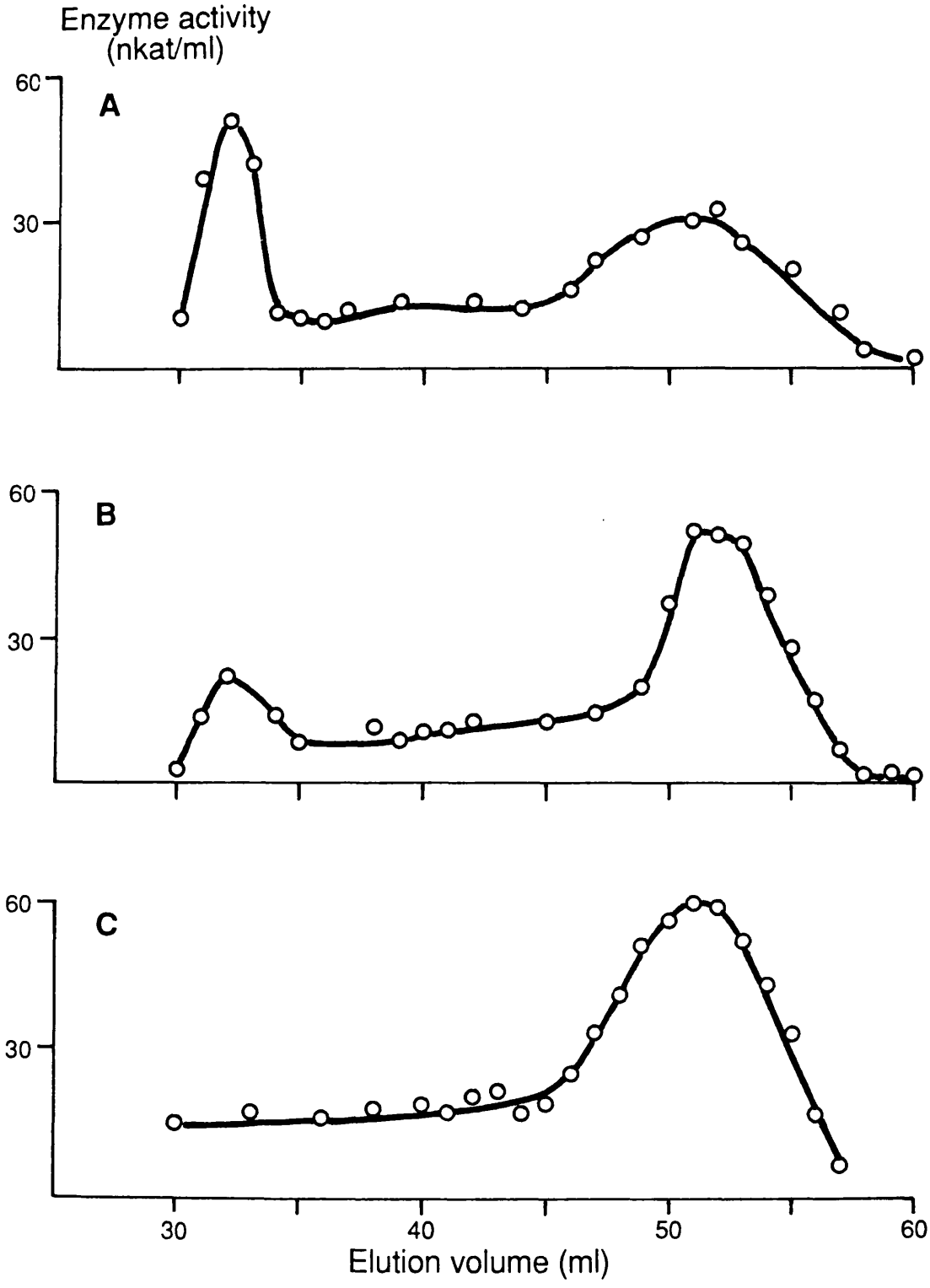

M_r (x 10^{-3})
94
67
43
30
E1
E1'
E3
E2
M
1
2
3
4
5
6

Fig. 4.7 Analysis of E3 release from proteolytically degraded OGDC

Bovine heart OGDC was proteolytically degraded with trypsin prior to gel filtration treatment. OGDC was diluted in 3mM NAD^+/ 2mM $MgCl_2$/ 0.2mM TPP/ 50mM potassium phosphate buffer pH 7.6. Digestions with trypsin were carried out at the following concentrations: panel A - 0.01%(w/w) trypsin for 3 min; panel B - 0.01%(w/w) trypsin for 6 min; panel C - 0.02%(w/w) trypsin for 6 min. All digestions were performed at 30°C and stopped by the addition of soya bean trypsin inhibitor. In each case the degraded complex was loaded immediately onto a Superose 12 gel filtration column (HR 16/50, bed volume 100ml) attached to a Pharmacia FPLC system. The column was equilibrated and eluted with 10mM NaCl/ 50mM potassium phosphate buffer pH 7.6 at a flow rate of 1ml/min. Collected fractions were assayed for E3 activity (o——o) as detailed in the Methods.


Enzyme activity
(nkat/ml)
60
30
A
B
C
30
40
50
60
Elution volume (ml)

association with the complex at this stage. However, a small amount of E3 activity was observed throughout the elution profile. This slow, steady elution of E3 activity probably represented E3 which dissociated slowly from the complex during its passage through the Superose 12 matrix. It should be stressed that this low background level of E3 release from the intact complex was distinct to the elution profile obtained for the free E3 pig heart control. E3 (M_r 110,000) eluted as a distinct peak (V_e 52ml) with between 95-100% of loaded activity being routinely recovered from the column. Using this method, the elution profiles obtained with OGDC and the free E3 enzyme were sufficiently different to allow for a distinction to be made between them. The reproducibility of the gel filtration column was such that the elution profiles presented in this chapter, represent an average of 3-4 separate column runs in which elution volumes differed by no more than +/- 1ml.

4.2.4 EFFECT OF TRYPTIC DIGESTION ON RELEASE OF E3 FROM OGDC

Before commencing these gel filtration studies, it was necessary to optimise conditions which would allow for the formation of the E1 to E1^l species while leaving E2 and E3 virtually intact. Fig. 4.6 shows the results of digesting OGDC under several different conditions. At levels of 0.01%(w/w) trypsin (tracks 1 and 2), although only a small amount of E1 was converted to E1^l, the bulk of E1 remained undegraded within the complex. By increasing the time of incubation with 0.01%(w/w) trypsin (tracks 3 and 4), a 50:50 E1 to E1^l conversion was possible. Approx. 80% conversion of E1 to E1^l could be accomplished by treating OGDC with 0.02%(w/w) trypsin (tracks 5 and 6), but at these concentrations of trypsin some minor proteolysis of the E2 enzyme was also observed as deduced by its appearance on SDS/polyacrylamide gels. In all of the tryptic digestions shown E3 remained intact.

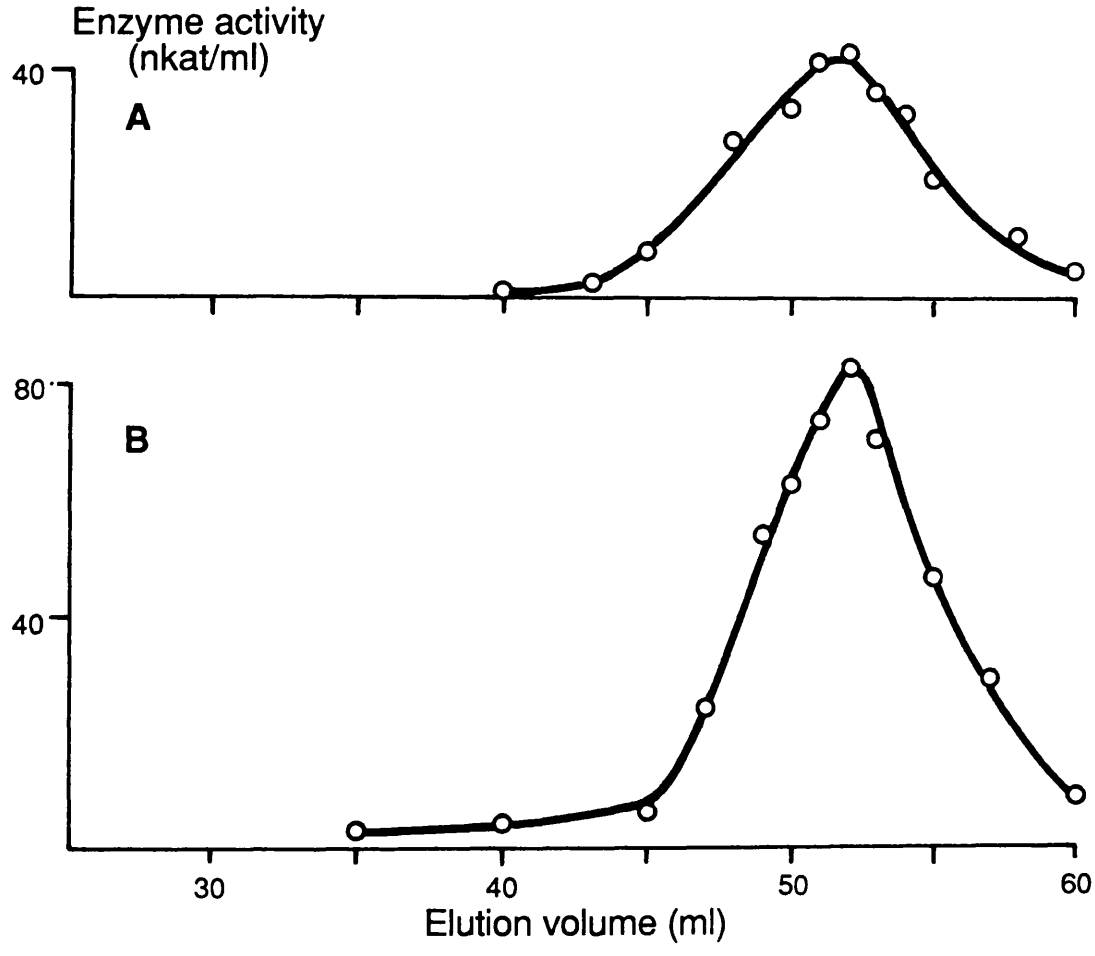
Fig. 4.7 shows the results obtained from the gel filtration of OGDC which had been digested with trypsin according to the conditions described above. Digestions

Fig. 4.8 Effect of protease arg C degradation on the release of E3 from OGDC

Gel filtration was performed using a Superose 12 gel filtration column (HR 16/50, bed volume 100ml) attached to a Pharmacia FPLC system. Conditions for each column run are detailed below.

Panel A - OGDC was incubated with NaCl at a final concentration of 1M for 1h at 4°C. The gel filtration column was equilibrated and eluted with 1M NaCl/ 50mM potassium phosphate buffer pH 7.6 at 1ml/min. The collected fractions were assayed for E3 activity.

Panel B - OGDC was digested with protease arg C at a final concentration of 9%(w/w) for 1h at 30°C; aliquots of 3%(w/w) arg C were added at times 0 min, 20 min, 40 min. Digestion was stopped at 60 min by addition of benzamidine to a final concentration of 1mM. The degraded complex was loaded immediately onto the gel filtration column and eluted with 10mM NaCl/ 50mM potassium phosphate buffer pH 7.6 at 1ml/min. The collected fractions were assayed for E3 activity as described in the Methods.


Enzyme activity
(nkat/ml)
40
A
80
B
40
30
40
50
60
Elution volume (ml)

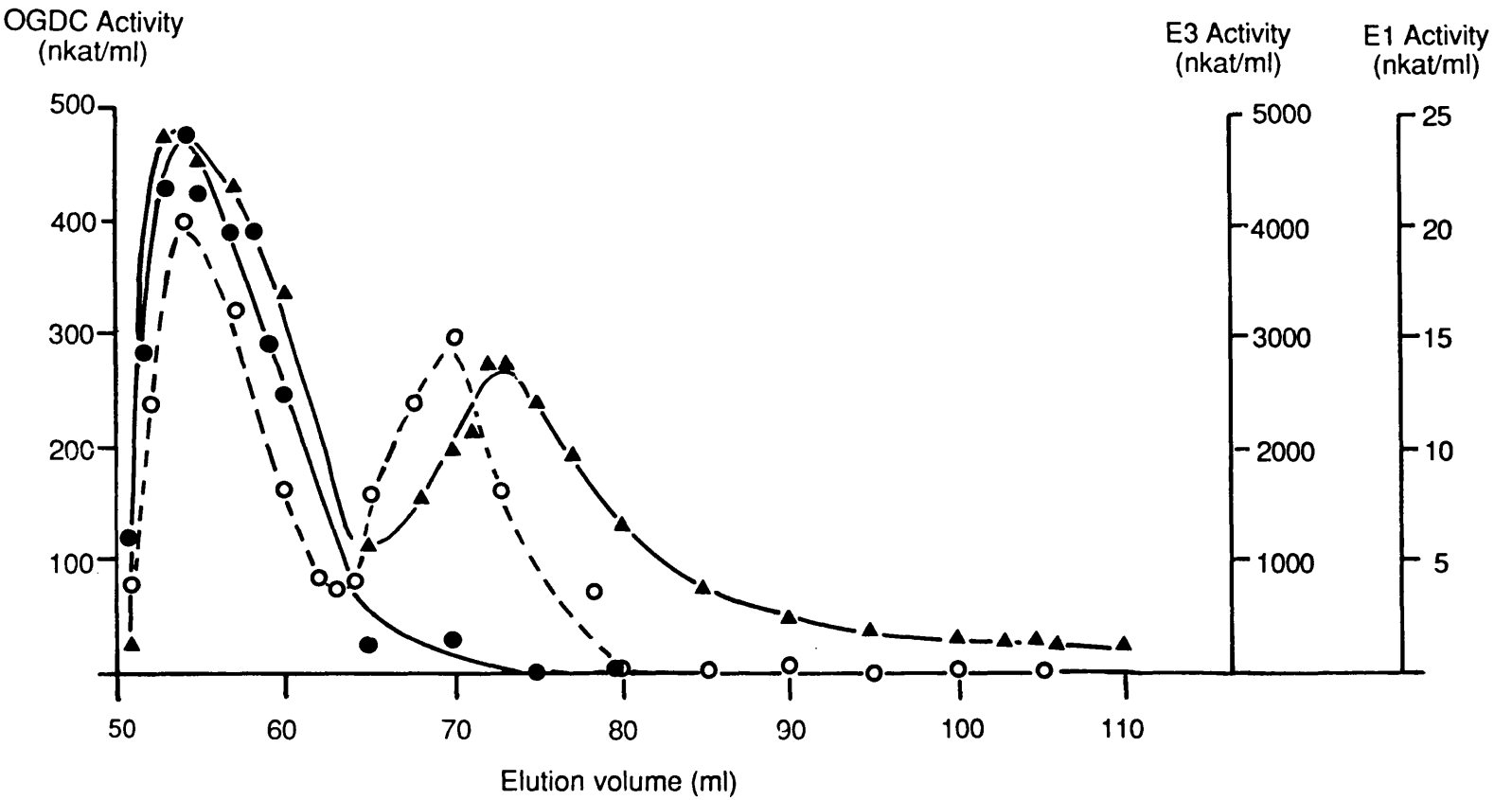
with 0.01%(w/w) trypsin for 3 min and 6 min are shown in panels A and B respectively. Both of the elution profiles obtained are characterised by the presence of two readily resolvable peaks of E3 activity at elution volumes of 32ml and 52ml. The peak eluting at 32ml corresponds to E3 activity which is still associated with the complex as was shown previously in Fig. 4.5. In comparison to this, the E3 enzyme activity eluting at 52ml represents E3 with an estimated M_r 110,000, corresponding to E3 which has become dissociated from the core. This E3 release occurs specifically as a result of cleavage of the N-terminal region of the E1 enzyme. The magnitude of the peak of released E3 activity eluting in this region appears to be related to the extent of E1 degradation such that, the greater the degree of E1 proteolysis the larger the peak of dissociated E3 activity present at 52ml. When OGDC was incubated with higher concentrations of trypsin for longer time intervals such as is shown in panel C of Fig. 4.7, approx. 80% of E1 was converted to $E1^l$ although some proteolysis of E2 had also begun to occur. On gel filtration of the digested complex most of the E3 activity eluted at 52ml. Although there was no peak of E3 activity at 32ml, a low level of activity persisted throughout the column suggesting that E3 may still retain a low affinity for the complex.

4.2.5 STABILITY OF E1 ENZYME INTERACTION WITH THE CORE STRUCTURE

Since it did not prove possible to effect complete conversion of E1 to $E1^l$ without causing minor proteolysis of E2, it is conceivable that breakdown of the core structure may be contributing to release of E3. Digestion of OGDC with protease arg C, which in contrast to trypsin extensively degrades E1 while leaving both the E2 core structure and E3 enzyme intact, was performed and the degraded complex subjected to gel filtration. The results of such an experiment are shown in Fig. 4.8. On assaying for E3 activity, only one major peak of activity was detected at an elution volume of 52ml corresponding to free E3 (M_r 110,000) which had become dissociated from the complex. The conclusion that can be drawn from this experiment is that E1 is

Fig. 4.9 Resolution of tryptic digested OGDC by Sephacryl S300 gel filtration.

OGDC diluted in 0.2mM TPP/ 2mM $MgCl_2$/ 3mM NAD^+/ 50mM potassium phosphate buffer pH 7.6 was digested with 0.01%(w/w) trypsin for 3 min at 30°C. Digestion was stopped by the addition of soya bean trypsin inhibitor and the OGDC loaded immediately onto a Sephacryl S300 gel filtration column (bed volume-120ml). Column was equilibrated and eluted with 10mM NaCl/ 50mM potassium phosphate buffer pH 7.6 at a flow rate of 25ml/h. The collected fractions were assayed for OGDC activity (●——●), E1 activity (○- - -○) and E3 activity (▲——▲).


OGDC Activity
(nkat/ml)
500
400
300
200
100
E3 Activity
(nkat/ml)
5000
4000
3000
2000
1000
E1 Activity
(nkat/ml)
25
20
15
10
5
50
60
70
80
90
100
110
Elution volume (ml)

involved either, with the direct binding or, in stabilising an interaction between E3 and the core structure. In this context, protease arg C has also shown itself to be capable of cleaving arg-lys and lys-lys bonds (Bousfield and Ward, 1988; Chartier *et al.*, 1989), in addition to cleaving arg-arg bonds.

Since it has been established that N-terminal cleavage of E1 present in OGDC results in dissociation of E3 from the core, some consideration should now be given to the nature of this association which is proposed to exist between E1 and E3. For example, on N-terminal cleavage of E1, the $E1^{l}$ species may undergo one of several fates either remaining associated with the core, being released as a distinct polypeptide or, if E1 is bound intimately to E3 in the intact complex, conversion of E1 to $E1^{l}$ may result in the release of a distinct $E1^{l}$/E3 subcomplex.

The approach employed to investigate the possible existence of a $E1^{l}$/E3 subspecies was similar to that described previously, such that OGDC was degraded specifically with trypsin to produce the $E1^{l}$ species and subjected to gel filtration. Both E3 and E1 enzyme activities were measured in the collected fractions. The Superose 12 column was not appropriate for use here since it was desirable to be able to detect M_r differences between polypeptides of similar size i.e. the native E1 and E3 enzymes, which may be released from the core as a result of tryptic digestion. A Sephacryl S300 HR column was used instead, as a set of protein standards showed reasonable separation over the M_r range of interest (1×10^{4}-1.5×10^{6}). On this column, intact OGDC eluted near the void volume which was 54ml; a peak of E1 activity eluted at 54ml with E3 enzymic activity at 53.5ml indicating that both the E1 and E3 enzymes remained associated with the core complex during elution of the column (not shown). In agreement with earlier experiments, a steady low level of E3 release was observed, representing dissociation of loosely bound E3 when OGDC was eluted from the column. In contrast to E3, no E1 release was observed with the control OGDC throughout column elution, the only peak of E1 activity representing that associated with the complex. Fig. 4.9 shows the result of digesting OGDC with 0.01%(w/w) trypsin for 3 min and subjecting it gel filtration on a Sephacryl S300 column; intact

OGDC activity together with the E1 and E3 enzymic activities were found to elute at 54ml, 54ml and 53.5ml, demonstrating that although the OGDC had been tryptic digested a considerable amount of both E1 and E3 remained associated with the complex. In addition to these peaks, two further peaks of enzyme activity were present at later elution volumes representing E1 and E3 eluting at 70ml and 73ml with apparent M_r values of approx. 200,000 and 100,000 respectively. Further analysis involved the acetone precipitation of protein from those fractions containing peak E1 and E3 activities and resolving them by SDS/polyacrylamide gel electrophoresis. The results obtained did not result in unequivocally proving that $E1^l$ and E3 associated with each other after digestion of OGDC with trypsin. From these results it would appear that in addition to E3, the $E1^l$ species formed as a result of tryptic digestion, also showed a marked tendency to dissociate from the complex. The full significance of these results will be discussed later in this chapter.

4.3 DISCUSSION

4.3.1 THE ABSENCE OF A CATALYTICALLY ACTIVE LIPOYL DOMAIN AT THE N-TERMINUS OF OGDC E1

Substrate loading studies involving treatment of OGDC with radiolabelled 2-oxoglutarate or pyruvate resulted in modification of E2 only. This result is explained by the E2 subunits of the 2-oxoacid dehydrogenase complexes containing lipoyl prosthetic groups bound to specific lysine residues, which in the presence of substrate and absence of CoASH are reductively acylated by the E1 subunits of the complex (Perham, 1991). The amino acid sequence surrounding the lysine residue to which the lipoyl group is attached has been well characterised and is found to be highly conserved (Yeaman, 1989). The lipoylation of the E2 polypeptides of these complexes can be inhibited by replacing the highly conserved lysine residue with another residue incapable of being lipoylated (Graham *et al.*, 1986). In contrast to the labelling observed with the E2 subunits, E1 of OGDC was not acylated by either

pyruvate or 2-oxoglutarate. Lack of incorporation of label into E1 might be explained by the absence of the region wherethe highly conserved amino acid sequence containing the lipoylated lysine residue is found. Alternatively, the lysine residue may have been replaced in the sequence by another residue which is incapable of being lipoylated. Analysis of the recently cloned amino acid sequence of OGDC E1 from a human source reveals the absence of the conserved sequence motif containing the lipoylated lysine residue. This confirms the result already deduced from the radiolabelling studies that is, that the sequence motif present at the N-terminus of OGDC E1 which is similar to that found on the E2 acetyltransferases, is not indicative of the presence of a complete acetylatable lipoyl domain.

When E1 of OGDC was cleaved with trypsin to form the $E1^{l}$ species, a rapid loss of overall complex activity was observed. This was in direct contrast to E1 which retained almost full enzymic activity. E1 degradation seemed to parallel the loss of OGDC activity; however, a more careful analysis of this result by densitometric scanning of SDS/polyacrylamide gels showed that the earlier stages of E1 to $E1^{l}$ conversion occurred at a rate faster than the loss of overall complex activity. The areas beneath the combined E1 and $E1^{l}$ peaks for each time point remained constant throughout the time course, suggesting that the $E1^{l}$ species formed was a stable intermediate of tryptic degradation. From this result it would appear that the cleaved N-terminal region of E1 is essential for maintenance of OGDC function. Since cleavage of E1 is likely to be a random process, initially resulting in the formation of $E1/E1^{l}$ heterodimers which may be able to maintain complex activity, it may be necessary to produce $E1^{l}$ homodimers before the ability of the individual E1 molecules to maintain overall complex activity is lost. Thus, in the early stages of tryptic digestion, loss of OGDC activity would occur at a slower rate than E1 to $E1^{l}$ conversion consistent with observations in Fig. 4.3.

4.3.2 ROLE OF OGDC E1 IN BINDING E3 TO THE CORE STRUCTURE

Genetic and biochemical studies have shown that protein X appears to be involved in binding and positioning the E3 enzyme with respect to the E2 core in PDC. In experiments with *S. cerevisiae*, the gene encoding protein X was disrupted and although the cells were still viable, PDC isolated from them was found to lack both the E3 and protein X subunits (Lawson *et al.*, 1991). The affinity of E3 for PDC isolated from bovine heart was also found to be decreased, when protein X in the intact complex was proteolytically degraded with protease arg C (Neagle and Lindsay, 1991). Both of these results can be explained by the existence of a small domain identified on protein X of PDC which shows distant but significant homology to an E3 binding domain; in *S. cerevisiae*, deletion of this putative E3 binding domain on protein X by genetic means resulted in the loss of E3 from the core structure (Lawson *et al.*, 1991). Similarly, protease arg C treatment of protein X in bovine heart PDC resulted in cleavage of a site at, or very close to, the boundary of the putative E3 binding domain causing the dissociation of E3 from the core complex (Neagle and Lindsay, 1991). The possibility that the N-terminal region of E1 was involved with binding E3 to the core was investigated in view of the sequence similarity between it and protein X from bovine heart PDC.

Evidence presented in this chapter has dealt primarily with effects induced by specific degradation of E1 of OGDC on the affinity of E3 for the core complex. It was shown that when E1 was degraded extensively using protease arg C the vast majority of E3 was released from the core. This result gave rise to the idea that a particular region within E1 may have been involved in promoting the binding of E3 to the core structure. Specific tryptic degradation of E1 to produce $E1^{l}$, as a result of N-terminal cleavage of E1, also had the net effect of causing E3 to show reduced affinity for OGDC. It was also observed that as the amount of E1 converted to $E1^{l}$ increased, so the extent of E3 release increased until eventually the E2 core itself began to show signs of proteolysis. It should be noted that both of these results which showed E3 to

be released from the core as a consequence of E1 degradation, were distinct from the small amount of non-specific E3 release which was commonly observed during these experiments. This represents E3 which is loosely associated with the core and was observed even when intact control OGDC was subjected to gel filtration on either Superose or Sephacryl gel matrices. In addition to examining dissociation of E3 from the complex, consideration was also given to the fate of E1 itself and its possible mode of association with the other enzyme subunits after its conversion to $E1^{l}$. Several possibilities were considered regarding the nature of the interaction between E1 and the other subunits of the complex; for example, the $E1^{l}$ species may be released on its own as a distinct polypeptide species, undergo associations with the E3 enzyme such that a stable $E1^{l}$/E3 subspecies is released from the complex, or it may remain associated with the core after tryptic degradation.

As shown in Fig. 4.9, E1 tryptic degraded OGDC (50% of OGDC activity remaining), was subjected to gel filtration on a Sephacryl S300 column during which E1 and E3 enzyme activities were resolved. The peaks of E1 and E3 activity which eluted near the void volume, represented enzyme which was still associated with the core even after tryptic digestion. In addition, two further peaks of E1 and E3 activity representing enzyme which had become dissociated from the core were detected at later elution volumes. It would appear that on tryptic digestion the $E1^{l}$ species shows a marked tendency to dissociate from the complex, as when untreated OGDC was resolved on the same column under identical conditions, E1 activity co-eluted with overall complex activity at the void volume. If $E1^{l}$ and E3 are not released from the complex as separate polypeptides following tryptic digestion, it may be postulated that they undergo some form of association with each other. The peak of E3 activity detected at later elution volumes representing enzyme which has become dissociated from the core did not co-elute with $E1^{l}$ enzyme activity. This suggests that $E1^{l}$ and E3 do not associate specifically with each other. However, the fact that these polypeptide species separated from each other during gel filtration does not rule out the possibility of an interaction between them as any existing association may be so weak as not to be

able to withstand passage down the length of the column. If a direct E1/E3 interaction is occurring within OGDC a similar situation may exist to that seen with icosahedral forms of PDC; in the complexes isolated from *B. stearothermophilus* (Packman *et al.*, 1988) or *A. vinelandii* (Hanemaaijer *et al.*, 1987), selective cleavage of the E3 binding domain results in the simultaneous release of E1 and E3 which both bind to the E2 subunit at or near the same site. This differs from the situation as it exists for OGDC and PDC isolated from *E. coli* where separate regions on E2 have been proposed for binding of the E1 and E3 subunits (Packman and Perham, 1986).

E3 binding domains are small folded domains of approx. 50 amino acids in length, involved with binding E3 to the E2 core, where limited proteolysis conditions used to remove this domain from the core also results in the release of E3 (Perham, 1991). Recent cloning and sequence analysis of rat heart E2 from OGDC has shown that the sequence motif for the putative E1/E3 binding site is absent from this subunit (Nakano *et al.*, 1991); the isolated cDNA clones showing considerable sequence similarity to the E2 subunits of OGDC from *E. coli* and *A. vinelandii* except in the region of the E1/E3 binding site. This region appeared to have been replaced with an unusual sequence motif which has not been detected to date in any of the other acyltransferases. If the E3 binding domain is not present on the E2 subunit of this complex, this raises the question as to the possible location of the E3 binding site in this complex. The possible existence and location of a putative E3 binding domain on E1 of OGDC is considered more fully in the final discussion chapter. Protein X of PDC and E1 of OGDC appear to be involved in assisting with binding E3 to the core complex, where removal of the putative E3 binding domain from protein X and the N-terminal region of the OGDC E1 enzyme, both result in dissociation of E3 from the E2 core. If this is the case, it would appear that in OGDC, a gene shuffling event has occurred such that structural elements involved in E3 binding are located on E1 of OGDC.

CHAPTER FIVE

FUNCTIONAL PROPERTIES OF THE MODIFIED E1 ENZYME OF OGDC

5.1 INTRODUCTION

The E1 enzyme of bovine heart OGDC is now known not to possess a lipoyl domain at its N-terminus. In the absence of an obvious catalytic function for this region, much consideration in this thesis has been given to the structural properties of E1; for example, it has been proposed that this component performs an integral role in assisting with the binding of E3 to the core complex.

The purpose of this chapter was to give closer consideration to any additional functions that E1 may perform on account of its close interaction with E3. In this regard, it has been proposed for PDC that protein X performs a role in shielding the active site of E3 (J. Neagle, unpublished results). In the light of this result, a similar role for E1 of OGDC was considered. Unfolding studies with the denaturant guanidinium hydrochloride (GdnHCl) on the intact E1 and tryptic derived $E1^{l}$ enzymes were also performed to assess the importance of the E1 N-terminal in maintaining E1 stability and assembly. Also, since E1 is functionally important within the complex being responsible for catalysing the rate-limiting reaction of the complex which is Ca^{2+} ion dependent, the effect of E1 tryptic degradation with regard to the Ca^{2+} ion sensitivity of the complex was also examined.

5.2 RESULTS

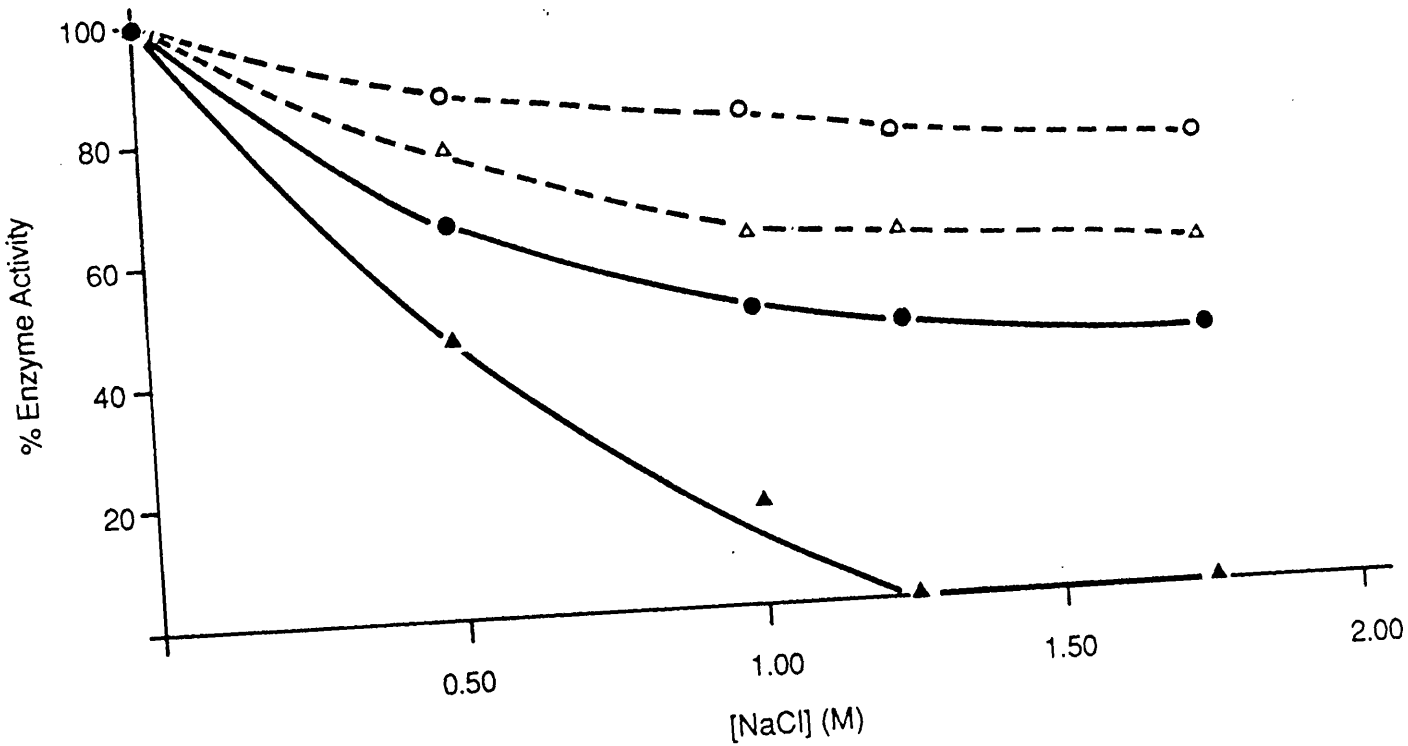
5.2.1 EFFECT OF HIGH IONIC STRENGTH ON OGDC ACTIVITY

When PDC is subjected to conditions of high ionic strength causing release of E1 and E3 from the core complex, no effect on overall complex activity is observed. This suggests that although the affinity of these enzymes for the core complex has been lowered, intimate interactions must still be capable of occurring between them so allowing the catalytic cycle to continue (Kresze and Steber, 1979). Careful treatment of PDC with protease arg C results in selective degradation of the protein X subunit, a

Fig. 5.1 Effect of high salt concentrations on overall complex activity of tryptic digested OGDC

OGDC was diluted in 0.2mM TPP/2mM $MgCl_2$/3mM NAD^+/50mM potassium phosphate buffer pH 7.6. Enzyme was digested with 0.01%(w/w) trypsin at 30°C for 0 min, 5 min, 10 min and 20 min, at which times aliquots were removed and digestion inhibited by the addition of soya bean trypsin inhibitor (x10). Samples (100μg) from each time point were then incubated with varying concentrations of NaCl at room temperature for 15 min, after which a sample of the enzyme was removed and assayed for OGDC enzyme activity as described in the Methods. All values shown represent the mean values of triplicate assays. For each time point of tryptic degradation, the 100% values were calculated from the residual activity remaining after digestion had gone to completion.

Untreated control OGDC (o- - -o), OGDC treated with trypsin for 5 min (Δ- - -Δ), 10 min (●——●) and 20 min (▲——▲) respectively.


100
80
60
40
20
% Enzyme Activity
0.50
1.00
1.50
2.00
[NaCl] (M)

consequence of which is the reduced affinity of E3 for the core. If PDC is subjected to high ionic strength after protease arg C treatment, a significant decrease in overall activity is observed; this can be explained by the affinity of E3 for the core being lowered further, such that overall complex activity becomes more susceptible to the presence of high concentrations of salt (Neagle and Lindsay, 1991).

In common with PDC, treatment of intact OGDC with high concentrations of salt has negligible effects on overall complex activity; the specific degradation of OGDC E1 component resulting in formation of the E1/E1^{l} subspecies, which causes E3 to be released from the core, was examined to determine if the complex showed an increase in its sensitivity to salt as a result of such treatment. Fig. 5.1 shows the results of exposing E1 trypsin degraded OGDC to conditions of high ionic strength; when intact OGDC is treated with increasingly higher salt concentrations, a small decrease in overall complex activity from 100% to approx. 80% was observed only at the highest salt concentrations. In contrast, OGDC incubated with trypsin causing E1 to be specifically degraded, resulted in the complex showing an increased sensitivity in conditions of high ionic strength; the residual enzyme activity remaining after tryptic digestion was 100%, 51%, 24% and 6% for the time points 0 min, 5 min, 10 min and 20 min respectively. For each time point of tryptic degradation, the 100% value in the absence of salt was taken with respect to the residual enzyme activity remaining after tryptic digestion had gone to completion; this allowed for comparison of the salt sensitivity of the complex which had been degraded to varying extents. Decreases in the overall complex activity could not be directly attributed to the degradation of E2 or E3 as determined by SDS/polyacrylamide gel electrophoresis (not shown).

5.2.2 EFFECT OF HIGH IONIC STRENGTH ON INHIBITION OF E3 ACTIVITY

In the presence of either pyruvate or NADH, the maleimides represent a class of compounds capable of inhibiting the 2-oxoacid dehydrogenase complexes. They do so by interacting specifically with free thiol groups present on the E2 dihydrolipoamide

groups, generated as part of the essential catalytic process of the complex. For example, treatment of PDC with N-ethyl[2,3-^{14}C]maleimide results in radiolabel being incorporated into both the E2 and protein X subunits (Hodgson *et al.*, 1986). Treatment of PDC with protease arg C or high concentrations of salt, promotes dissociation of E1 and E3 from the core, allowing N-ethyl[2,3-^{14}C]maleimide to modify the E3 enzyme of the complex in an NADH dependent manner (J. Neagle, unpublished results). It was postulated from such analyses that on dissociation of E3 from the PDC, the active site redox-active disulphide bridge was unmasked, and in the presence of NADH was rendered susceptible to modification by N-ethylmaleimide.

To determine if a similar situation existed for OGDC, the complex was treated in conditions known to cause E3 dissociation from the complex, to establish if the active site of E3 was capable of being modified in an NADH dependent manner by thiol group specific compounds. Since NADH is an end product of the OGDC reaction sequence, it is responsible for product inhibition of the complex; it was necessary to establish conditions to determine the level of NADH which when added to the complex would not affect E3 activity. In early experiments, addition of NADH at concentrations between 50μM-0.5mM effected decreases in E3 activity of approx. 50-90% (not shown). Removal of NADH by centrifugal gel elution as described by Penefsky (1977), resulted in 95% recovery of activity in the elutant. Despite the obvious advantages of this method, it proved to be impractical for the purposes of the experiment on account of the number of samples which could easily be processed. It had been reported in the literature that preparations of NADH invariably seemed to contain impurities, some of which inhibited dehydrogenase activity (Fawcett *et al.*, 1961); it appears that NADH is unstable and forms a large variety of degradation products either under acidic conditions or on prolonged exposure to phosphate buffers at pH 7.6. The formation of certain enzyme inhibitors have also been reported to form in frozen solutions and even damp powder (Margolis et al., 1976). Close inspection of NADH used at the start of this study suggested that it had undergone considerable

Fig. 5.2 Effect of N-ethylmaleimide on E3 enzyme activity in the intact and salt treated complex

OGDC was diluted in 50mM potassium phosphate buffer, pH 7.6, to a final concentration of 1mg/ml. Complex was then treated as follows: NADH was added to samples at final concentration of 0.5mM for 10 min prior to the addition of N-ethylmaleimide, which was also present at a final concentration of 0.5mM. In all cases, 20mM mercaptoethanol was added 10 mins prior to assaying enzyme activity. In cases where it was necessary to incubate OGDC in conditions of high ionic strength, the complex was incubated with 0.25M $MgCl_2$ for 1h at 4°C and further additions made similar to those outlined above. Unless otherwise indicated, values shown represent the enzyme activity of the samples after 2h.

Results shown represent the mean value of triplicate assays.

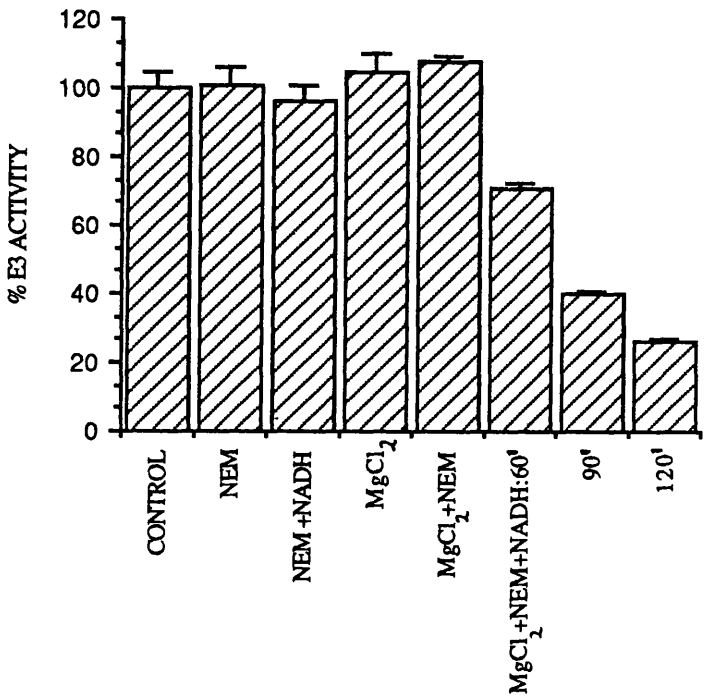

% E3 ACTIVITY
120
100
80
60
40
20
0
CONTROL
NEM
NEM +NADH
MgCl2
MgCl2+NEM
MgCl2+NEM+NADH:60'
90'
120'

Fig. 5.3 Effect of p-aminophenyldichloroarsine on E3 enzyme activity in the intact and salt treated complex

OGDC was diluted in 50mM potassium phosphate buffer, pH 7.6, to a final concentration of 1mg/ml. NADH was added to samples at a final concentration of 0.5mM for 10 min prior to addition of 0.5mM p-Aminophenyldichloroarsine (termed inhibitor in the figure). In cases where it was necessary to subject the complex to high ionic strength, OGDC was incubated with 0.25M $MgCl_2$ for 1h at 4°C and then treated in a similar manner to that described above.

Unless otherwise indicated, values shown represent the enzyme activity of the samples after 2h.

Results shown represent the mean value of triplicate assays.

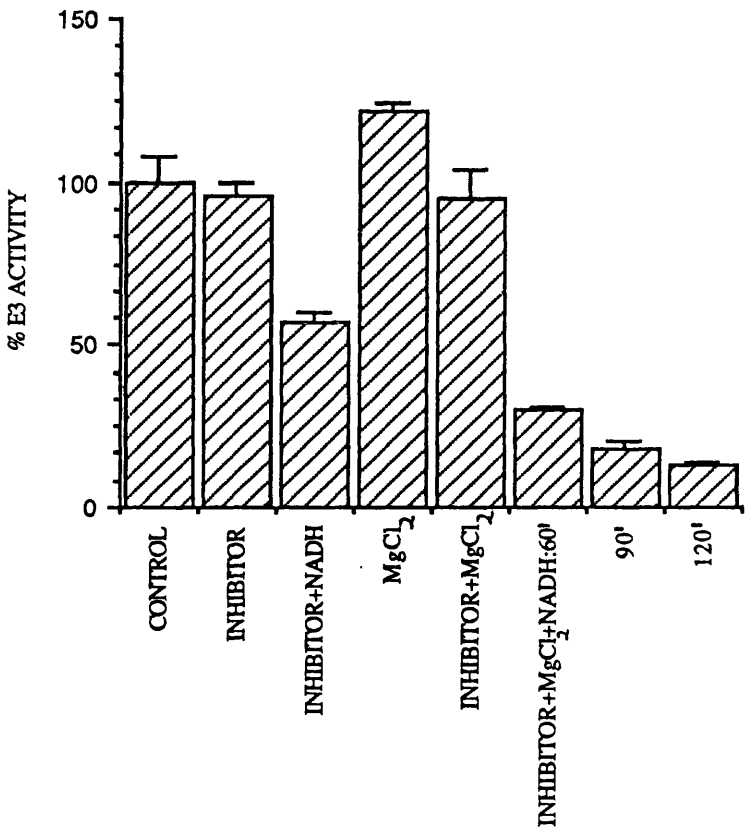
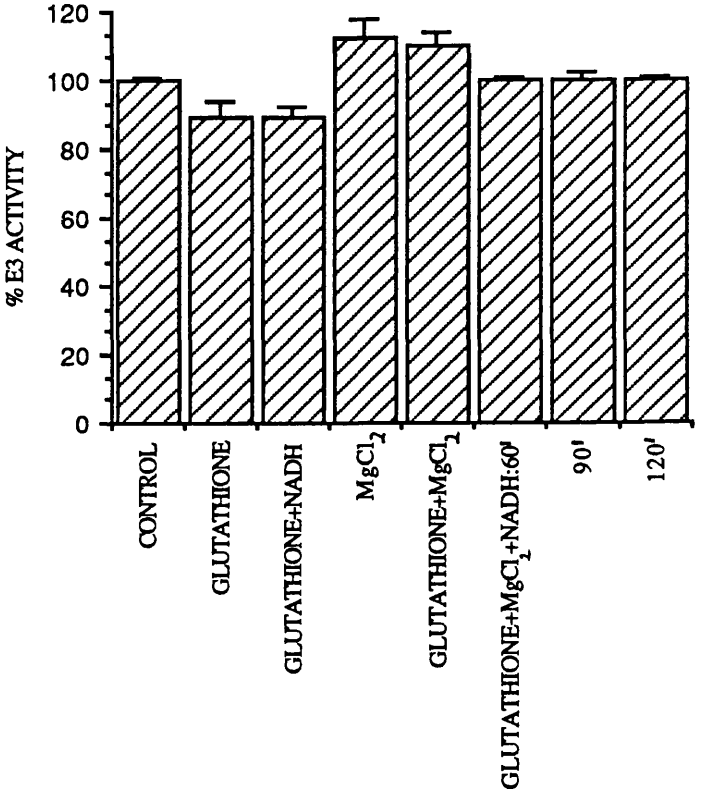

% E3 ACTIVITY
150
100
50
0
CONTROL
INHIBITOR
INHIBITOR+NADH
MgCl2
INHIBITOR+MgCl2
INHIBITOR+MgCl2+NADH:60'
90'
120'

Fig. 5.4 Effect of glutathione on E3 enzyme activity in the intact and salt treated complex

OGDC was diluted in 50mM potassium phosphate buffer, pH 7.6, to a final concentration of 1mg/ml. NADH was added to samples at a final concentration of 0.5mM for 10 min prior to the addition of 0.5mM glutathione. In cases where it was necessary to subject the complex to high ionic stength, OGDC was incubated with 0.25M $MgCl_2$ for 1h at 4°C and then treated in a similar manner to that outlined above.

Uless otherwise indicated, values shown represent the enzyme activity of the samples after 2h.

Results shown represent the mean value of triplicate assays.


% E3 ACTIVITY
120
100
80
60
40
20
0
CONTROL
GLUTATHIONE
GLUTATHIONE+NADH
MgCl2
GLUTATHIONE+MgCl2
GLUTATHIONE+MgCl2+NADH:60'
90'
120'

degradation; therefore in subsequent experiments undegraded NADH was prepared just prior to use in Tris-HCl, pH 7.2 and was never stored for use in later experiments.

In the studies which follow, OGDC was treated with conditions of high ionic strength, known to cause E3 to dissociate from the core, while not affecting overall complex activity. Using the appropriate controls, several different thiol group specific reagents were incubated with the salt treated complex in conjunction with NADH to determine if E3 activity was affected by such compounds. Fig. 5.2 shows the effect of N-ethylmaleimide both on untreated complex and OGDC dissociated with 0.25M $MgCl_2$; when the intact complex was treated with N-ethylmaleimide only, or NADH in the presence of N-ethylmaleimide, there was no appreciable effect on E3 activity even after 2h. It would appear that E3 is not susceptible to inactivation by N-ethylmaleimide in the native (intact) complex. In contrast, treatment of OGDC with high concentrations of salt appeared to cause E3 inhibition by N-ethylmaleimide in a time dependent manner. Fig. 5.2 shows that E3 activity, in the dissociated complex, was inhibited to 70% of its original activity after 1h incubation with N-ethylmaleimide in the presence of NADH. Further decreases in E3 activity were observed at later time intervals.

p-aminophenyldichloroarsine ($H_2NPhAsCl_2$), is an arsenical compound which exhibits a strong affinity for dithiols with a redox-active disulphide pair and has been shown to be capable of chemically modifying the E3 enzyme at its active site (Danson *et al.*, 1987). Fig. 5.3 shows the result of treating OGDC with this compound (termed inhibitor in the legend). When added to the intact complex in the absence of NADH there is no observable effect on E3 activity; however, in contrast to the previous result obtained with N-ethylmaleimide, addition of the arsenical compound in conjunction with NADH resulted in a significant decrease to approx. 50% of the original activity. Treatment of OGDC with high salt concentrations to cause dissociation of the complex resulted in further inhibitions of E3 activity to approx. 15% of the original activity after 2h. The final experiment performed in this section illustrates the effect of glutathione, (oxidised and reduced forms), on E3 activity. From Fig. 5.4, it is apparent that within

Fig. 5.5 Proteolytic degradation of OGDC and the effect of N-ethylmaleimide on E3 enzyme activity

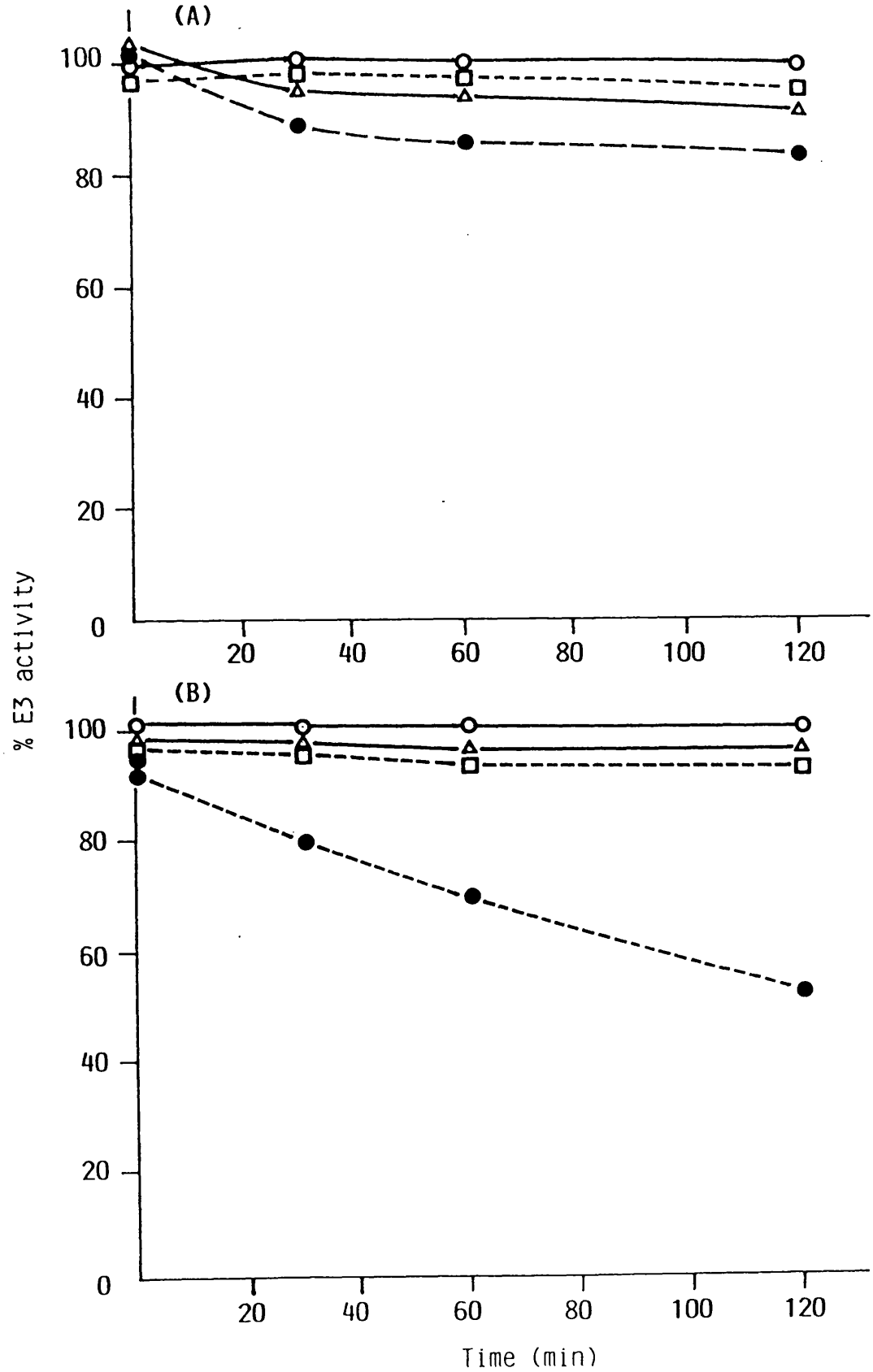
OGDC was diluted with 50mM potassium phosphate buffer, pH 7.6, to a final concentration of 1mg/ml. Enzyme was then subjected to proteolytic degradation as follows:

Panel A- OGDC was digested with 0.01%(w/w) trypsin for 3 min at 30°C, after which soya bean trypsin inhibitor (x10) was added.

Panel B- OGDC was digested with 9%(w/w) protease arg C for 1h at 30°C; protease arg C was added in 3%(w/w) aliquots at times 0 min, 20 min and 40 min respectively. Digestion was inhibited after 1h by the addition of benzamidine to a final concentration of 1mM.

After digestion, aliquots of enzyme were removed from the initial digest and treated as follows: NADH added to a final concentration of 0.5mM (Δ——Δ), N-ethylmaleimide added to a final concentration of 0.5mM (□- - -□), NADH and N-ethylmaleimide both present at concentrations of 0.5mM (●- - -●) where NADH was added 10 min prior to the addition of N-ethylmaleimide. 20mM mercaptoethanol was added to all samples 10 min prior to assaying enzyme activity. In addition to the above samples, a control sample representing untreated OGDC was assayed throughout the duration of the experiment (o——o). E3 activity was measured as described in the Methods.

All values shown represent the mean value of triplicate assays.


(A)
100
80
60
40
20
0
20
40
60
80
100
120
(B)
100
80
60
40
20
0
20
40
60
80
100
120
% E3 activity
Time (min)

the limits of experimental error, glutathione has no significant effect on E3 activity, either in the intact or dissociated complex.

It has been shown that treatment of OGDC with conditions of high ionic strength has a negligible effect on overall complex activity, demonstrating that under such conditions the dissociated subunits of the complex are still able to associate with each other. From the experiments performed above, it is apparent that the redox-active disulphide group forming the active site of E3 can be inhibited by N-ethylmaleimide in an NADH dependent manner, but only when the complex has been salt treated. This result implies that when E3 becomes dissociated from the E2 core the active site of E3 which is normally shielded in the native complex, is unmasked and becomes susceptible to inhibition by N-ethylmaleimide.

Further analyses were performed to determine if specific degradation of E1 was capable of exposing the active site of E3, causing it to become susceptible to inhibition by N-ethylmaleimide in an NADH dependent manner; If E1 and E3 associate with each other in the native complex as suggested by earlier experiments, degradation of E1, in addition to promoting E3 dissociation from the core assembly, may also expose the E3 active site so allowing it to be modified by thiol group specific reagents. As shown in Fig. 5.5, OGDC was treated with either 0.01%(w/w) trypsin or 9%(w/w) protease arg C, these levels of protease known to degrade E1 specifically; after such treatment, the E1 degraded complex was incubated with N-ethylmaleimide in the presence of NADH and the E3 activity was measured. Panel A shows the results obtained for tryptic digestion; initially, after a small decrease in E3 activity within 20 min, the enzyme activity remained unchanged for the remainder of the experiment suggesting that N-terminal cleavage of E1 was not sufficient to unmask the E3 active site. However, in the case of protease arg C (panel B), in the presence of both N-ethylmaleimide and NADH a decrease in E3 activity was observed throughout the time course of the experiment, such that approx. 50% inhibition of E3 activity was achieved within 2h. The cause of this steady decrease is unclear as it cannot be attributed to an increase in the inhibitory actions of either NADH or N-ethylmaleimide during the course of the

experiment, as they were seen to have no effect on the E3 activity of the control samples. Thus, treatment of OGDC with protease arg C but not trypsin, appears to render the E3 enzyme more susceptible to inhibition by N-ethylmaleimide but only in an NADH dependent manner. It may be postulated therefore, that E1 protects the active site redox-active disulphide pair of E3 from the NADH dependent inhibition caused by N-ethylmaleimide. However, this experiment does not allow for the exact region of E1 responsible for such protection of the E3 active site to be directly determined.

5.2.3 EFFECT OF GUANIDINIUM HYDROCHLORIDE TREATMENT ON E1 ACTIVITY OF OGDC

The effect of cleaving OGDC E1 specifically with trypsin was investigated with respect to the subsequent re-folding of the enzyme after denaturation with GdnHCl. This was performed to determine if trypsin cleavage of E1 caused significant changes in the susceptibility of the denatured enzyme to re-fold and regain its native conformation after GdnHCl treatment.

The native conformational states of proteins can usually be reversibly unfolded by the addition of denaturants such as GdnHCl to the purified protein in solution. Multi-domain proteins usually unfold stepwise, with the domains either unfolding individually (Privalov, 1982) or with varying degrees of interaction existing between them (Brandts *et al.*, 1989). Many purified proteins can spontaneously refold *in vitro* after being completely unfolded, so the final three dimensional structure must be determined to a large extent by the primary structure. However, not all aspects of the primary structure are required for folding as experiments with proteins whose N-terminal sequences were altered from linear to circularised orientations, were shown to fold at nearly normal rates to the same folded conformation (Goldenberg and Creighton, 1983; Luger *et al.*, 1989). From such experiments the conclusion was drawn that the termini of neither the original nor the altered primary structures were crucial, so folding need not proceed from the N-terminus of the polypeptide chain.

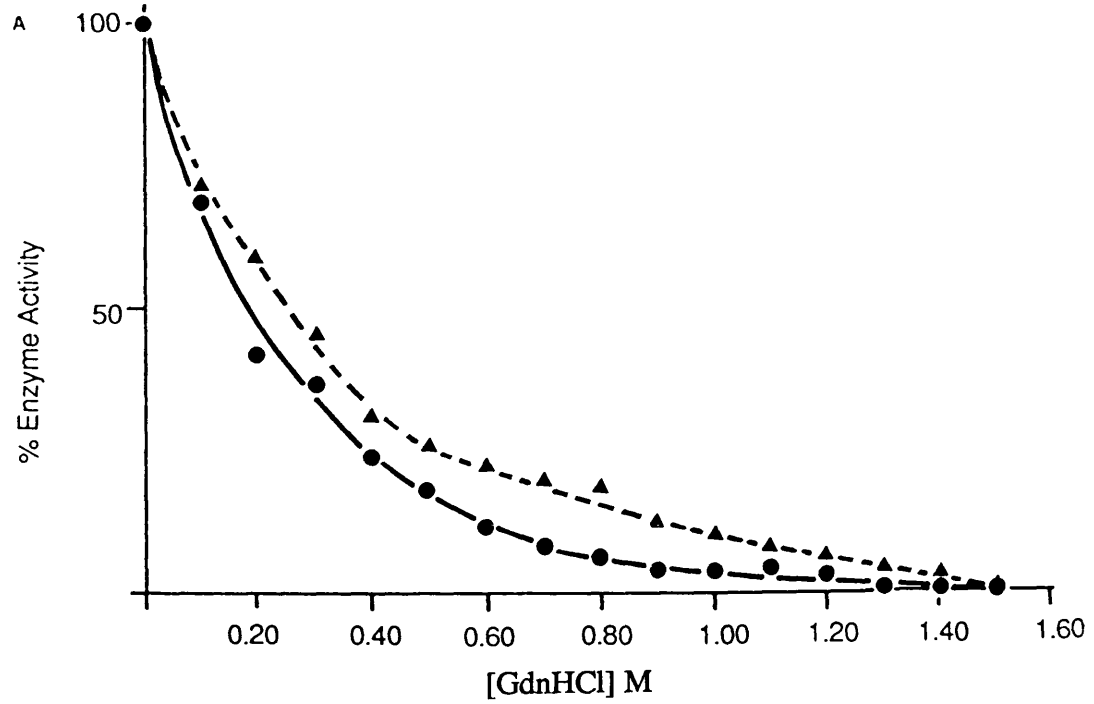
Fig. 5.6 Effect of guanidinium hydrochloride treatment on the E1 enzyme activity of OGDC

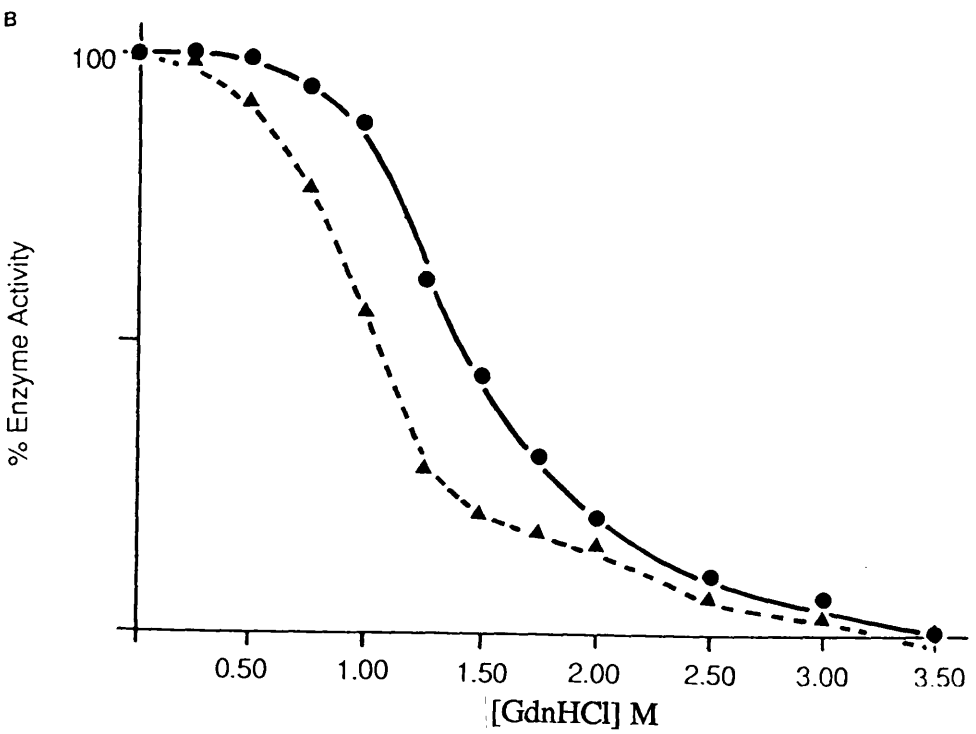
Intact OGDC was diluted with 50mM potassium phosphate buffer, pH 7.6, and then incubated at 4°C for 15 min with the appropriate concentration of guanidinium hydrochloride (GdnHCl). For tryptic degradation, OGDC was incubated with 0.01%(w/w) trypsin for 5 min after which soya bean trypsin inhibitor (x10) was added.

Panel A- intact and tryptic degraded OGDC were incubated at 4°C for 15 min with differing concentrations of GdnHCl. Prior to assay, the enzyme was diluted approx. 30 fold in assay mix containing the same concentration of GdnHCl as was present in the initial incubation mix. After 2 min at 30°C, E1 activity was assayed as described in the Methods.

Panel B- intact and tryptic degraded OGDC were incubated at 4°C for 15 min with varying concentrations of GdnHCl. Prior to assay, the enzyme was diluted approx. 30 fold in assay mix which contained no additional GdnHCl. After 2 min at 30°C, E1 activity was assayed as described in the Methods.

E1 activity measured for the intact complex (●——●), E1 activity measured for the tryptic degraded $E1^{l}$ complex (▲- - -▲).


A
100
50
% Enzyme Activity
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
[GdnHCl] M


B
100
% Enzyme Activity
0.50
1.00
1.50
2.00
2.50
3.00
3.50
[GdnHCl] M

When OGDC is incubated with GdnHCl, total complex activity is progressively lost on incubation of the complex with increasing concentrations of denaturant (not shown); total recovery of complex activity can be achieved by diluting out GdnHCl, up to concentrations of approx. 0.2M, above which 100% activity is no longer regained and the degree of reactivation observed diminishes rapidly. In experiments where the effect of such denaturation on E1 activity was measured, E1 activity was found to recover much more efficiently than previously observed for OGDC activity (not shown). Fig. 5.6 shows the results of using trypsin to degrade specifically E1 and the effect that this had on the unfolding of this enzyme. Panel A shows the profile obtained when loss of E1 enzymic activity was plotted against increasing concentrations of GdnHCl, panel B depicting the recovery of E1 activity as the concentration of denaturant was decreased. From panel A it is apparent that both E1 and $E1^{l}$ species are equally sensitive to GdnHCl inactivation, a 50% loss of activity for both species occurring in the range 0.2-0.25M of denaturant; examining the effect that removal of denaturant had on the recovery of enzyme activity revealed that $E1^{l}$ recovered much less efficiently than E1, where a 50% recovery of activity was achieved at 1.4M and 1.0M of denaturant for the E1 and $E1^{l}$ species respectively. It would appear therefore, that although $E1^{l}$ is capable of re-folding, its capacity to do so is significantly diminished when compared to the activity displayed by the re-folding of native E1. This suggests that although the cleaved region is not absolutely required for enzyme activity, it has a minor effect on the efficiency of the re-folding of E1.

5.2.4 EFFECT OF TRYPTIC DIGESTION ON THE CALCIUM SENSITIVITY OF OGDC

An increase in the concentration of mitochondrial Ca^{2+} ions in the range 0.1-1.0μM, is known to be a common mechanism for enhancing the rate at which respiratory fuels are metabolised in mammalian tissues (McCormack and Denton, 1979). This proposal is based on observations that Ca^{2+} ion concentrations in this range greatly alter the activity of the PDC phosphatase in relation to the associated PDC

Table 5.1 Effect of tryptic digestion on Ca^{2+} ion sensitivity of OGDC

OGDC was diluted in 50mM potassium phosphate buffer, pH 7.6, and treated with 0.01%(w/w) trypsin for 10 min. Digestion was terminated by addition of soya bean trypsin inhibitor and placing the sample on ice. The K_m values shown were calculated by measuring the rates of reaction at varying 2-oxoglutarate concentrations and conventional Michaelis-Menten reciprocal plot analysis. E1 assays were performed as described in the Methods except that 50mM MOPS pH 7.0 buffer was used instead. Where Ca^{2+} ions are present in the buffer, 5mM EGTA/5mM $CaCl_2$ was added; this corresponds to a free Ca^{2+} ion concentration of approx. 20-33μM.

Km VALUE FOR 2-OXOGLUTARATE (mM)

	+ Ca^{2+} ions	- Ca^{2+} ions
INTACT E1	0.16	0.30
TRYPSIN DEGRADED E1	0.15	0.32

kinase. The effects of calcium within the cell are important as many regulatory hormones and external stimuli acting upon mammalian cells, are capable of increasing the cytoplasmic concentration of this ion (Rutter and Denton, 1989). Ca^{2+} ions are also capable of affecting enzyme activity. For example, NAD^{+}-isocitrate dehydrogenase (Denton *et al.*, 1978) and OGDC, both representing important sites for regulation of the citric acid cycle, are known to be stimulated by increases in intracellular Ca^{2+} ion concentration. Experiments performed with purified OGDC demonstrated that the activity of isolated E1 is sensitive to the Ca^{2+} ion concentration (Lawlis and Roche, 1981b). It was postulated that Ca^{2+} binding sites located on E1 cause an increase in overall enzyme activity when occupied (Rutter and Denton, 1989). Analyses were performed to determine if the putative Ca^{2+} ion binding site thought to be present on E1, was located within the N-terminal region of E1 which is cleaved as a result of tryptic degradation. Since the complete amino acid sequence of this enzyme from a mammalian source was unknown at the time of these experiments, the exact location of the putative Ca^{2+} ion binding site was a matter for speculation.

In order to control the free concentration of calcium ions used in this experiment, free Ca^{2+} ions in the form of $CaCl_2$ were not added directly to the assay medium; instead EGTA buffers were used (Denton *et al.*, 1978; Portzhel *et al.*, 1964). A stock solution containing EGTA and $CaCl_2$, each present at 150mM, was prepared for use in regulating the free ion concentration for the experiment. The use of separate stocks of EGTA and $CaCl_2$ was avoided since the release of H^{+} that accompanies the binding of the metal ions to EGTA is minimised. Care was taken in the preparation of the 150mM EGTA/150mM $CaCl_2$ stock to ensure that they were both present in equimolar proportions; this was achieved by monitoring the release of H^{+} that occurred on addition of $CaCl_2$ to EGTA at pH 7.0. Enzyme measurements were performed in the presence of 5mM EGTA/5mM $CaCl_2$, corresponding to a free Ca^{2+} ion concentration of approx. 20-33μM as described by McCormack and Denton (1979). The results of this experiment are shown in Table 5.1; OGDC was treated with a level of trypsin sufficient to specifically degrade E1 to the $E1^{l}$ species. The K_m values of the intact and

trypsin-modified enzymes, were calculated by measuring enzyme activity at varying concentrations of substrate and plotting the reciprocals of the velocity of the reaction against substrate concentration . This was performed both in the presence and absence of Ca^{2+} ions (conc. approx. 30μM). Results obtained show that similar K_m values (approx. 0.15-0.16mM), were observed for both the intact and tryptic degraded E1 enzyme in the presence of Ca^{2+} ions. In the absence of Ca^{2+} ions, a two-fold increase in the K_m value (approx. 0.30-0.32mM) was observed; this was true for both the intact and $E1^l$ species.

5.3 DISCUSSION

5.3.1 ROLE OF E1 IN SHIELDING THE E3 ACTIVE SITE REGION

The experiments in this section were performed to examine more closely the nature of the association existing between E1 and E3 in the complex, in particular the possible involvement of E1 in the active site protection of E3.

It has been shown that dissociation of the enzyme subunits from each other using above physiological concentrations of salt has no effect on overall activity; the complex only becoming inhibited if after salt treatment, it is subjected to gel filtration chromatography resulting in the physical separation of the subunits from each other. In contrast to the effects of salt treatment alone, if OGDC is degraded specifically with trypsin, treatment of the degraded complex with increasing salt concentrations results in a corresponding decrease in overall activity. This result implies that as E1 is cleaved at its N-terminus, the subunits are unable to associate with each other such that the complex becomes increasingly inhibited in conditions of high ionic strength.

In the salt treated complex it can be shown that E3 activity is unaffected; however, it is possible to inhibit E3 activity by the use of certain thiol group specific compounds in the presence of NADH. For example, when OGDC dissociated by treatment with

$MgCl_2$ was incubated with N-ethylmaleimide in the presence of NADH, a decrease in E3 activity was observed. Similarly, the arsenical compound p-aminophenyldichloroarsine was capable of inhibiting E3 activity in the intact complex when NADH was present thus, the E3 active site appears to be directly accessible to this reagent. The difference between the results obtained with the arsenical compound and N-ethylmaleimide, probably reflects differences in the reactivity and accessibility of these reagents with the redox-active disulphide pair, present at the E3 active site.

Treatment of OGDC with concentrations of salt capable of causing dissociation of the complex resulted in E3 becoming susceptible to inhibition by N-ethylmaleimide, but only in an NADH dependent manner. The manner in which the E3 active site in the native complex was protected from N-ethylmaleimide inhibition remains unclear. Experiments involving the proteolytic degradation of E1 with either trypsin or protease arg C, were performed to determine if E1 was responsible for protecting the E3 active site such that it became susceptible to inhibition in the proteolytically degraded complex. Tryptic degradation of OGDC resulted in no NADH dependent inhibition of E3 activity in the presence of N-ethylmaleimide. As was shown from earlier gel filtration experiments, the $E1^l$ species formed as a result of tryptic digestion showed a tendency to dissociate from the complex. It is possible that on tryptic digestion, the $E1^l$ species remains associated with E3 so that its active site remains inaccessible; this result would imply that the N-terminal region of E1 is involved directly in shielding the E3 active site. In contrast, when E1 was non-specifically degraded using protease arg C, a decrease in E3 activity by N-ethylmaleimide in the presence of NADH was apparent. This result suggests that E1 and E3 do associate with each other in the complex such that the active site of E3 is protected by E1, although this function cannot be assigned to a particular region on the E1 enzyme. It would appear that protease arg C treatment of OGDC has a similar effect to that seen when the complex is treated in conditions of high ionic strength , where E3 becomes inhibited by the thiol group specific compound N-ethylmaleimide in an NADH dependent manner. This result is similar to that observed with PDC, the specific degradation of protein X with protease

arg C rendering the complex susceptible to inhibition by N-ethylmaleimide in the presence of NADH. An explanation as to why the E3 active site needs to be protected by either E1 of OGDC or protein X of PDC is lacking. It may represent a means of shielding the active site from potentially toxic compounds capable of affecting enzyme activity, such as free radicals or peroxides within the cell.

5.3.2 ROLE OF THE N-TERMINAL REGION OF E1 IN Ca^{2+} SENSITIVITY OF OGDC

The binding of Ca^{2+} ions to both OGDC and NAD^+-isocitrate dehydrogenase has been examined previously in pig heart (Rutter and Denton, 1989). The stoichiometry of binding obtained (3.5+/- 0.07mol/mol complex) was less than expected for a site on each of the individual molecules of the complex. Since both E1 and E3 are homodimers, the results obtained were consistent with a single Ca^{2+} binding site on either of these enzymes. As the reaction catalysed by the E1 enzyme is the rate-limiting step of the overall reaction sequence catalysed by OGDC (Walsh *et al.*, 1976), this enzyme would be the most logical site at which to regulate complex activity. Supporting this idea, Lawlis and Roche (1981b) found that E1 activity, but not E3 activity, was sensitive to Ca^{2+} ions *in vitro*. Ca^{2+} ions act to stimulate OGDC activity by decreasing the K_m of the enzyme for 2-oxoglutarate, the substrate for the rate-limiting E1 enzyme of the complex. From such experiments, the data obtained suggested that Ca^{2+} ions probably bind to the E1 subunits. However, the methodology used in these experiments did not allow for E1 and E2 to be separated. From the results presented earlier in Table 5.1, it is apparent that tryptic degradation of the E1 enzyme to form $E1^l$ has no significant effect on the ability of the enzyme to be stimulated by Ca^{2+} ions; this appears to suggest that the N-terminal region of E1 is unlikely to be directly involved with the Ca^{2+} ion dependent regulation of OGDC activity. The K_m values as calculated from this experiment show only a 2-fold increase when measured in the presence and absence of Ca^{2+} ions. These results differ in some

respects from those obtained by McCormack and Denton (1979), who achieved an approx. 4-5 fold activation of pig heart OGDC activity in the presence of Ca^{2+} ions.

The results obtained here may be explained by the failure to include 5mM EGTA in those samples where Ca^{2+} ions should have been absent. Thus endogenous Ca^{2+} ions in the buffers or tightly-associated with the enzyme itself may not have been totally removed. More detailed analysis of these preliminary findings is required in particular, as regards the inclusion of chelating agents in those samples where Ca^{2+} ions should be rigorously excluded. However, in spite of the obvious limitatons of this experiment, it seems likely that the N-terminal region of the E1 enzyme is not directly involved with the Ca^{2+}-dependent regulation of OGDC activity.

A feature known to be diagnostic of calcium binding sites in proteins is the EF hand structure; this region is composed of a linear sequence of between 30-35 amino acids, where the N- and C-terminal helical regions flank a 12 residue Ca^{2+} binding loop. By alignment of the primary sequences of the EF sites from several proteins stimulated by Ca^{2+} ions common features were identified, the most conserved being a strong β-turn forming region in the Ca^{2+} ion binding loop (Gariepy and Hodges, 1983). From analysis of the OGDC E1 sequence from human foetal liver as deduced by Koike *et al.* (1992), no evidence could be found for the existence of such a Ca^{2+} ion binding region on the bovine heart OGDC E1 enzyme.

CHAPTER SIX

DISCUSSION

6.1 INTRODUCTION

The 2-oxoacid dehydrogenase multienzyme complexes are responsible for catalysing the oxidative decarboxylation of a variety of 2-oxoacids in the cell (Perham, 1991). They perform a central role in metabolism being responsible for catalysing several of the key regulatory reactions in carbohydrate and amino acid metabolism. These large multimeric structures are composed of three distinct subunits termed E1, E2 and E3, were the E2 subunits bind to each other forming a large core structure around which E1 and E3 bind non-covalently. It has been shown that PDC isolated from eukaryotic sources also contains an additional polypeptide, commonly referred to as protein X. The exact functioning of this protein within the complex is still a matter of some debate, although it is generally believed to be involved in a structural capacity assisting with binding E3 to the core. A major problem in proposing such a role for protein X is that OGDC and BCOADC, which both contain the identical E3 gene product to that in PDC, do not possess an analogous protein X subunit. It would appear therefore, that protein X is the product of a specific gene only in PDC.

Direct protein sequencing of the N-terminal region of E1 of bovine heart OGDC, revealed surprising sequence similarity with the N-terminal region of both the eukaryotic E2 acetyltransferases and the protein X component of PDC. It is postulated that a protein X like sequence which is present as a distinct subunit in PDC, has become incorporated onto one of the integral subunits of OGDC as the result of a gene shuffling event.

6.1.1 ROLE OF THE N-TERMINAL REGION OF E1 IN BOVINE HEART OGDC

Lipoyl domains present at the N-terminal region of the E2 acyltransferases and protein X of PDC, can be removed by proteolysis using specific proteases; the cleaved lipoyl peptides have an apparent M_r 15,000 as determined by their migration in SDS/polyacrylamide gels. When the N-terminal amino acid sequence of E1 from bovine

heart OGDC was obtained by direct protein sequencing it showed sequence similarities, also in the N-terminal region, to both the eukaryotic E2 acetyltransferases and protein X.

The amino acid sequence determined for E1 of OGDC possessed a slightly modified form of the sequence PSLSPTM, which precedes the highly conserved sequence motif containing a lipoylatable lysine residue. It was thought originally that the existence of such a sequence at the N-terminus of E1 might signify the presence of a lipoyl domain; this assumption was based not only on the presence of this sequence at the N-terminus, but on its ability to be removed by trypsin similar to lipoyl domains, although yielding a smaller peptide of M_r approx. 8,000. To determine if this subunit possessed a catalytically active lipoyl domain at its N-terminus, OGDC was treated with radiolabelled pyruvate, a substrate known for its ability to incorporate [^{14}C] acetyl groups into the lipoic acid groups of the E2 acetyltransferases and protein X subunits of PDC. Radiolabelling experiments performed with OGDC revealed no incorporation of [^{14}C] groups into E1; this suggested that the lipoyl-like sequence present on this subunit was not catalytically active. Experiments performed with the radiolabelled substrate 2-oxoglutarate, yielded similar results with no incorporation of succinyl groups into E1 observed. These results were later confirmed when the sequence for OGDC E1 from human foetal liver was published; inspection of this sequence showed the absence of the sequence containing the critical lysine residue.

From examination of the effects of proteolytic degradation on complex activity, it appeared that the 'lipoyl-like' domain present at the N-terminus of OGDC E1 was essential for the maintenance of overall activity; cleavage of this region resulted in the rapid loss of overall enzyme activity, while E1 activity remained unaffected. Cleavage of E1 by trypsin to form the E1^{l} species is probably a random process resulting initially in the formation of E1/E1^{l} heterodimers; observations that E1 cleavage proceeds more rapidly in the initial stages of digestion indicate that it may be necessary to produce E1^{l} homodimers before the ability of the E1 subunits to maintain overall activity is lost. It would appear, therefore, that the N-terminal region of E1 is involved in maintaining overall OGDC activity. In view of the sequence similarity between E1 of OGDC and

protein X of PDC from bovine heart, the results obtained for OGDC are in contrast to those for PDC, where protein X can be cleaved to remove the lipoyl domains only without any significant effect on complex activity.

Although protein X from eukaryotic PDC contains a lipoyl group which is capable of being acetylated, its catalytic role within the complex is unclear. When the protein X gene from *S. cerevisiae* was sequenced (Behal *et al.*, 1989), the clone was found to lack the highly conserved acetyltransferase active site sequence motif normally present in the C-terminal region. They assumed that protein X, in contrast to the E2 acetyltransferases, was incapable of catalysing the transfer of acetyl groups between S-acetyldihydrolipoamide and CoA. Sequencing of a cDNA clone obtained for the C-terminal region of protein X from bovine heart PDC, conflicted with the results obtained for *S. cerevisiae*; the clone isolated from bovine heart was shown to possess a variation on the conserved motif, His-Xaa-Xaa-Xaa-Ser-Gly, which is located near the C-terminus and is essential for acetyltransferase activity (J. Neagle, unpublished results). It would appear that protein X of bovine heart PDC, may retain the capacity to participate in acetyl transfer reactions within the complex. Since the sequence of the *S. cerevisiae* protein X clone is distinct from that obtained for the partial clone of protein X from bovine heart, it suggests that the clones may differ from each other in their C-terminal regions. Collagenase treatment of PDC preferentially removes the lipoyl domains from the N-terminus of E2, while leaving those on protein X intact (Rahmatullah *et al.*, 1989). Treatment of bovine heart PDC with collagenase to cause total removal of lipoyl domains from the E2 subunits only, resulted in the complex retaining approx. 10-20% of its original overall activity. This demonstrates that in addition to the lipoyl domains on E2 interacting with E1, those present on protein X are also capable of interacting with E1 so allowing overall complex activity to be maintained. In mutagenesis experiments performed by Russell and Guest (1990), substitution of the acetyltransferase active site histidine residue of *E. coli* PDC E2, resulted in a total loss of enzymic activity; this result was in direct contrast to Niu *et al.* (1990), who claimed to be able to mutate the corresponding acetyltransferase active site histidine for E2 of

S. cerevisiae without any resultant loss of activity. This result is puzzling since a high degree of sequence conservation has been shown to exist between the E2 enzymes.

6.1.2 ROLE OF THE E1 N-TERMINAL REGION IN BINDING E3 TO THE CORE COMPLEX

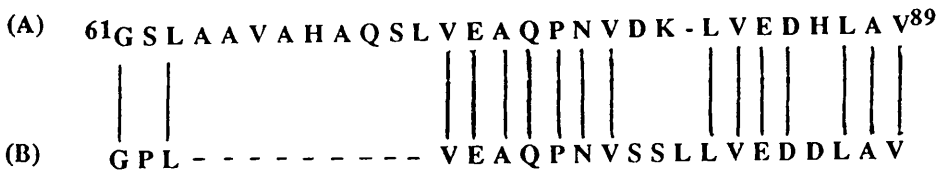
Treatment of PDC with protease arg C resulting in removal of the lipoyl domain from the N-terminal region of protein X, causes E3 to show a reduced affinity for the E2 core structure such that it can be preferentially dissociated (Neagle and Lindsay, 1991). Protease arg C degradation of PDC resulted in protein X forming two polypeptides of M_r 35,000 and 15,000, reduced E3 binding is apparent after release of the lipoyl domain. N-terminal amino acid sequence of this M_r 35,000 peptide was obtained and compared with the sequences corresponding to the linker regions and E3 binding domains of the E2 enzymes from the 2-oxoacid dehydrogenase complexes (S. Sanderson and J. Neagle, unpublished results). By identifying the site at which protease arg C cleaved, it was discovered that the protease cleaves within the linker region at, or very close to, the boundary of a putative E3 binding domain present on protein X of bovine heart PDC. Thus, cleavage of protein X with protease arg C and the subsequent release of E3, may reflect the instability of the putative E3 binding domain thought to be present on protein X; at longer time intervals of incubation the M_r 35,000 species can be seen to be degraded further producing a polypeptide M_r 30,000; cloning studies of *S. cerevisiae* protein X (Lawson *et al.*, 1991), revealed the presence of a small domain (approx. M_r 5,000) situated between residues 144-180, which showed distant but related homology to the E3 binding domain. Thus, production of the lower M_r 30,000 species is most probably related to cleavage of the putative E3 binding domain region.

When bovine heart OGDC was treated with trypsin, the proteolytically sensitive E1 enzyme was cleaved at the N-terminus as evidenced by protein sequencing. This digestion resulted in production of a peptide approx. M_r 8,000 to form a lower M_r species termed E1^{l}. During trypsin treatment of OGDC, the lipoyl domains on E2 were removed at a slower rate, the E3 enzyme remaining intact. In contrast to trypsin,

treatment of OGDC with protease arg C caused extensive degradation of the E1 enzyme, with both the E2 and E3 remaining undegraded. However, in common with tryptic degradation, protease arg C treatment of OGDC effected preferential dissociation of E3 from the core structure; as in the case of trypsin, the newly formed $E1^{l}$ species exhibited a marked tendency to dissociate from the complex. The proteolytic degradation of E1 and subsequent dissociation of E3 from OGDC would appear to suggest that an interaction between these two enzymes exists within the complex.

Sequence motifs for an E3 binding domain have been located on the E2 subunits of the 2-oxoacid dehydrogenase complexes; however, when the E2 enzyme of OGDC was sequenced from rat heart by Nakano *et al.* (1991), no sequence which could be identified as representing an E3 binding domain was found to be present. It is possible, that the novel sequence which was found instead at the expected location of the E3 binding domain, was involved with binding E3. Alternatively, a distinct E3 binding domain may exist elsewhere in the complex. A possible location for the E3 binding domain if not on E2, may be E1 as suggested from results presented earlier in this thesis. As stated previously, when OGDC was treated with trypsin and then subjected to gel filtration, in addition to E3 dissociation, the $E1^{l}$ species also showed a marked tendency to dissociate from the core. The mode of association between $E1^{l}$ and E3 after tryptic degradation of the complex is subject to speculation; for example, $E1^{l}$ may be released on its own as distinct species or, $E1^{l}$ and E3 may remain in direct association with each other. The latter case is unlikely as both $E1^{l}$ and E3 were found to elute at distinct volumes on the gel filtration profile with M_r values corresponding to the free enzymes; this indicates that they do not associate with each other, although it is possible that any interaction existing between them is very weak so as not to be detectable on gel filtration analysis.

Fig. 6.1 Comparison of the OGDC E1 enzyme isolated from bovine heart and human foetal liver



(A) Amino acid sequence of OGDC E1 from human foetal liver deduced from the nucleotide sequence

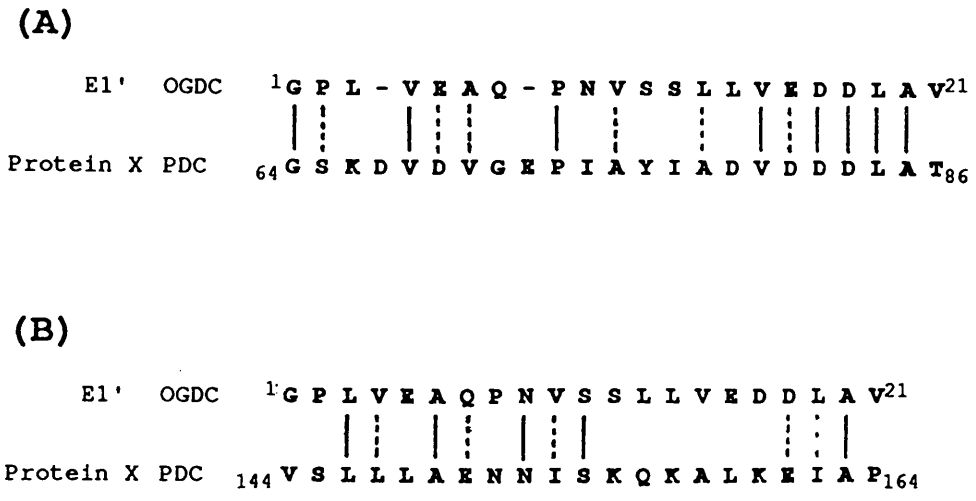
(B) Amino acid sequence of OGDC E1 from bovine heart deduced from direct protein sequencing. Protein sequence shown is continuous with gaps inserted for the purposes of matching.

6.1.3 EVIDENCE FOR A PUTATIVE E3 BINDING DOMAIN ON E1 OF BOVINE HEART OGDC

Prior to starting this project the complete amino acid sequence of E1 OGDC from a mammalian source was unknown. As a result, many of the experiments presented in this thesis were performed before publication of the human foetal liver OGDC E1 sequence (Koike *et al.*, 1992). It is known that the specific cleavage of E1 from OGDC results in dissociation of E3 from the core; this result is similar to that observed in PDC on the specific degradation of protein X , which also results in release of E3 from the core complex. Protein X of *S. cerevisiae* is thought to contain a small domain of approx. M_r 5,000 which has been implicated in the binding of E3. It has not proved possible to identify a similar subunit binding domain on the protein X subunit of PDC from bovine heart, since cloning of a full length copy of the gene has not as yet been achieved. However, it is assumed that protein X from bovine heart contains a putative E3 binding domain on the basis of the results of proteolysis experiments; cleavage of protein X in bovine heart PDC using protease arg C resulting in formation of a M_r 35,000 species as a result of lipoyl domain cleavage. The cleavage site has been identified as occurring at, or near, the boundary of a putative E3 binding domain (S. Sanderson, unpublished results). In view of the sequence similarity shared by E1 of OGDC and protein X of PDC from *S. cerevisiae*, the existence of a putative E3 binding domain on E1 of OGDC was considered.

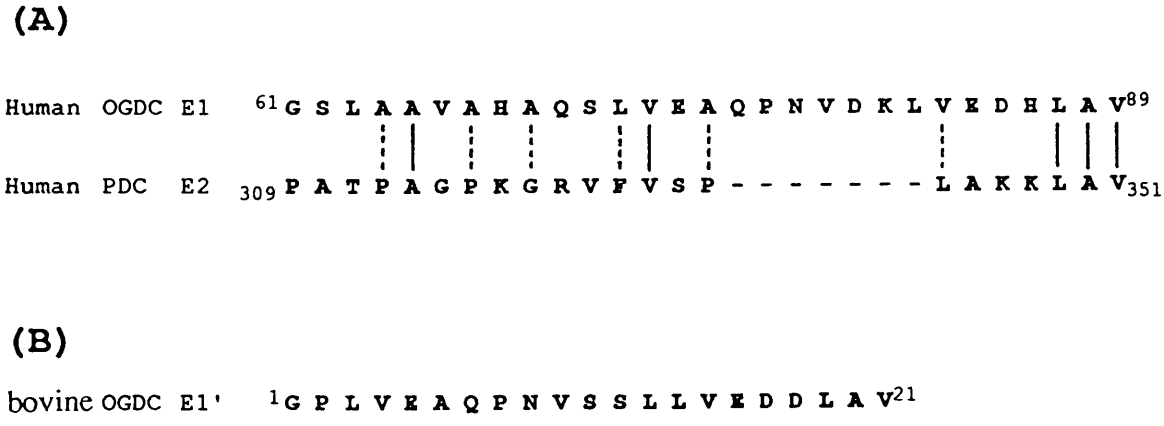
Comparison of the amino acid sequences for E1 from bovine heart and human foetal liver OGDC, revealed the presence of the sequence motif PFLSGTS at their N-termini. Fig. 6.1 shows the result of further matching between the $E1^l$ species from bovine heart with the human E1 enzyme; the GPL motif present in the bovine sequence can be matched with the human E1 enzyme at the sequence GSL (residues 61-63). Matching the sequences at this position presumes that a deletion of approx. 9 amino acid residues has occurred in the bovine sequence; alternatively, insertion of additional residues into the human E1 enzyme may have occurred instead. This sequence alignment shows that a degree of similarity exists between the E1 enzyme of OGDC isolated from both human

ig. 6.2 Sequence alignment between the E1′ species of bovine heart OGDC and protein X of PDC from *S. cerevisiae*



The match shown in panel A is a result of visually matching the sequences to each other. The match in panel B was obtained using the computer programme SWISSPROT.

Fig. 6.3 Sequence alignment between human OGDC E1 and the E3 binding domain as determined from human PDC E2



Panel A shows the alignment when human OGDC E1 sequence was probed with the E3 binding domain motif from human PDC E2 (residues 309-351).

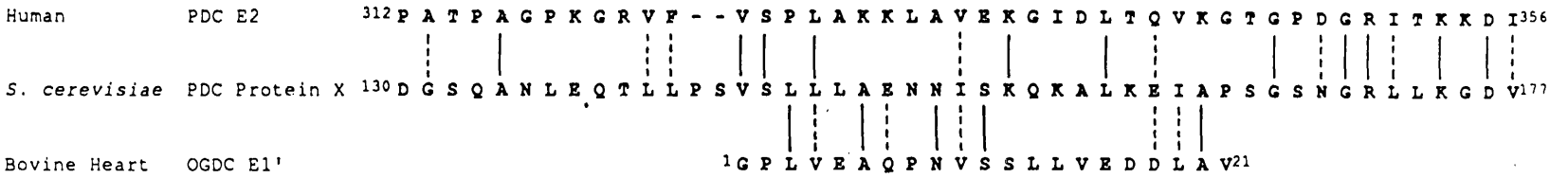
Panel B depicts the region of $E1^{l}$ formed as a result of tryptic degradation of bovine heart OGDC E1. This region was matched earlier to residues 61-89 of human OGDC E1 (see Fig. 6.1).

and bovine sources. The matching of the bovine heart OGDC E1^{l} sequence to this region of the human sequence corresponds closely to the expected site of tryptic cleavage in the bovine heart E1 enzyme, since a peptide of approx. M_r 8,000 is cleaved from the N-terminus of E1 to form the E1^{l} species. The validity of the protein sequence obtained for the E1^{l} species is not in doubt as this particular region of the protein was independently sequenced several times.

When the N-terminal region of bovine heart OGDC E1 was originally sequenced, similarity was observed with the N-terminus of protein X from *S. cerevisiae*. It was postulated that these protein species were performing similar functions in their respective complexes by mediating in an interaction between E3 and the core structure. If this is true, it may be assumed that in addition to similarity at the N-terminus, additional regions of internal sequence similarity may also exist between the proteins. Following this reasoning, the protein X sequence from *S. cerevisiae* was matched with the E1^{l} amino acid sequence of bovine heart OGDC E1, the results shown in Fig. 6.2. In panel A the match was performed visually in contrast to panel B where a best fit was calculated using the computer programme SWISSPROT. It is evident that the region to which the E1^{l} sequence maps is dependent on the method used for matching; when the alignment was performed visually, the two sequences mapped to a region between residues 64-86 of the protein X sequence, with the computer alignment matching E1^{l} with residues 144-164 of protein X.

As discussed earlier, proteolytic degradation of bovine heart OGDC E1 by trypsin results in the dissociation of E3 from the complex, the lower M_r E1^{l} species formed as a result of digestion showing a marked tendency to dissociate from the core. The possibility of the existence of an E3 binding domain on E1 of OGDC was investigated by computer alignment of the human E1 OGDC sequence with the human E3 binding domain present on the E2 enzyme of mammalian PDC (Thekkumkara *et al.*, 1989). The results of such a search are shown in Fig. 6.3. Panel A shows the results of probing the entire length of the human E1 OGDC sequence with the human E3 binding domain (PDC E2 residues 309-351) using the computer programme SWISSPROT. This search

Fig. 6.4 Alignment of *S. cerevisiae* protein X and bovine heart OGDC E1with the E3 binding domain present on human PDC E2



Bovine heart OGDC E1 was aligned with protein X; this region was probed with the E3 binding domain as deduced from human PDC E2. Computer alignments were performed according to the SWISSPROT programme.

results in alignment of the human E3 binding domain with residues 61-89 of the human OGDC E1 sequence. Although gaps have been inserted into the E3 binding domain sequence to allow for alignment, out of 963 residues which comprise the entire length of the E1 enzyme, this particular region was matched with the E3 binding domain. In Fig. 6.3, panel B shows the $E1^{l}$ sequence of bovine heart which was aligned in an earlier search with residues 61-89 of the human E1 sequence (see Fig. 6.1). The finding that the $E1^{l}$ sequence of bovine heart OGDC aligns with the region of human E1 which shares homology with the E3 binding domain, implies that a putative E3 binding domain is present on the bovine heart OGDC E1 enzyme. Thus, E1 of bovine heart OGDC has been shown to possess sequence similarity to protein X; E1 differs from protein X in that it appears to contain a residual form of a lipoyl domain at its N-terminus which is non-functional. This is closely followed by a region proposed to be involved with E3 binding, such that cleavage of E1 by trypsin results in release of E3 from the core.

When PDC is treated with protease arg C, the protein X subunit is degraded within the complex resulting in removal of the lipoyl domain from the N-terminus in addition to which, E3 dissociates from the complex. Fig. 6.4 shows a computer sequence alignment performed with the E3 binding domain from human PDC E2, the bovine heart OGDC E1 enzyme and protein X from *S. cerevisiae.*; this alignment shows that the human E3 binding domain maps to a region within the *S. cerevisiae* protein X subunit, which in turn maps to the region of the bovine heart OGDC E1 enzyme which has previously been postulated to contain an E3 binding domain. The existence of an E3 binding domain within protein X has been proposed previously by Lawson *et al.* (1991). Since protein X aligns to a region within bovine heart OGDC E1 which is proposed to be involved in E3 binding and is strong evidence that these two enzyme species are involved in promoting similar structural roles within their respective complexes.

Assuming that both protein X and the N-terminal region of the E1 enzyme of OGDC are involved in promoting an interaction of E3 with the complex, it is possible to theorise about how they may interact with the core assembly; PDC is thought to contain six homodimers of E3 and between 6-12 mols of protein X, although current estimates put this

value closer to twelve subunits (S. Sanderson, unpublished results). OGDC contains six homodimers each of E1 and E3. Since both of the interacting species are present within the complex in equimolar amounts, the potential exists for a 1:1 stoichiometric interaction between them. Whatever type of association that protein X and the E1 enzyme exhibit with the E2 core, treating the complexes with strongly dissociating conditions (4M urea) has been shown to be necessary to remove them from the core complex (Reed and Willms, 1969). In PDC, the E3 enzyme has been shown to exhibit an affinity for the E2 core (Gopalakrishnan et al., 1989), meaning that in addition to binding the E3 enzyme, protein X may also be involved in stabilising low affinity interactions of E3 with the core although by what mechanism this occurs is unknown.

6.2 FUTURE WORK

Future work would involve the isolation of a full length cDNA clone for the bovine heart OGDC E1 enzyme; this would allow not only for a direct sequence comparison to be made with the human E1 enzyme, but also to determine conclusively if an E3 binding domain motif was present. In addition, it would be desirable to clone and sequence OGDC E2 from other mammalian sources to determine if the E3 binding domain was present on this enzyme or, if it had been replaced by the novel sequence as found in rat heart OGDC E2. Once the E3 binding domain had been located within the complex, it would then be possible to perform studies where this domain could be deleted and the effect on complex stability investigated; for example, the mode of association between E3 and E1 within the complex could be studied in particular, to determine if the truncated $E1^{l}$ species and E3 enzyme associated intimately with each other or were present as distinct species on their release from the complex.

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