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IMMUNOLOGY AND BIOSYNTHESIS OF THE MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX

Thesis submitted for the Degree of Doctor of Philosophy

by OLGA G. LAMBERTUS DE MARCUCCI

Department of Biochemistry
University of Glasgow

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FOR ETTORE

One has but to renounce the false view that glorifies the effect of the activity of the heroes of history in warfare in order to discover the unknown quantity, x. X is the spirit of the army, the greater or less desire to fight and to face the dangers on the part of the men composing the army.....
To define and express the significance of this unknown factor, the spirit of the army, is the problem of Science.

Leo Nikolayevitch Tolstoy,

War and Peace

Abbreviations

The abbreviations recommended by the Biochemical Journal in its Instructions to Authors (1985) (Biochem. J. 225, 1-26) have been used throughout this Thesis with the following additions:

AcSCoA,	acetyl coenzyme A
BRL,	Buffalo rat liver cells
BSA,	bovine serum albumin
CCCP,	carbonyl cyanide, m-chlorophenylhydrazone
DMSO,	dimethylsulphoxide
DNP,	2,4-dinitrophenol
DTNB,	5,5'-dithio(bis) 2-nitrobenzoic acid (Ellman's reagent)
DTT,	dithiothreitol
EDTA,	ethylenediamine tetracetic acid
EGTA,	ethyleneglycol-bis-(3-amino-ethyl ether)N,N' tetracetic acid
FCCP,	carbonyl cyanide, 4-(5 fluoromethoxy) phenylhydrazone
Hepes,	4-(2-hydroxyethyl)1-piperazine ethanesulphonic acid
Leupeptin,	propionyl-L-leucyl-L-arginal
Mops,	4-morpholinopropanesulphonic acid
NBL-1,	bovine kidney cells
NEM,	N-ethylmaleimide
PBS,	phosphate buffered saline (see section 2.2.11. e)
PDC,	pyruvate dehydrogenase complex
PK-15,	pig kidney cells
PMSF,	phenylmethylsulphonyl fluoride
PPO,	2, 5-diphenyloxazole

SDS,	sodium dodecyl sulphate
TCA,	trichloroacetic acid
TEMED,	N,N,N',N'-tetramethylethylenediamine
TLCK,	N- α -ρ-tosyl-L-lysine-chloromethyl ketone
TPCK,	L-1-tosylamide-2-phenylethylchloromethyl ketone
TPP,	thiamine pyrophosphate
Tween 20,	polyethylene sorbitan monolaureate

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SUMMARY

The mammalian pyruvate dehydrogenase complex, located in the inner membrane-matrix compartment of mitochondria, is a large multi-molecular aggregate, M_r 8.5 x 10^6, containing multiple copies of its three constituent enzymes, pyruvate dehydrogenase (E1), dihydrolipoyl acetyltransferase (E2) and lipoamide dehydrogenase (E3).

High-titre, monospecific, polyclonal antibodies against the native complex and its individual components are characterised and employed to study the events involved in the biosynthesis and import of the components of this complex into mitochondria.

Antisera are examined with respect to their ability to inhibit the overall activity of the complex and of the intrinsic protein kinase. Monospecificity of all antisera is demonstrated when challenged with crude cell extracts and subsequent immunoblotting analysis. The antiserum to native PDC exhibits high reactivity against all components of the complex, except E3, which cannot be explained by a low content of E3 in the purified antigen or by its inaccessibility to the immune system. The significance of the low immunogenicity of E3 is probably related to the conservation of its primary sequence and tertiary structure during evolution.

A major observation from this study is that ox heart PDC contains an additional polypeptide, M_r 51,000 ± 1,000 of unknown function. This protein is called component X and constitutes approx. 6% of the total complex. Detailed immunological studies suggest that component X is a normal cellular component, located in the mitochondrial compartment and does not represent a fragment of subunit E2 or the intrinsic protein kinase of the complex.

Additional studies on the subcellular localisation of PDC suggest that this enzyme is associated with the inner membrane of rat liver and ox heart mitochondria. These and other studies also indicate that component X is an integral component of the complex and not a membrane protein, which becomes associated with the PDC during its isolation. The individuality of protein X is also demonstrated by comparison of the peptide maps of the ^{125}I and ^{14}C-labelled subunits E2 and X obtained with several proteases. Protein X seems to be tightly-associated with the E2 core of the complex and is at least partially exposed on the surface of the native assembly, as it is accessible to proteases and to antibodies directed against it.

Further studies reveal that, after incubation of the complex in the presence of [2-^{14}C] pyruvate, ^{14}C-label, probably in the form of acetyl groups is incorporated rapidly into both E2 and component X. Phosphorylation of the complex causes a parallel decrease in the acetylation of both proteins, indicating the involvement of the E1 component in the acetylation of these groups. Similarly, the transfer of acetyl groups from E2 and X onto CoA is observed to occur in a parallel fashion from both proteins. Studies on the effects of NEM on the incorporation of ^{14}C-labelled acetyl groups also reveal interesting features of the acetylation reaction, which suggest the existence of secondary NEM-sensitive acetylation sites on the complex. Additional studies on the nature of the 'acetylatable' group in protein X and the physiological acceptor of acetyl groups are required to clarify the function of this component in the acetylation reactions of the complex.

Studies on cultured bovine kidney, rat liver and pig kidney cells, incubated with [^{35}S] methionine in the presence of uncouplers of

oxidative phosphorylation, demonstrate the accumulation of larger M_r precursor polypeptides to the subunits E2, E3, El α and El β of the complex. Precursor forms for the individual subunits of the PDC are identified by immunoprecipitation techniques using antisera against the native complex and its SDS-denatured subunits followed by fluorographic analysis.

These precursors, possessing M_r values 2,000-8,000 larger than their mature counterparts in the mitochondria, are relatively stable in the cytoplasm of the cells when monolayers are incubated in the presence of uncouplers for several hours. Removal of the uncoupler and subsequent chase shows that these precursors are processed into their mature forms with a similar lag time. Complete processing is observed within 30 min.

Chapter 1: INTRODUCTION

1.1 Multienzyme complexes

Enzymes are biological catalysts which usually function sequentially by promoting consecutive chemical reactions linked by intermediate products. They range in organisation and complexity from enzymes consisting of a single folded polypeptide chain to the most highly-organised systems of membrane-bound enzymes such as the electron transport chains of mitochondria or microsomes.

The assembly of enzymes in multienzyme complexes is a consequence of the possession of quaternary structure by enzymes. These complexes represent an answer to some of the problems of organisation and control in intermediary metabolism. Multienzyme complexes are aggregates of functionally-linked enzymes which catalyse two or more steps in a metabolic sequence. Their physical association produces an enzyme mosaic which possesses characteristic morphology.

There are several advantages in this type of enzyme organisation: enhancement of catalytic or regulatory efficiency and substrate channelling. (Perham, 1975). Complex formation provides a means of concentrating enzymes in a cellular compartment rather than having them randomly distributed. Interaction of the component polypeptides may produce particles that have catalytic properties not present in the separate chains. Aggregation of interrelated enzymes also makes it physically easier for the products of one enzyme to act as substrate for the next enzyme in the sequence. This may well increase the efficiency of the overall process, even if the intrinsic catalytic activity of each component is not altered by their association.

The increased efficiency of a complex as compared to that of the separate enzymes dispersed at random is most obvious when all intermediate products are strongly-bound to the complex. In such a case, reactions of the intermediate products of one component with

the next are much more probable. Moreover, substrate can be channelled through a series of reactions to avoid competition with other enzymes for the intermediate products or to reduce sensitivity to inhibitors in the surrounding medium. Such a system would function as a mechanism for selecting the metabolic fate of the substrate. Another possible reason for 'channelling' could be that an intermediate product in a reaction sequence might prove to be unstable in aqueous solution and therefore not survive the passage between the active sites if diffusion was required.

It is now thought that a substantial part of the enzyme complement of cells may be present as discrete multienzyme complexes. Such organisation may give rise to increased efficiency and new three dimensional arrangements with elaborate control mechanism. Isolation procedures for the resolution of mixtures of enzymes cannot be assumed to preserve the integrity of these aggregates. Therefore, it is possible that their natural occurrence is more frequent than reported (Reed & Cox, 1966). It is also possible that enzymes may exist as smaller aggregates bound to membranes and in the course of isolation these aggregates become detached, forming large complexes which are not normal cellular components.

Isolated multienzyme complexes consist of 2-6 interrelated enzymes with huge M_r values. Some of the most elaborate enzymes of this type are the tryptophan synthetase (E. coli), the 2-oxo-acid dehydrogenase multienzyme complexes of bacteria and mammals, and the fatty acid synthetase from yeast and mammals. A full account of several other multienzyme complexes may be found in Reed & Cox (1966).

1.2 2-oxo-acid dehydrogenase complexes

Multimeric enzymes that catalyse oxidative decarboxylation of pyruvate, 2-oxoglutarate and branched-chain 2-oxo acids have been

purified to a homogeneous state from microbial and eukaryotic cells, as functional assemblies of high M_r (Reed, 1974; Koike & Koike, 1976; Reed & Oliver, 1982).

Each complex catalyses a coordinated sequence of reactions that can be represented by the overall reaction:

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

where R= CH_3 (pyruvic acid) or $HO_2C\text{-}[CH_2]$ (2-oxoglutarate) or the 2-oxo-acid derived from the metabolism of valine, isoleucine and leucine (α -oxoisovaleric, α -oxoisocaproic acid and α-oxo-β-methylvaleric acids).

Each assembly contains a substrate-specific dehydrogenase (E1), a dihydrolipoyl acyltransferase (E2), specific for each species of complex and a lipoamide dehydrogenase (E3), a flavoprotein which is a common component of the three types of complex. The acyltransferase components constitute the core of the complexes to which the other enzymes are attached by noncovalent bonds

The pyruvate and 2-oxoglutarate dehydrogenase complexes, which are about the size of ribosomes, contain 48 to over 190 polypeptide chains in highly-organised assemblies. These complexes are found in aerobic prokaryotes and in eukaryotes where they occupy key positions in carbon and energy metabolism by funnelling the main product of glycolysis (pyruvate) into the citric acid cycle and by performing an essential role within this cycle. The preparations from E. coli are the most well-studied multienzyme complexes. They were separated into their three oligomeric components, and reassembled into particles resembling the native complexes, some years before similar studies were performed on the mammalian enzymes (Reed, 1974).

1.3 The mammalian pyruvate dehydrogenase complex

The mammalian PDC catalyses the intramitochondrial oxidative decarboxylation of pyruvate to AcSCoA. This reaction is essentially irreversible in cells, occupying a key position in the cellular metabolism by controlling the supply of acetyl groups in mitochondria arising from the oxidation of carbohydrates and amino acids (Fig. 1.1).

In many tissues, including heart, muscle, brain and kidney, the AcSCoA generated is mainly oxidised via the citric acid cycle, but in other tissues such as liver, adipose tissue and mammary gland, a significant proportion is utilised in the synthesis of fatty acids and sterols. Regulation of this step is of critical importance to the general metabolic balance and fuel economy in animals. It is perhaps not surprising that a range of different regulatory mechanisms appear to have evolved to ensure appropriate control of its activity. Fig. 1.2 shows, schematically, how PDC carries out the successive reactions involved in the oxidation of pyruvate: decarboxylation of pyruvate and subsequent reductive acetylation of the lipoyl moieties by the pyruvate dehydrogenase component (EC 1.2.4.1) (E1), transfer of the acetyl groups by the dihydrolipoyl acetyltransferase component (EC 2.3.1.12) (E2) and reoxidation of the lipoyl moieties with NAD^+ as the ultimate electron acceptor by lipoamide dehydrogenase (EC 1.6.4.3)(E3).

The intermediate acetyl dihydrolipoamide possesses an energy-rich thioester bond which is conserved when the acetyl group is transferred to CoA by the acetyltransferase component (see also Fig. 7.1).

Five different coenzymes and prosthetic groups are involved: TPP, FAD, CoA, NAD^+ and lipoic acid. The core of the cluster, the E2 component, contains lipoic acid bound in amide linkage to the ε-amino groups of specific lysine residues located near the active site of the subunits (Reed, 1974). Movement of the lipoyl groups of the core enzyme among the catalytic sites of E1 and E3 permits thiol-disulphide

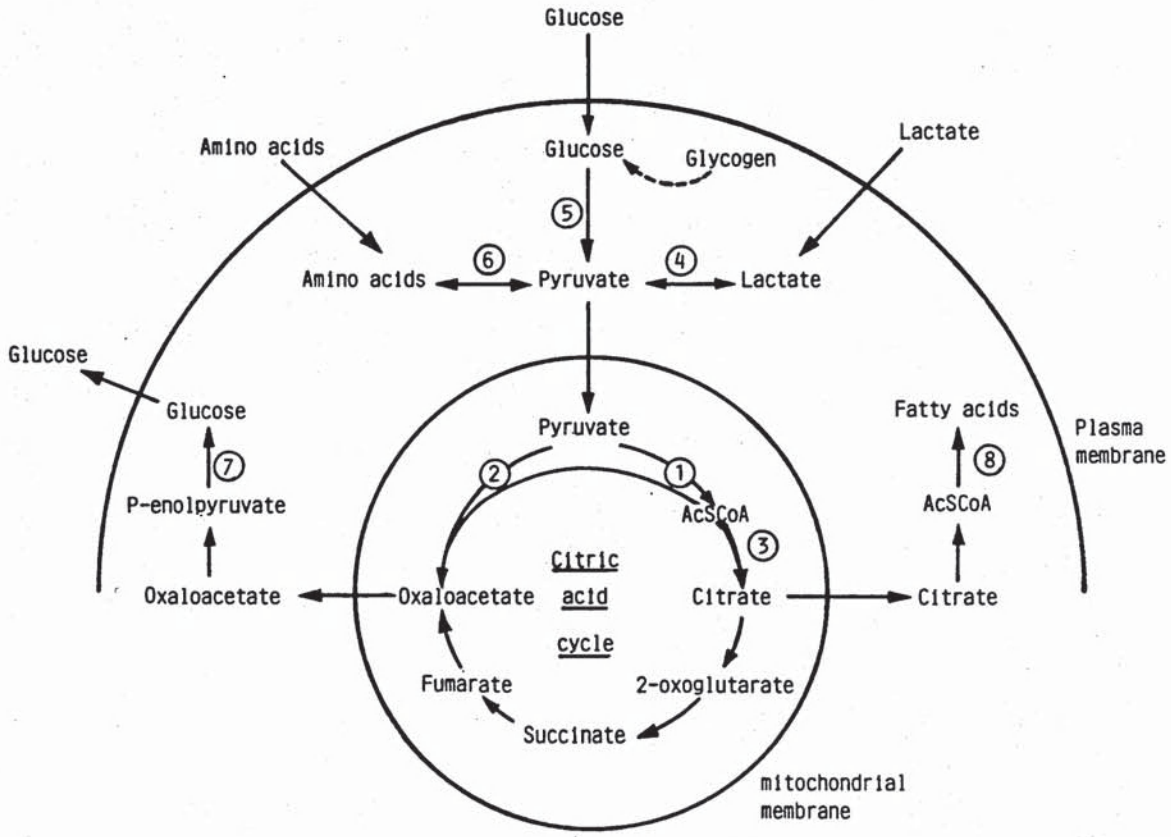
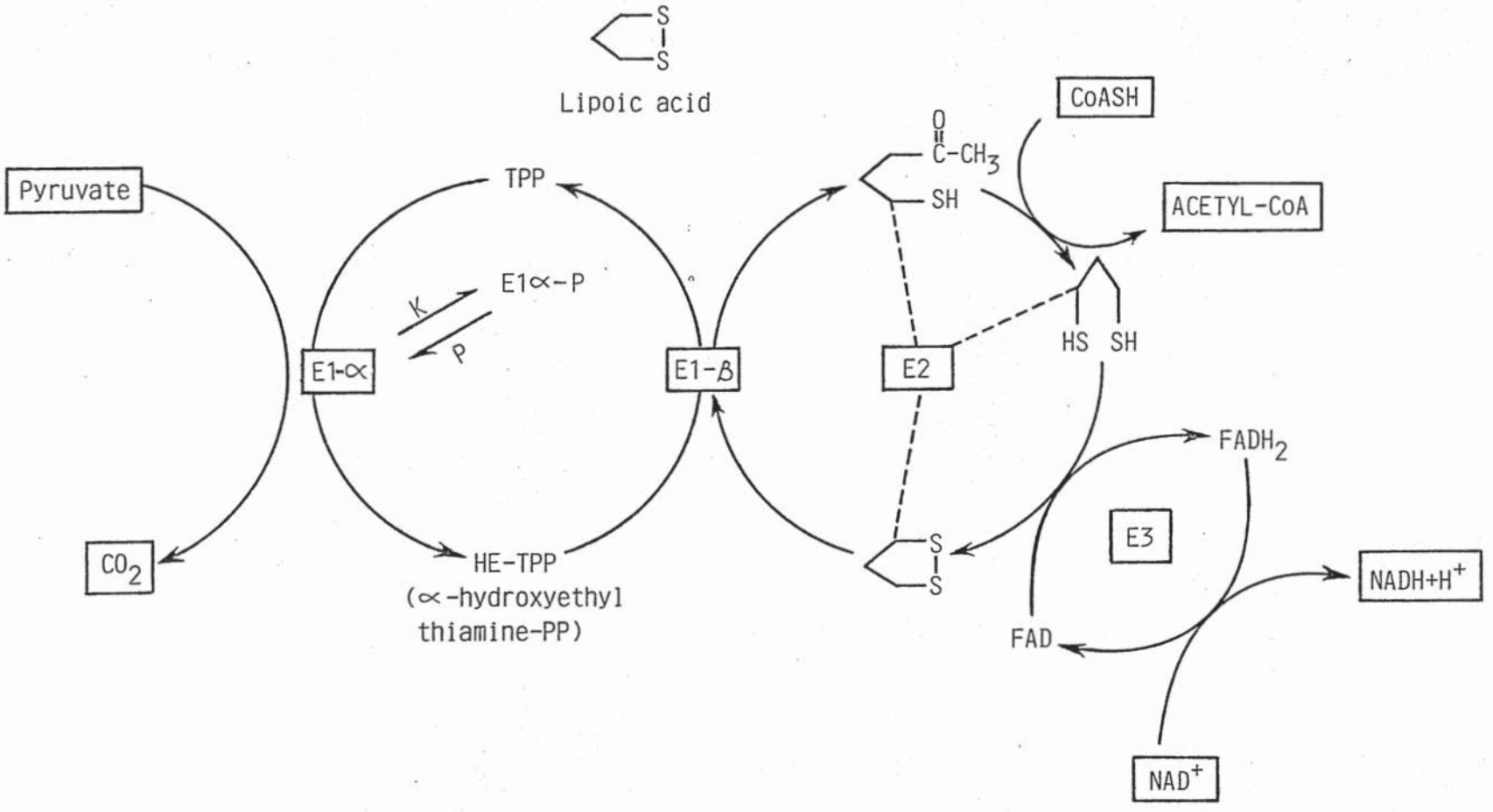


Fig. 1.1. Major routes of pyruvate metabolism in mammalian cells.

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



Sequence of Reactions Catalyzed by the Mammalian Pyruvate Dehydrogenase Complex

exchange and acetyl transfer reactions between the component proteins. These interactions occur within an arrangement in which movement of the individual enzymes is limited and from which intermediates do not dissociate. Highly-favourable positioning of the three enzyme components and their prosthetic groups would permit efficient coupling of these reactions.

1.3.1 Structure of the mammalian PDC

Based largely on the insight and experience gained with the E. coli pyruvate and 2-oxoglutarate dehydrogenase complexes, the mammalian complexes were initially purified from pig heart (Hayakawa et al., 1966) and bovine kidney mitochondria (Ishikawa et al., 1966). Many later studies resulted in improved purification procedures and resolution of the pig heart and ox heart and kidney complexes (Hayakawa et al., 1969; Linn et al., 1972). Furthermore, novel biochemical features of the mammalian PDC were discovered, including the subunit composition of the pyruvate dehydrogenase component and its regulation by phosphorylation/dephosphorylation (Linn et al., 1969a; 1969b; Barrera et al., 1972).

When the geometric organisation of the 2-oxo-acid dehydrogenase complexes was studied by their appearance in the electron microscope, two polyhedral forms of the E2 component were observed, the cube and the dodecahedron, both designs based on cubic point group symmetry.

The former design is exhibited by the E2 components of the pyruvate and 2-oxoglutarate components of E. coli and the mammalian 2-oxoglutarate dehydrogenase complex. In these enzymes, the transacetylase and transsuccinylase components are similar in size and cube-like appearance as demonstrated by sedimentation equilibrium analysis and electron microscopy (Reed & Oliver, 1968). Although

there is some disagreement regarding the number of E1 and E3 subunits that are combined with E2 in the E. coli PDC (Danson et al., 1979), Reed and his colleagues have proposed a structural model in which the 24 identical subunits of the E2 components are arranged with octahedral (432) symmetry (Reed, 1974; Reed & Oliver, 1982). In this model, eight morphological units formed by trimeric clusters of E2 subunits would be centred at the vertices of a cube. Pyruvate dehydrogenase (24 subunits) or 2-oxoglutarate dehydrogenase (12 subunits) would be uniformly distributed along the twelve edges of the core. Six E3 dimers are thought to be located on the faces of the E2 core bound diagonally to opposed units of E2.

This model points to many basic similarities between the morphological features of these complexes. The two major differences appear to be the presence of 12 additional subunits and 24 lipoyl moieties on the E. coli PDC as compared with the microbial or mammalian 2-oxoglutarate dehydrogenase complexes. An octahedral design is also exhibited by the mammalian branched-chain 2-oxo-acid dehydrogenase complex.

On the other hand, the appearance of the E2 component of PDC from bovine heart and kidney, avian tissues, Neurospora, yeast and some bacteria shows a different design, i.e. icosahedral (532) symmetry. In this case, the sixty subunits of the transacetylase component would be arranged in groups of three about the 20 vertices of a pentagonal dodecahedron, with 30 molecules (tetramers) of E1 and 6 flavoprotein dimers aligned on the 30 edges and 12 faces of the dodecahedron, respectively (Reed & Pettit, 1981).

The interpretation of the structure and composition of the pig heart PDC, based on biochemical data and electron microscopic studies, are matter of dispute. From data on the subunit composition and coenzyme content of the component enzymes of PDC, Koike & Koike (1976)

concluded that the pig heart complex is of the octahedral type, containing a core of 24 identical E2 polypeptide chains, to which about 24 tetramers (24 - 30) of E1 and 12 chains of E3 (6 dimers) are attached noncovalently. Sugden & Randle (1978) suggested a similar subunit composition on the basis of studies on the pig heart complex by the radioamidination procedure and by quantitative densitometric scanning of Coomassie Blue-stained polyacrylamide gels.

These data suggested octahedral symmetry and, as mentioned above, do not correlate with electron microscopic evidence showing similarities with the bovine complex (Junger & Reinausen, 1972; Koike & Koike, 1976). The controversy over the structure of the pig heart complex may be partly attributed to methodological differences and dissimilar values for the M_r of the E2 components of the bovine and pig heart enzymes. Hamada et al. (1975) determined an M_r of 1.8×10^6 for the E2 component of the pig heart complex, as compared with the value of 3.2×10^6 for the bovine enzyme (Barrera et al., 1972). Moreover, the former authors reported an M_r value for the enzyme subunit of 70,000-74,000 by SDS/polyacrylamide gel electrophoresis and gel filtration in 6M-guanidinium-HCl. This value was quoted by Sugden & Randle (1978) and by Machicao & Wieland (1980) to propose the same stoicheiometry. An M_r value of 52,000 has been obtained by sedimentation equilibrium analysis (Barrera et al., 1972) or gel filtration in the presence of guanidinium-HCl (Kresze et al., 1980). The anomalous migration of the E2 component on SDS/ polyacrylamide gels apparently causes an overestimation of its M_r value. This has been attributed to the extended structures of the lipoyl containing regions of these polypeptide chains (see section 1.3.3). Therefore, the mass of evidence from electron microscopic, sedimentation equilibrium analysis and limited proteolysis argues against the proposal that mammalian acetyltransferases consist of 24 subunits and favours the model

proposed by Reed and his colleagues. However, the problem of the structure of the pig heart complex has not yet been solved. The low content of E2 in the preparations of this complex is too low for strict icosahedral symmetry, even if it is assumed to be present as dimers.

1.3.2 Subunit composition of the mammalian PDC

PDC isolated from bovine kidney and heart have M_r values approx. 7×10^6 and 8.5×10^6, respectively (Linn et al., 1972). The M_r values of the various polypeptide chains and the proposed stoicheiometry of the bovine heart complex are presented in Table 1.1. The E1 component consists of nonidentical polypeptide chains α, M_r 42,000 and β, M_r 36,000, which form $\alpha_2\beta_2$ tetramers of M_r 154,000. The core enzyme consists of 60 identical polypeptide chains of M_r about 52,000. It is generally accepted that each monomer contains one lipoyl residue (White et al., 1980; see also Chapter 7, section 7.1). E3 has an M_r 110,000 and contains two identical subunits M_r 55,000, each containing a molecule of FAD.

The bovine kidney PDC contains approx. 20 E1 tetramers and 6 E3 dimers, whereas the heart complex contains about 30 E1 tetramers and 6 dimers of E3. This accounts for the difference in M_r values between the two types of complex. The kidney complex can bind 10 additional E1 tetramers but neither complex can bind additional E3 dimers.

The stoicheiometries of the pyruvate dehydrogenase kinase and phosphatase are not exactly known. In bovine kidney the number is between 3 and 5 moles/mol complex (Pettit et al., 1982a, 1982b). The kinase and the phosphatase have been purified to homogeneity. Each of these enzymes consists of two subunits which have M_r values of 47,000 and 45,000 for the kinase (designed as α and β, respectively) and 97,000-110,000 and 50,000 for the phosphatase (Pratt et al., 1982; Stepp et al., 1983).

Table 1.1

Subunit Composition of Bovine Heart Pyruvate Dehydrogenase Complex

Enzyme	M_r	Subunits No.	Subunits M_r	Subunits per molecule of complex
Native Complex	8,500,000			
E_1	154,000	4		
E_1 α		2	41,000	60
E_1 β		2	36,000	60
E_2	3,100,000	60	52,000	60
E_3	110,000	2	55,000	12
Kinase	100,000	1	47,000	
		1	45,000	
Phosphatase	150,000	1	97,000	
		1	50,000	

Reed & Pettit (1981); Stepp et al. (1983)

1.3.3 Functional domains in PDC

Studies of limited tryptic digestion of the E. coli acetyltransferase have revealed novel aspects of its structure (Bleile et al., 1979). These authors cleaved the E2 subunit into two types of domain: a compact domain of M_r 30,000 that possessed all the intersubunit binding sites as well as the catalytic site and another peptide M_r 32,000, which contained the lipoyl moieties. For these experiments lipoyl moieties were labelled after incubation of the complex with [2-^{14}C] pyruvate and N-ethyl [2,3-^{14}C] maleimide. Under these conditions, the lipoic acid becomes acetylated and the thiol groups generated in the S-acetyl dihydrolipoamide are then susceptible to selective modification with NEM (Brown & Perham, 1976; see also Chapter 7, section 7.1). Results indicated that limited tryptic digestion of the complex released polypeptide chains containing the radioactively-labelled lipoyl moieties. These fragments were separated from the residual modified complex by gel filtration or by ultracentrifugation. Electron microscopy showed that, after release of the lipoyl groups, the gross cube-like appearance of the complex was not altered, which suggested that this assembly conferred quaternary structure on the acetyltransferase and provided specific binding sites for E1 and E3 (subunit-specific domain).

Kresze and co-workers have examined the effects of several proteases on the overall and component activities of the bovine kidney PDC. They concluded that a group of proteases which includes papain, elastase and the leupeptin-sensitive lysosomal protease (inactivase), inactivate the complex by cleavage of the E2 core, thereby inducing the disassembly of the complex into the indivual enzyme components (Kresze & Steber, 1979). Proteases of this group do not cleave E1 α

subunit or degrade it very slowly. A second class of proteases which includes trypsin, chymotrypsin and clostripain cleave E1 α much faster than E2 and disassembly occurs more slowly than inactivation of E1. With all the proteases studied, a fragment of approx. M_r 26,000 was formed which contained the acetyl transferase activity (Kresze & Ronft, 1980). This fragment formed a high M_r aggregate and did not contain acetyl binding sites. On elastase digestion, a fragment of M_r 45,000-42,000 (possibly a duplet) was formed, which was resistant to elastase but sensitive to trypsin to give fragments of M_r 36,000 or lower. This fragment contained the lipoyl domains.

Subsequently, Bleile et al. (1981) further characterised the fragments arising from limited tryptic digestion. These authors observed fragmentation of subunit E1 α and the cleavage of E2 to a fragment of the same size as E1 β (M_r 36,000) and another fragment of M_r 27,000. Release of the lipoyl domain did not affect the state of aggregation of the E2 subunit as shown by sedimentation equilibrium analysis. In contrast with the results in the E. coli complex, this treatment affected the ability of the residual core to bind E1 and E3. Apparently, intact E2 subunits, possessing both binding and lipoyl domains were required to bind E1 and E3. These two fragments were separated by gel filtration chromatography on Sepharose 6B columns. Acetyl-transferase activity was retained in the peak of modified E2 whereas the lipoyl domains were inactive. The M_r value for lipoyl domains was about 37,000 as estimated by SDS/polyacrylamide gel electrophoresis. This value is larger than that estimated by sedimentation analysis of 28,000.

The apparent isoelectric points for the lipoyl domain and the residual E2 subunit were estimated by isoelectric focusing in the presence of urea as 4.6 and 7.7, respectively. Amino acid composition

analysis showed a high content of acidic residues, a high content of prolyl residues and a low content of histidine, arginine and tyrosine in the lipoyl domain. This fragment also stained very slightly with Coomassie Blue. The acidic nature of this fragment may be responsible for the anomalous electrophoretic migration and Coomassie Blue staining properties of the E2 component.

From the sedimentation coefficient and the M_r values, a frictional coefficient value of 1.9 was calculated, which indicated a swollen or extended structure for the lipoyl domains. These architectural features of the bovine kidney E2 are remarkably similar to those found in the E. coli acetyltransferase and transsuccinylase by Bleile et al. (1979).

1.3.4 Models of PDC structure

Bates et al. (1977) and Collins & Reed (1977) have presented evidence that lipoyllysine "swinging arms" can participate in two types of reaction. Each E1 dimer can interact with the lipoic acid residues of a small number of E2 chains, followed by transfer of acetyl groups between the lipoic acid moieties of neighbouring E2 chains in the E. coli complex. These rapid internal reactions or "transacetylations" would occur by means of a network of interacting lipoyl moieties through acetyl and electron (thiol-disulphide exchange) transfer reactions. This system would allow efficient coupling of the sequential reactions of the complex, even when the substrates are present in low concentrations (Danson et al., 1978)

The lipoyl-lysine residues in the E2 chains act as "swinging arms" in the mechanism of the complex. These groups reside in portions of the E2 chain that protrude from an inner part of the core (Bleile et al., 1979), which contain conformationally mobile polypeptide chains.

The sites of attachment of the lipoyl groups are therefore not included in the folding domains but are likely to be forming exposed flexible loops of the polypeptide chain, susceptible to proteolysis by trypsin (Hale & Perham, 1979). Mobility of lipoyl-peptides is thought to increase the effective radius of the lipoyl-lysyl swinging arms in carrying the intermediates between the different catalytic centres of the 3 enzymic components and between different acetyltransferase subunits in the complex.

Nearly half of the lipoyl moieties in E. coli PDC can be removed before there is appreciable loss of activity (Ambrose-Griffin et al., 1980; Stepp et al., 1981). This has been also found for the bovine kidney complex (Stanley et al., 1981). This has been explained by assuming that the highly-mobile regions of the E2 polypeptide or lipoyl domains detected in n.m.r. studies (Perham & Roberts, 1981; Perham et al., 1981) enable a given lipoyl group to serve more than one E1 active site. Removal of one of the two lipoyl moieties that serve a particular E1 site by lipoamidase or trypsin allows the catalytic cycle to continue via the remaining lipoyl domain(s). An active site coupling (or multiple random coupling) mechanism has been proposed to explain the current experimental data on the structure and function of the E. coli PDC (Roberts et al., 1983; Hackert et al., 1983).

It is currently believed that the ox kidney PDC also possesses a comparable system of interacting lipoic acid residues (Wawrzynczak et al., 1981). Roche, Cate and co-workers have presented evidence that high levels of acetylation with pyruvate are achieved in conditions in which only a few E1 subunits are functional. For these experiments, the bovine kidney PDC was inhibited by treatment with thiamine thiazolone pyrophosphate, an essentially irreversible inhibitor

of E1. After adding TPP, they observed a fast incorporation of acetyl groups into the complex. They have also examined whether exchange of E1 components contributed to these high levels of acetylation by conducting "mixing experiments" using active PDC mixed with inactive PDC. PDC was inactivated by treatment with thiamine thiazolone pyrophosphate or by phosphorylation with ATP. After removal of the inactivating reagent, the levels of acetylation achieved were greater than the additive levels of acetylation measured with active and inactive PDC assayed individually. They have explained these results as due to rapid transfer of acetyl groups between E2 subunits and migration of the E1 subunits from one molecule of complex to the other. They have also suggested that these movements of E1 components are too slow to make a contribution to the catalytic mechanism of the complex, but appear to be fast enough to be important in the regulation of the complex by pyruvate dehydrogenase kinase (Cate & Roche 1978; 1979).

Gene sequence analysis of the pyruvate and 2-oxoglutarate dehydrogenase complexes of E. coli have been reported (Stephens et al, 1983; Guest et al., 1984; Packman et al., 1984). These studies have solved some of the controversial features regarding the size and number of domain structures of the acetyltransferase, and have confirmed that the apparent M_r for the E2 component as inferred from SDS/polyacrylamide gel electrophoresis was significantly overestimated. These studies have also helped to clarify the view that there are 3 lipoylated segments in E. coli PDC, highly-homogeneous in amino acid sequence and connected by intervening regions of high-mobility. Mobility of the polypeptide chains is associated with alanine and proline-rich sequences. Lipoyl domains are linked to the subunit-binding domains located near the C-terminal half of the E2 chain. (Packman et al., 1984).

At present, it also seems clear that there is heterogeneity in the quaternary structure of the PDC complex. Since the overall complex reactions can be catalysed by complexes deficient in (although not completely lacking) E1 or E3 components, it is evident that structurally complete complex is not necessary for biological activity (Perham, 1975). These considerations suggest that PDC is not necessarily an exact homogeneous structure in terms of fixed chain ratios although the structure is governed by its octahedral or icosahedral symmetry (Packman et al., 1984). This property differentiates 2-oxo-acid dehydrogenase complexes from simpler oligomeric proteins where subunit composition is invariant.

1.3.5 Functions of pyruvate dehydrogenase (E1)

Linn et al. (1972) and Barrera et al. (1972) reported the purification and properties of the bovine kidney and heart E1. These authors demonstrated that purified E1 catalysed the incorporation of ^{14}C-labelled acetyl groups derived from pyruvate into isolated E2 component. Bleile et al. (1981) obtained similar results with isolated lipoyl domains. These results support the conclusion that E1 catalyses both the decarboxylation of pyruvate and the subsequent reductive acetylation of the lipoyl moiéties bound to E2.

The reaction catalysed by this enzyme can be described as follows:

$$CH_3COCO_2H + TPP \longrightarrow CH_3CHOH\text{-}TPP + CO_2 \quad (1)$$

$$CH_3CHOH\text{-}TPP + LipS_2 \longrightarrow CH_3CO\text{-}S\text{-}LipSH + TPP \quad (2)$$

Roche & Reed (1972) obtained evidence that phosphorylation inhibited the ability of the enzyme to catalyse reaction 1 with [1-^{14}C] pyruvate as substrate, but not with [α-^{14}C] hydroxyethyl TPP as substrate (reaction 2). Since phosphorylation occurs at the E1 α subunit and not in the β subunit, they suggested that subunit α

catalyses reaction 1 and subunit β , reaction 2. However, this proposal has been questioned by Walsh et al. (1976) who reported that phosphorylation of the pig heart enzyme inhibits the NADH-dependent transfer of acetyl groups from enzyme-linked acetyldihydrolipoamide to TPP (reversal of reaction 2). These results await clarification, perhaps when isolated active α and β subunit are available.

Denton et al. (1975) have shown that, besides the α-hydroxyethyl TPP, other products of decarboxylation of pyruvate may be formed, including acetyldehyde, acetoin and other byproducts. However, the extent to which these compounds are formed in vivo is not known. The equilibrium of the overall PDC reaction is largely in favour of its products and would be expected to be irreversible. All partial reactions except decarboxylation (reaction 1) are reversible. This step has probably the largest negative value of ΔG° and thus may be the rate-limiting step. However, Cate et al. (1980), using flow-quench rapid reaction studies, determined that the initial rate of decarboxylation of pyruvate proceeds at a much faster rate than the incorporation of acetyl groups. They concluded that reductive acetylation and not decarboxylation is the rate-limiting step in the overall reaction.

1.3.6 Studies on the function of lipoamide dehydrogenase (E3)

Lipoamide dehydrogenase belongs to a class of flavoproteins which catalyse the transfer of electrons between pyridine nucleotides and disulphide compounds. Other members of this group are glutathione reductase and thioredoxin reductase with many properties in common (Williams, 1976; Arscott et al., 1982).

Lipoamide dehydrogenase contains in addition to FAD, a reactive cysteine disulphide, which functions in catalysis. The two half-cystine are separated in the polypeptide chain by only four residues. Evidence

for the involvement of this group in catalysis comes from studies in the presence of arsenite and NADH, in which the flavin is oxidised completely. As arsenite quite specifically reacts with vicinal dithiols, these data suggest that the disulphide group participates in the catalysis.

Upon reduction, lipoamide dehydrogenase accepts only two electrons from the substrate, but in the presence of arsenite, this intermediate is not stable and a 4-electron reduction results, which completely inactivates the enzyme. A mechanism of action of lipoamide dehydrogenase has been proposed by Massey & Veeger in which the catalytic intermediate is one in which the enzyme accepts two electrons and these are shared bewteen the FAD group and the reactive disulphide (reviewed by Williams, 1976).

Lipoamide dehydrogenases have been isolated from many sources, both eukaryotic and prokaryotic; they are remarkably resistant to heat inactivation, urea treatment and proteolysis. The E. coli and Saccharomyces cerevisiae enzymes also resemble the pig heart enzyme in size and amino acid composition (Williams, 1976; Heinrich et al., 1983).

Considerable sequence homology has been found between peptides of the pig heart enzyme and the primary sequence of the E. coli lipoamide dehydrogenase estimated from nucleotide sequence data (Stephens et al., 1983). These similarities strongly favour a common ancestry. Also, the E1-E2 subcomplexes from mammalian and bacterial sources retain sufficient homology to permit exchange of their E3 components. Furthermore, the pig heart lipoamide dehydrogenase couples with the E. coli subcomplex to generate overall complex activity and assembles into a hybrid multienzyme complex (Perham, 1975; Guest, 1978).

The mammalian enzyme seems to be present in various forms, while the E. coli enzyme is a single species encoded by a structural gene

(Stephens et al., 1983). The physiological significance, number and origin of these electrophoretically-separable forms have been the topic for considerable controversy. The basis for the diversity of isoenzymes of E3 (2-13) has been discussed by Guest (1978). Gene multiplicity has not yet been discarded although the biochemical differences between the isolated forms are very small (Sakurai et al., 1970; Kenney et al., 1972). The existence of conformational isomers or the generation of secondary isozymes by post-translational modification are other possible mechanisms.

1.4 Regulation of mammalian PDC

1.4.1. Introduction

The regulatory properties of mammalian PDC have been studied extensively. The conversion of pyruvate in AcSCoA catalysed by PDC serves both bioenergetic and biosynthetic roles. In animals, AcSCoA cannot be used as a source of carbohydrate and thus metabolism of pyruvate through this step represents net losses of carbohydrate reserves (see Fig. 1.1).

Garland & Randle (1964) proposed a mechanism for regulation of the complex by end product inhibition with high ratios of [AcSCoA]/[CoA] and [NADH]/[NAD^+]. The end-product inhibition of the complex was first observed with a partially-purified preparation of the pig heart PDC. It was found that if PDC activity was measured spectrophotometrically in a system which allowed the products to accumulate, the rate of reaction declined to 5-10% of the original value. Addition of carnitine and carnitine acetyltransferase to remove the accumulated AcSCoA, caused an increase in the rate to about 70% of the initial value. Subsequent studies demonstrated that AcSCoA and NADH are effective competitive inhibitors of CoA and NAD^+, respectively (Tsai et al., 1973). This mechanism of regulation is very important in the control of pyruvate

oxidation by heart and diaphragm tissue (Denton et al., 1975).

A second type of regulation was reported by Linn et al. (1969a ; 1969b). These workers showed that the complex exists in an inactive phosphorylated form and an active non phosphorylated form. Interconversion of the two forms was catalysed by an ATP-requiring kinase and a phosphatase associated with the complex (Fig. 1.3).

PDC from several mammalian and avian tissues, Neurospora crassa and plants and the mammalian branched-chain 2-oxo-acid dehydrogenase complex are regulated by covalent modification (Wieland, 1983). There is no evidence that PDC in prokaryotic cells or yeast or the 2-oxo-glutarate dehydrogenase complex in eukaryotic or prokaryotic cells undergo phosphorylation and dephosphorylation.

Changes in the [AcSCoA]/[CoA] and [NADH]/[NAD^+] ratios also influence regulation of PDC by phosphorylation/dephosphorylation. The relative importance of these two differing mechanisms of control depends, at least in part, on the nature of the oxidisable substrate available to the tissue (Wieland et al., 1971).

1.4.2 Pyruvate dehydrogenase kinase. Isolation and properties

Pyruvate dehydrogenase kinase has been purified about 2,700-fold to apparent homogeneity from extracts of bovine kidney mitochondria (Stepp et al., 1983). Kidney mitochondria contain at least 4 times as much pyruvate dehydrogenase kinase as heart mitochondria and are the preferred source for isolation of the enzyme. The amount of kinase present is small, and only 2-4 mg are recovered from about 12 Kg kidney cortex.

The kinase is tightly-bound to the E2 component and co-purifies with the complex. Treatment of the core with p-hydroxymercuriphenyl sulphonic acid at alkaline pH promotes release of the kinase (Linn et al., 1972). Highly-purified preparations of the kinase show a doublet on

SDS/polyacrylamide gel electrophoresis, provided that proteolysis is minimal. (Stepp et al., 1983). The two subunits α and β have M_r values of 47,000 and 45,000, respectively. This finding and the sedimentation coefficient ($s_{20,w}$) of 5.5S indicates that kinase has a subunit composition $\alpha\beta$.

Limited proteolysis with chymotrypsin modified the kinase subunit selectively and was accompanied by loss of kinase activity; whereas limited tryptic digestion cleaved the β subunit selectively without affecting the activity. These observations and peptide mapping studies suggest that the two proteins are different and that the catalytic activity resides in the α subunit. The function of the β subunit remains to be established. An attractive possibility is that it functions as a regulatory subunit.

1.4.3 Sites of phosphorylation

Phosphorylation and concomitant inactivation of pyruvate dehydrogenase (E1) occurs on three serine residues in the α subunit (Yeaman et al., 1978; Sugden et al., 1979).

Tryptic digestion of ^{32}P-labelled pyruvate dehydrogenase from bovine kidney and heart yielded 3 phosphopeptides, a monophosphorylated (site 1) , a diphosphorylated (sites 1 and 2) tetradecapetide and a monophosphorylated nonapeptide (site 3).

Phosphorylation at site 1 proceeds markedly faster than at sites 2 and 3;inactivation (approx. 95%) is accomplished by phosphorylation of only one site (site 1) per tetramer ($\alpha P \cdot \beta_2$) and then 2 further moles of phosphate may be incorporated ($\alpha P_3 \cdot \beta_2$) (Sugden & Randle, 1978; Yeaman et al., 1978).

Randle and co-workers reported that phosphorylation at sites 2 and 3 on E1, in addition to site 1, markedly inhibited the rate of its reactivation by pyruvate dehydrogenase phosphatase (Sugden et al.,1978;

Kerbey & Randle, 1979). Partially-phosphorylated complex was reactivated more rapidly than fully-phosphorylated complex. These authors have proposed a role for multisite phorphorylation as a mechanism for regulating the conversion of inactive complex into active complex in rat mitochondria. Hutson et al. (1978) proposed that phosphorylation of these additional sites was responsible for the lower rate of reactivation of the complex by pyruvate dehydrogenase phosphatase in heart mitochondria from diabetic and starved rats (reviewed by Randle et al., 1981).

This proposal has been criticised by Reed and his colleagues (Reed et al., 1980), since they are at variance with the results of Teague et al. (1979), who observed that the presence of phosphoryl groups at sites 2 and 3 on bovine kidney E1 did not significantly affect the rate of reactivation by pyruvate dehydrogenase phosphatase.

These authors obtained evidence that dephosphorylation at sites 1, 2 and 3 is random and that the relative rates of dephosphorylation were in the order site 2 > site 3 > site 1. For these experiments, [γ -^{32}P] ATP and adenosine 5'-[γ -thio] triphosphate ATP (ATP γ S) were used to distinguish between the effects of phosphoryl groups at sites 2 and 3. The presence of thiophosphoryl groups at sites 2 and 3 did not inhibit the release of phosphoryl groups from site 1. However, the dephosphorylated enzyme was inactive. This indicated that phosphorylation at sites 2, and possibly at site 3, function, as well as site 1, as inactivating sites on the enzyme. In additional experiments, ^{32}P-labelled groups were incorporated into sites 1 and 2 and a thiophosphoryl group was inserted at site 3. The phosphatase released the phosphoryl groups from sites 1 and 2 and the enzyme was active, suggesting that phosphorylation at site 3 is not inactivating. The rate-limiting step is apparently the

dephosphorylation at site 1. The physiological significance of phosphorylation at site 2 in unknown.

The basis for the inconsistencies between the results from the two laboratories, although not apparent, has been attributed to methodological differences e.g. the molar ratios of phosphatase to phosphorylated E1 and differences in the media of incubation (Randle et al., 1981; Reed & Yeaman, 1985). However, it remains an important unanswered question.

Phosphorylation of E1 results in essentially total loss of its enzymic activity. No allosteric activators of the phosphorylated enzyme have yet been reported, in spite of a wide search (Hucho et al., 1972).

1.4.4 Pyruvate dehydrogenase phosphatase. Isolation and properties

Pyruvate dehydrogenase phosphatase has been purified from bovine heart and kidney mitochondria to apparent homogeneity (Teague et al., 1982; Pratt et al.,1982), and partially-purified from pig heart (Siess & Wieland, 1972). Heart mitochondria contain at least 3 times as much phosphatase as kidney mitochondria and are the preferred source for isolation of the enzyme. The purification procedure is based on binding of the phosphatase to E2 coupled to Sepharose in the presence of Ca^{2+} and subsequent release in the presence of EGTA. The phosphatase has a sedimentation coefficient of about 7.4S and an M_r of about 150,000 as determined by several methods. The phosphatase consists of two subunits of M_r about 100,000 and 50,000 in SDS/polyacrylamide gels. Phosphatase activity resides in the smaller subunit, which is sensitive to proteolysis (Teague et al., 1982; Pettit et al., 1982b). FAD is apparently associated with the larger subunit, the function of which is still unknown.

1.4.5 Control of kinase and phosphatase activities

Pyruvate dehydrogenase kinase activity is stimulated by AcSCoA and by NADH, products of pyruvate oxidation, in the presence of K^+ or NH_4^+ ions. (Cooper et al., 1974; Pettit et al., 1975; Cooper et al., 1975). The coenzyme, TPP and other pyrophosphate compounds, inhibit kinase activity, presumably by binding at the catalytic site of E1 and thereby altering the conformation about phosphorylation site 1 so that the serine hydroxyl group is less susceptible to the kinase (Roche & Reed, 1972).

When E1 and its kinase are bound to the core enzyme, the rate of phosphorylation increases markedly, apparently because of favourable topographical positioning of E1 with respect to the kinase or possibly to a change of conformation of either the kinase or E1 or both enzymes induced by binding to the transacetylase (Hucho et al., 1972; Pettit et al., 1982a).

Treatment of highly-purified kinase from bovine heart and kidney with excess NEM resulted in a loss of kinase activity, but had little effect on the oxidation of pyruvate to AcSCoA. It appears that the kinase contains a thiol group (or groups) involved in maintaining the conformation of the enzyme that facilitates phosphorylation of E1. It is not known if modulation of the activity by thiol-disulphide exchange has a physiological role.

Pyruvate dehydrogenase kinase is highly-specific for its substrate. It exhibits little activity towards casein, approx. 0.5% of the activity obtained with pyruvate dehydrogenase (Hucho et al., 1972). Mg^{2+} is required by both the kinase and the phosphatase. However, the apparent K_m of the phosphatase for Mg^{2+} (approx. 2 mM) is about 100 times higher than the K_m of the kinase for the same ion, Ca^{2+} markedly stimulates phosphatase activity (Denton et al., 1972;

Pettit et al., 1972) (K_m in the order of micromolar) provided that E2 is present (Pettit et al., 1972; Siess & Wieland, 1972). However, phosphatase activity towards phosphopeptide substrates is not affected by Ca^{2+}, whether or not E2 is present. These observations suggest that Ca^{2+} is not directly involved in phosphatase catalysis. In the presence of Ca^{2+}, the phosphatase binds to E2 and its K_m for phosphorylated E1 is decreased about 20-fold. Favourable positioning of the phosphatase and phosphorylated E1 on the core apparently facilitates the Mg^{2+} dependent phosphorylation. Because pyruvate dehydrogenase phosphatase and PDC are located in the mitochondrial matrix, changes in the intramitochondrial Ca^{2+} concentration could play an important role in the regulation of PDC activity (Randle et al., 1974; Denton et al., 1981).

At saturating concentrations of Mg^{2+}, the polyamines spermine, spermidine and putrescine stimulated the activity of the phosphatase (Damuni et al., 1984). This effect may be relevant to stimulation of the complex by insulin in adipose tissue. Pyruvate dehydrogenase phosphatase activity is inhibited by NADH and the inhibition is reversed by NAD^+ (Pettit et al., 1975). It exhibits little activity toward phosphorylase a and phosphorylated branched-chain 2-oxo-acid dehydrogenase complex. A broad-specificity phosphatase M_r 34,000 from rabbit liver cytosol showed significant activity towards phosphorylated PDC from bovine kidney (Reed et al., 1980).

The pyruvate dehydrogenase system is well designed for fine regulation of its activity. Interconversion of the active and inactive forms of E1 is a process that leads to the establishment of steady states, in which the fraction of phosphorylated E1 can be varied over a wide range by changing the concentrations or molar ratios of the effectors that regulate the activities of the kinase and phosphatase

(Reed & Pettitt,1981). The steady state activity of the complex is sensitive to the ratios [ATP]/[ADP], [AcSCoA]/[CoA] and [NADH]/[NAD^+]. Pyruvate dehydrogenase and its two converter enzymes comprise an interconvertible enzyme cascade. The mechanism whereby AcSCoA, CoA, NAD^+ and NADH modify the activity of the kinase is an intriguing problem. It is possible that the kinase or its substrate bears regulatory binding sites for these effectors but equally it is possible that these compounds act through binding to their substrate sites on E2 and E3. This mechanism might involve conformational changes, in view of the importance of the attachment to the acetyltransferase for kinase activity. Fig.1.3 summarises the control of kinase and phosphatase activities by effectors observed with the purified enzymes.

A novel mechanism is that lipoate may act as the transmitter of regulatory interactions. This suggestion was originally made by Kerbey et al. (1976) and subsequently supported by Cate, Roche and co-workers. These latter authors have postulated a mechanism in which the regulatory effects on pyruvate dehydrogenase are mediated through changes in the distribution of the lipoyl moieties of E2 between the oxidised, reduced and acetylated forms. Reduction of lipoyl moieties bound to E2 would activate the kinase and AcSCoA stimulation of the kinase would require acetylation of the lipoyl groups (Roche & Cate, 1977).

The activating effects of low pyruvate concentrations in the presence of TPP could thus be explained in terms of acetylation or reduction of lipoyl groups (Cate & Roche, 1978; 1979). However, this suggestion is in contradiction with the findings of Reed et al. (1980) with highly-purified pyruvate dehydrogenase kinase and its dephospho-tetradecapeptide substrate. The rate of phosphorylation was stimulated by AcSCoA and NADH and inhibited by ADP and pyruvate. As E2 was absent in the preparations of enzyme, these results indicate that these

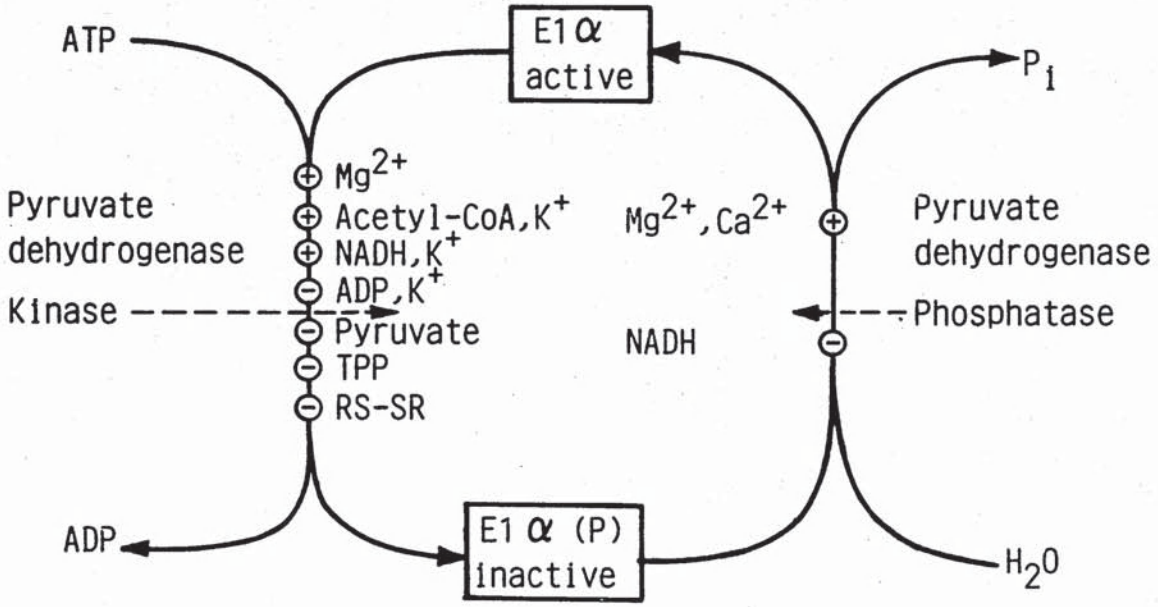


Fig. 1.3

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

effectors act directly on the kinase, and not via lipoyl moieties bound to E2. However, the controversy has not yet been solved, since Roche and co-workers were unable to reproduce these data (Rahmatullah & Roche, 1985).

It is not yet clear how a few molecules of pyruvate dehydrogenase kinase, tightly-bound to the core enzyme, can inactivate the full E1 complement of the complex. In this respect, Brandt & Roche (1983) have proposed that E1 molecules migrate on the surface of the E2 core to the fixed kinase subunits (see also section 1.3.4).

1.4.6 Role of PDC in the regulation of glucose oxidation

The oxidative decarboxylation of pyruvate by PDC is of special importance to glucose homeostasis in man and other animals. Owing to the irreversibility of the PDC reaction, an unregulated flux of pyruvate through this step would seriously jeopardise the balance of body glucose (Wieland, 1983). Glucose conservation is a physiological necessity during starvation since glucose reserves are limited. This necessity is developed to a pathological extent in diabetes where rates of glucose oxidation are inappropriately small for the prevailing glucose concentration in the extracellular fluids.

In diabetes and starvation, there is a marked decrease in the activity state of the complex in heart, liver and kidney (Wieland et al., 1971). Re-feeding or administration of insulin or inhibitors of fatty acid oxidation reverse effects of starvation and diabetes. These effects on PDC activity have been attributed to stimulation of the activity of pyruvate dehydrogenase kinase or increased synthesis of a protein activator of the kinase or the kinase itself (Hutson & Randle, 1978; Kerbey & Randle, 1982).

The two best-studied acute effects of hormones on the activity of PDC are those of positive inotropic agents such as adrenalin in

the heart and the effect of insulin on the enzyme in several tissues (Denton & Hughes, 1978; McCormack & Denton, 1984).

In perfused rat heart, it has been demonstrated that the initial PDC activity is increased about 4-fold after the administration of adrenaline and other inotropic agents (McCormack & Denton, 1981). These effects have been attributed to increased transport of Ca^{2+} ions into mitochondria and consequent activation of the pyruvate dehydrogenase phosphatase. Similarly, stimulation of PDC activity in liver by vasopressin, angiotensin and adrenaline (α -adrenergic action), which act via the formation of inositol 1,4,5-trisphosphate and mobilisation of cytoplasmic Ca^{2+} may be due to increases in the intramitochondrial Ca^{2+} concentration (Berridge & Irvine, 1984). The most extensively studied hormonal effects on PDC activity are those on control of its activity in adipose tissue by insulin. Physiological concentrations of insulin, increase the basal activity of PDC by 60-200% after 5-10 min incubation of fat pads with insulin in the presence of glucose (or fructose). This increase is accompanied by dephosphorylation of the E1 α subunit. (Denton et al., 1975).

Elucidation of the mechanism by which insulin exerts its acute effects on key target enzymes such as PDC, remains one of the major problems in metabolic regulation. The literature on this subject is vast as these aspects have attracted considerable attention (Denton et al., 1981).

There have been proposed many hypotheses to explain how a signal arising from the interaction of insulin with its receptor in the plasma membrane is transmitted to mitochondria. Besides Ca^{2+} ions, many other molecules have been proposed to act as mediators of the insulin action, e.g. H_2O_2 and various soluble peptide mediators (Paetzke-Brunner et al., 1980; Saltiel et al., 1981; Thomson et al., 1984).

At present, it has been difficult to demonstrate the physiological importance of these mediators since their complete structure must be elucidated and the purified molecule must be shown to produce insulin effects in target tissues. For reviews on this subject, refer to Wieland (1983) and Reed & Yeaman (1985).

1.5 Aims and importance of the present study

The large number of research groups involved in studies on short-term effects of hormones on PDC activity and other regulatory aspects of the enzyme contrasts with the few reports on the mechanism that influences PDC levels in the cells (Knowles & Ballard, 1974; Weinberg & Utter, 1979). Other aspects of the structure, function and regulation of the mammalian PDC still remain to be examined. Little is known about biosynthesis, assembly and turnover of the multienzyme within the mitochondria. Moreover, the spatial arrangement of the complex in situ is not yet understood, bearing in mind the size of the isolated complex as estimated by electron microscopy exceeds by far the thickness of the mitochondrial inner membrane plus the width of the intracristal space. Studies on the organisation of the complex inside the mitochondrion would help to clarify this problem.

This study was undertaken to address the problem of the biosynthesis of this multimolecular aggregate in the cytoplasm and the mechanism of import of the individual polypeptides into the mitochondria for the assembly of the complex. Studies on the biosynthesis of 2-oxo-acid dehydrogenase complexes in eukaryotic cells have not been reported.

Although association of enzymes into complexes offers a number of advantages for biological organisation (section 1.1) it raises new problems of its own. First, because of the disproportionate polypeptide composition, biosynthesis of the individual components

must be coordinated for the different polypeptide chains to be in the appropriate amounts. In microorganisms, clustering of functionally-related genes in operons are a special form of genetic organisation that is assumed to provide efficient and coordinate regulation of gene expression. The specific components of PDC from E. coli are encoded by two structural genes which form an operon. The complex is independently regulated by pyruvate and inhibited (but not repressed) during anaerobic growth. The E3 component is encoded by a single gene linked to this operon (Stephens et al., 1983).

Less is known about the control of gene expression in eukaryotic cells where stable mRNAs emerge from a separate organelle (the nucleus). Most mitochondrial matrix proteins are synthesised in the cytoplasmic ribosomes in the form of larger M_r precursors which are specifically recognised and imported inside the organelle. Translocation is an energy-dependent process and processing of the precursors is mediated by a specific protease located in the mitochondrial matrix.

Studies on the assembly of multienzyme complexes, are important in themselves. The role of nucleation and stable intermediates in the process has been recognised as important elements in the assembly of many oligomeric proteins. In the case of PDC, studies of the self-assembly of the structure inside the mitochondrion from the individual components are an important issue.

1.6 Synthesis of mitochondrial proteins

1.6.1 Introduction

The eukaryotic cell is organised by a variety of membranes: the plasma membrane which encloses the cell and various intracellular membranes which determine cell compartmentation and delimit organelles. These organelles perform specialised functions and this is reflected

in their characteristic polypeptide compositions. This organised distribution of polypeptides is necessary because, with only few exceptions, the proteins of the various organelles and their membranes are not synthesised in the same compartments in which they perform their functions. Most of them are translated on cytoplasmic ribosomes and have to be selectively translocated across these diverse membranes. Organelle membranes must not only have devices for the specific recognition of proteins which are destined for the compartments they enclose, but also have mechanisms to translocate these proteins across the lipid bilayer; this is quite remarkable, as many proteins are very large and hydrophilic.

1.6.2 Mechanisms of transport of proteins in the eukaryotic cell

According to our present knowledge, there are two basic mechanisms for the transfer of proteins across biological membranes. In the co-translational mechanism, proteins can be inserted into membranes in a synthesis-dependent fashion. This appears to be the case for secretory proteins and proteins destined for the plasma membrane and for lysosomes. In contrast, import into organelles such as chloroplasts, mitochondria and peroxisomes is apparently the result of a post translational process. Some proteins of the endoplasmic reticulum, such as cytochrome b_5 and NADH cytochrome b_5 reductase also appear to utilise this latter type of transport (Kreibich et al., 1985).

Many proteins are translated on polysomes tightly-associated with the rough endoplasmic reticulum and co-translationally inserted into the membranes. This process, often termed 'vectorial translocation' is coupled to elongation of the polypeptide chain. The finished proteins are sequestered on the opposite site of the membrane and once released from the ribosomes may either remain as integral membrane proteins or be released into the lumen. A detailed mechanism of co-translational transport was first proposed by Blobel &

Dobberstein (1975a; 1975b) in the so called, 'signal hypothesis', which has been modified in response to a large number of data (Meyer, 1982). The nascent peptides are thought to interact with the appropriate membranes through signal peptides, an amino-terminal presequence of 15-20 amino acids residues. As the signal sequence emerges from the ribosome, it binds an oligomeric 'signal recognition particle' (SRP) a ribonucleoprotein complex, which arrests further elongation of the polypeptide. The SRP-polysome complex then interacts with an SRP receptor or 'docking protein' at the membranes of the endoplasmic reticulum. This releases the arrest of elongation and the nascent polypeptides penetrate the membrane as they grow. The signal sequence is cleaved off the polypeptide before elongation is completed. This is accomplished by a 'signal peptidase' an integral membrane protein, which is probably located on the luminal side of the membranes of the endoplasmic reticulum.

The SRP thus sorts proteins by performing the dual functions of preventing inappropriate synthesis in the cytoplasm and by assuring that synthesis of signal-bearing proteins occurs only on ribosomes attached to the endoplasmic reticulum. The pathway taken for proteins within the Golgi <u>en route</u> to secretory granules, plasma membrane and lysosomes is becoming clearer as the role of the various types of Golgi cisternae is emerging. These proteins reach their final locations by transport mechanisms which involve addition of oligo-saccharides, flow of membrane and shuttling between organelles and plasma membrane. The intercompartmental transfer of proteins is accompanied by secondary sorting decisions. However, it is not yet known how these proteins signal their route or how information is encoded in the many kinds of vesicles that are required to carry out membrane traffic (Rothman & Lenard, 1984).

The second mechanism of translocation of proteins through membranes is a synthesis-independent process. In contrast with the co-translational transport mechanism, posttranslationally transferred proteins are synthesised essentially on free polysomes and released into the cytosol as complete polypeptide chains. The primary translation products differ in structure and properties from their mature counterparts. In particular, precursors to most mitochondrial proteins carry pre-sequences. The newly-synthesised proteins enter the cytosolic pool of free precursors from which they are rapidly cleared by uptake into the appropriate organelles. These precursors interact directly with their target membranes, apparently via specific receptors on the surface of the organelles. During translocation the precursors are processed to the mature protein (Neupert & Schatz, 1981; Hay et al., 1984). The most characteristic feature of posttranslational transport is that synthesis of the precursors on the polysomes and their translocation are two clearly separate events.

1.6.3 Biogenesis of mitochondria

Mitochondria and chloroplasts are organelles which possess their own limited genetic systems. The biosynthesis of the proteins for these organelles depends on the coordinated interplay between the two genetic systems located in the organelle and the nucleus. (De Vries & van't Sant, 1983).

It is estimated that mitochondria contain several hundred proteins. The mitochondrial genome from various species contains roughly the same information for a set of 12 proteins and for the mitochondrial tRNAs and rRNAs. (Chomyn et al., 1985). An interesting feature is that the products of the mitochondrial genome are mostly hydrophobic components of complexes which contain products of both organelle and nuclear genes.

However, it is obvious that the majority of mitochondrial proteins (about 95%) are specified by nuclear genes, synthesised on cytoplasmic ribosomes and then transported into mitochondria.

Ever since the presence of a mitochondrial biosynthetic system was discovered, basic questions were posed: Why are some genes in the organelle DNA and others in the nucleus?. How do genes in organelle and nucleus work together in making mitochondria ?. The answers to these and other questions are not known, but considerable progress has been achieved in sequencing mitochondrial genes and obtaining the transcription maps. By genetic manipulation, it should be possible e.g. to transfer mitochondrial genes to the nucleus and to test whether mitochondrial genes encode proteins that can be synthesised in the active form but cannot be imported into the organelle.

Another interesting aspect of mitochondria is that these organelles are surrounded by two differing membranes, whereas only single membranes surround most of the other organelles. The two mitochondrial membranes divide the mitochondrion into four different locations: the outer membrane, the inner membrane, the intermembrane space, which lies between the two membranes, and the matrix, which is enclosed by the inner membrane.

Biogenesis of mitochondria occurs by insertion of individual components into pre-existent mitochondria, i.e. mitochondria multiply by growth and division. (Yaffe & Schatz,1984b). The transfer of hundreds of mitochondrial proteins through the mitochondrial membranes to reach their various locations is a complex process. Several aspects of the mechanisms involved are known.

It can be divided conceptually into five steps:

i) synthesis of the polypeptide, usually as a larger precursor

ii) recognition and binding of the precursors at the mitochondrial surface

iii) translocation of the precursor across or into one or both mitochondrial membranes

iv) cleavage and/or other processing modifications of the polypeptide chain to the mature protein

v) assembly into functional units

These steps are schematically shown in Fig. 1.4.

Degradation of mitochondrial proteins is not a well-known process. The proteolytic systems involved are not yet understood; however, some studies indicate that mitochondrial proteins have different half-lives, which suggests the existence of mechanisms that regulate their degradation (Mori et al., 1981c).

1.6.4 Characteristics of precursor polypeptides

Two different experimental approaches have been used to study the synthesis of mitochondrial proteins. The first relies on experiments performed in vitro, employing cell-free translation. The second is based on experiments performed in vivo. (see also Chapter 8, section 8.1). Although precursors are difficult to isolate in appreciable amounts, it has been shown by pulse-labelling of spheroplasts, fibroblasts or hepatocytes and subsequent immunoprecipitation, that many of the cytoplasmic precursors to mitochondrial proteins are made as larger polypeptides. This difference in size is frequently shown by a slow migration on SDS/polyacrylamide gel electrophoresis of the precursor polypeptide as compared with the mature form. Such larger precursors can also be detected in vitro after analysis of the translation products.

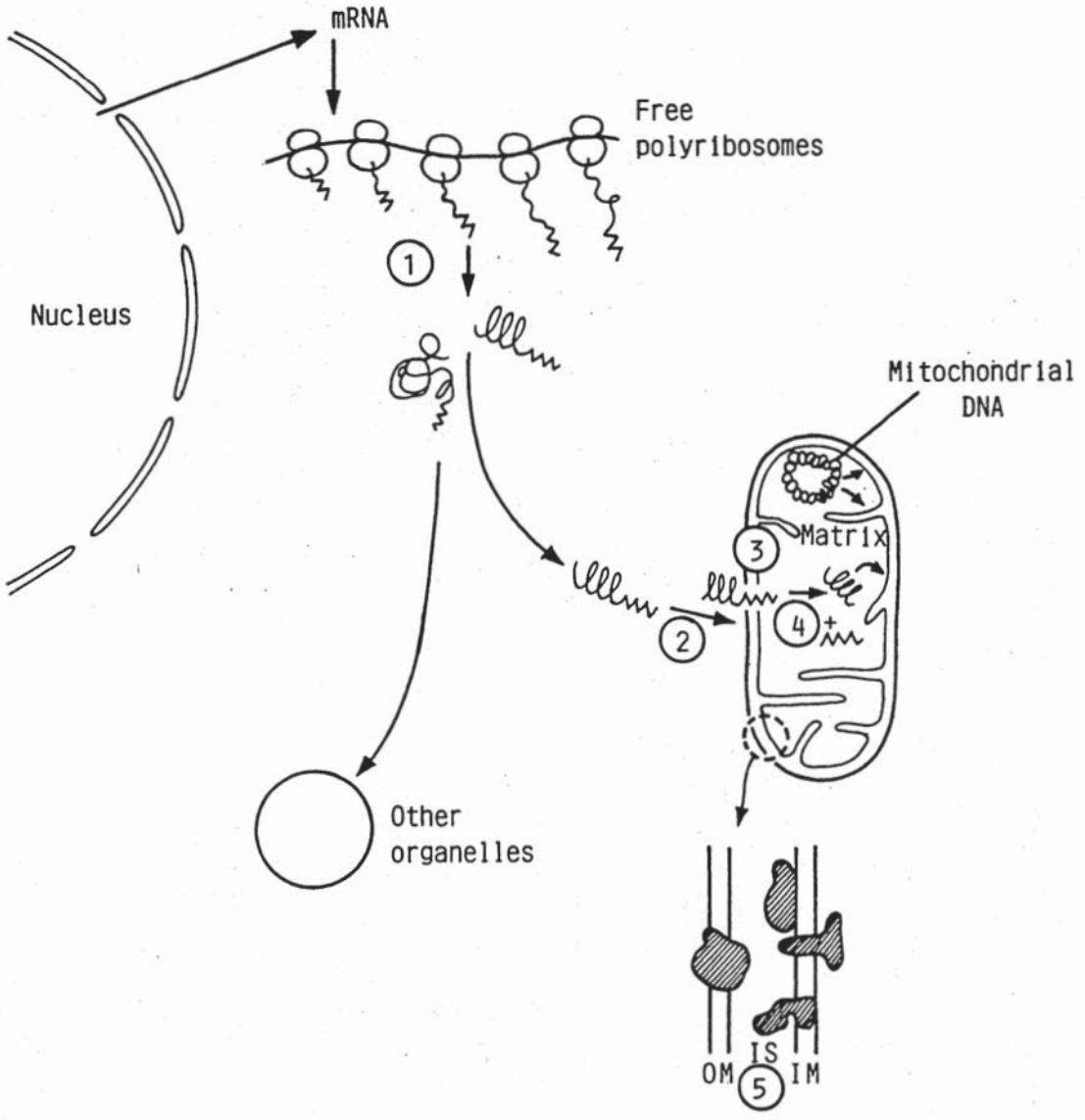


Fig. 1.4 Stages on mitochondrial biogenesis

1. Biosynthesis of precursors.
2. Recognition and binding.
3. Translocation.
4. Processing.
5. Assembly into functional units.

Table 1.2. CYTOPLASMIC PRECURSORS OF MITOCHONDRIAL PROTEINS

Protein location	protein	organism	Apparent M_r mature	Apparent M_r precursor	Ref.
Matrix	aspartate aminotransferase	chicken	44,500	47,000	Jaussi *et al.* (1982)
	F_1-ATPase α subunit	yeast	58,000	64,000	Macchechini *et al.* (1979); Lewin *et al.* (1980)
	β subunit	yeast	54,000	56,000	
	γ subunit	yeast	34,000	40,000	
	carbamyl phosphate synthetase	rat	160.000	165,000	Mori *et al.* (1981a); Raymond & Shore (1981)
	malate dehydrogenase	rat	37,000	38,000	Aziz *et al.* (1981); Mihara *et al.* (1982)
	methyl malonyl CoA mutase	rat	77,500	80,500	Fenton *et al.* (1984)
	ornithine transcarbamylase	rat	39,000	43,000	Mori *et al.* (1981a); Oda *et al.* (1981)
	fumarase	rat	45,000	50,000	Ono *et al.* (1985)
Inner Membrane	adenine nucleotide transporter	N. crassa	32,000	32,000	Zimmermann & Neupert (1980)
	F_1-F_o ATPase subunit IX	N. crassa	8,200	14,000	Zimmermann *et al.* (1981)
	cytochrome c_1	yeast	31,000	37,000	Teintze *et al.* (1982)

Table 1.2. (Cont.).

Protein location	protein	organism	Apparent M_r mature	Apparent M_r precursor	Ref.
	cytochrome c	N. crassa	12,000	12,000	Zimmermann et al. (1981)
Intermembrane space	cytochrome c peroxidase	yeast	33,500	39,500	Reid et al. (1982)
	cytochrome b_2	yeast	58,000	68,000	Daum et al. (1982a); (1982b)
	porin	yeast	29,000	29,000	Gasser & Schatz (1983)
Outer membrane	70 Kd-protein	yeast	70,000	70,000	Hay et al. (1984)

The table summarises the proteinsdiscussed in this study.

Cell-free translation is frequently employed to analyse the properties of precursors as they are released from the ribosomes. In such studies, the mRNA can be obtained from heterologous systems, and mitochondria are absent during protein synthesis. This avoids post-translational modification of the primary translation products which is part of their biogenesis. Standardised preparations of lysed reticulocytes or extracts of wheat germ are used for most studies since efficient translation of mitochondrial proteins is generally achieved.

It has become apparent that the majority of mitochondrial proteins are formed as precursors, which exhibit M_r 500-10,000 larger than their corresponding mature proteins, on the basis of their electrophoretic mobility on SDS/polyacrylamide gels.

Cytosolic precursors for many mitochondrial proteins in many species have been identified. Table 1.2 summarises the proteins discussed in this study. For an extensive list of precursors refer to Hay et al. (1984).

There is no apparent correlation between the size of the N-terminal extension and the final location of the mature protein within the mitochondrion. Furthermore, precursors to subunits of the same enzyme complex do not possess 'signal' peptides of identical size or amino acid composition. Several imported mitochondrial proteins are synthesised with no N-terminal extensions. Among these are porin, which appears to be present in all mitochondrial outer membranes so far studied and probably forms aqueous channels in the membrane, (Gasser & Schatz, 1983), the adenine nucleotide transporter of Neurospora (Zimmermann & Neupert, 1980) and cytochrome c, an intermembrane space protein (Zimmermann et al., 1979).

The amino acid sequence of the amino-terminal extensions of several mitochondrial precursors have been elucidated from the nucleotide sequence of the corresponding genes (Reid, 1985). Among these are the subunit IX of the Neurospora ATPase and the yeast cytochrome c peroxidase (Viebrock et al., 1982; Kaput et al., 1982). It has been suggested that the sequence of 66 amino acids of the precursor to the subunit IX of ATPase renders the precursor more basic and more hydrophilic than the mature protein.

The observation that precursors, from which the prepiece has been removed in vitro by addition of solubilised matrix protease, can no longer be imported into mitochondria (Gasser et al., 1982b) supports early suggestions (Schatz, 1979) that this sequence contains information for targetting the attached protein to its correct intramitochondrial location. This has been further demonstrated by DNA recombination methods (section 1.6.8). Since some proteins, without transient N-terminal extensions, can be imported into mitochondria, it is possible that these proteins contain other 'signals' in the polypeptide structure which allow their specific uptake by the organelle. Physicochemical and immunological differences have been detected between precursor and mature proteins, e.g. between the cytochrome c and its precursor, apocytochrome c. (Hennig & Neupert, 1983). Conformational differences are also suggested by intermolecular associations between precursor molecules e.g. rat liver ornithine transcarbamylase is a trimeric enzyme which sediments at 6S; the in vitro synthesised precursor sediments at 14S although its M_r is only 3000-4000 larger than the mature protein (Miura et al., 1981 Kalousek et al., 1984). Aggregation of precursors has also been reported for other proteins e.g. the adenine nucleotide translocator (Zimmermann & Neupert, 1980) and carbamyl phosphate synthetase (Miura et al., 1981).

Recent evidence suggests that cytosolic or lysate factors are required for import of precursors (Argan et al., 1983; Miura et al., 1983; Ohta & Schatz, 1984). Such factors could catalyse the formation of precursor aggregates, either for their protection in the cytosol or for their proper presentation to the mitochondrial surface.

Regarding the subcellular site of synthesis of mitochondrial precursors, several lines of evidence indicate that while the membranes of the endoplasmic reticulum may be in some case contiguous with the mitochondrial outer membrane, there is yet no conclusive evidence that this association is related to the biosynthesis of mitochondrial proteins (Ades, 1982; Hay et al., 1984). Answers to this question have been obtained by employing cell-free translation systems programmed with polysomes (free or membrane-bound) or mRNA extracted from these polysomes. The translation products have been analysed by immunoprecipitation procedures and the site of synthesis for a number of mitochondrial proteins has been found in free polysomes (Raymond & Shore, 1979; Mihara et al., 1982).

1.6.5 Mitochondrial import receptors

Shortly after synthesis, cytoplasmic precursors to mitochondrial proteins find their way into the organelle. This step requires interaction of precursors with the cytoplasmic face of the outer membrane . The interaction may involve specialised receptors, which recognise precursors destined for mitochondria.

Alternatively, translocation may not require the presence of specific receptors on the outer mitochondrial membrane. The presence of extra peptides may introduce features into the precursor which simply allow the proteins to diffuse through the mitochondrial membrane(s). This mode of translocation would accord with the membrane-triggered folding model proposed by Wickner (1979).

The existence of the proposed receptors has been tested by incubating the precursors, recently-synthesised in vitro, with isolated mitochondria. In all cases studied, the precursor can be recognised and transported into the organelle. In order to study the interactions between precursors and mitochondria, procedures have been employed to inhibit translocation. Binding of precursors to mitochondria appears to be less dependent on temperature than the translocation or insertion into the membrane. Another approach takes advantage of the observation that the import of most proteins into mitochondria requires an electochemical potential across the inner membrane. When the membrane potential is dissipated, transfer is halted although precursors remain bound to the mitochondrial outer surface. However, under these conditions, some precursors bind poorly to mitochondria (Zwizinski et al., 1984).

From the work of Neupert's group on the biosynthesis and import of cytochrome c, evidence for the existence of mitochondrial import receptors has been obtained (Hennig & Neupert, 1983). These studies exploited the fact that large amounts of the precursor, apocytochrome c, can be prepared by chemical means by removing the covalently-bound haem group from cytochrome c. Several characteristics of the binding of precursors have emerged from these and similar studies:

i) Binding is rapid and reversible. The rate of binding is sufficient to account for the rate of transport into mitochondria

ii) Binding is specific for precursor molecules. Apocytochrome c is tightly-bound but can be released by addition of excess unlabelled apocytochrome c, whereas excess cytochrome c has no effect.

iii) Binding is specific for the outer membrane or for outer membrane vesicles and depends on one or more polypeptides exposed on the cytoplasmic face of the outer membrane. Treatment with trypsin (or proteinase K) reduces dramatically the ability of the particles to bind precursors or to import them. (Riezman et al., 1983; Zwizinski et al., 1984).

iv) Binding is not energy-dependent nor is it accompanied by proteolytic cleavage of the precursor. The precursor to Neurospora adenine nucleotide translocator binds to de-energised mitochondria and becomes internalised when the membrane potential is restored. As precursor does not dissociate from the mitochondrial surface before translocation, these results suggest also that transport occurs from those sites (Zwizinski et al., 1983).

v) Even when the criteria of saturability has not yet been met in all cases because of the technical problem of the small quantities of precursors that can be synthesised in vitro, there is evidence of a limited number of different and evolutionarily conserved recognition mechanisms.

Transfer in vitro of most proteins studied so far, does not exhibit species specificity, i.e. precursors to mitochondrial proteins from Neurospora can be transferred into yeast or rat liver mitochondria (Schleyer et al., 1982). Also, precursor proteins from one type of cell, e.g. precursor to ornithine transcarbamylase, a urea cycle enzyme from rat liver, can be imported into mitochondria from rat kidney and heart, which do not contain this protein (Morita et al., 1982; Côte & Boulet, 1985).

At present, the existence of at least 3 mitochondrial receptor systems mediating import is suspected on the basis of competition experiments: one for import into outer membrane (insensitive to prior protease treatment) (Gasser & Schatz, 1983), the apocytochrome c receptor (Hennig et al., 1983) and a receptor for intermembrane space and matrix proteins (Riezman et al., 1983: Mori et al., 1985).

Evidence for the existence of receptor molecules is very strong. The next step must be the isolation of these proteins and detailed analysis of their properties and their role in import. Identification of receptor-like polypeptides could be achieved in the near future after solubilisation and separation of the outer membrane polypeptides which contain binding activity. Some progress in this direction has been already reported (Riezman et al., 1983; Schmidt & Neupert, 1984; Ono & Tuboi, 1985).

1.6.6 Translocation and processing of mitochondrial proteins

Once bound to a receptor site on the mitochondrial surface, precursor polypeptides must be transported into or across the mitochondrial membranes. This step i.e. translocation into one of the mitochondrial compartments, is presumably of considerable complexity.

It is not yet clear how many steps are involved, but it is obvious that different pathways exist. It has been repeatedly speculated that transport of proteins across the mitochondrial membranes takes place in sites where the two mitochondrial membranes come into close contact or may even be fused. These 'contact points' have been observed by electron microscopy of sectioned mitochondria. However, it is not known whether these structures are really related to protein transport. An understanding of the precise mechanism of transfer would require exact knowledge of the conformation of precursors and their changes during interaction with the membrane(s).

a) Energy requirement for import

Posttranslational import of precursors across the inner mitochondrial membrane, unlike import into the outer membrane, is an energy-dependent process. Detailed studies have been made by using *in vitro* import systems, in which radiolabelled precursors are incubated with isolated mitochondria. After incubation, mitochondria are re-isolated from the suspension by centrifugation. Proper processing of the precursors is assayed by immunoprecipitation and SDS/polyacrylamide gel electrophoresis. To confirm that the imported polypeptides are inside the mitochondria, their sensitivity to externally-added proteases is examined; internalised polypeptides should be inaccessible to the protease because of the physical barrier imposed by the mitochondrial membranes.

In elegant experiments, Schleyer *et al*. (1982) and Gasser *et al*. (1982a) demonstrated that, whether supported by substrate respiration or ATP hydrolysis, the ability of mitochondria to import proteins correlated with experimental conditions where the electrochemical potential gradient would be expected to be high, regardless of the intramitochondrial ATP concentration. Thus, it appears that import of proteins to the mitochondrial matrix or inner membrane depends on an electrochemical potential across the inner membrane, contrary to early suggestions (Nelson & Schatz, 1979) that ATP was the direct energy source. However, it is not known which is the specific role of membrane potential in mitochondrial protein import. One possibility is that it provides energy for translocation.

It appears that the N-terminal regions of precursors so far investigated are predominantly basic in nature; therefore it is possible that transport is initiated by movement of a cluster of positively charged residues of a precursor polypeptide towards the more electronegative

mitochondrial matrix. Another possibility is that the electrochemical potential may induce interactions between the two membranes or favour a protein conformation which in turn would allow the polypeptide to cross the lipid bilayer, perhaps involving changes in membrane lipid conformation (Schatz & Butow, 1983).

The insertion of mitochondrial proteins into the outer mitochondrial membrane seems to be independent of a membrane potential or ATP. These features are also shared by cytochrome c. However, unlike cytochrome c, the major outer membrane proteins such as porin do not require covalent modification during its insertion in this membrane. (Suissa & Schatz, 1982; Freitag et al., 1982, Gasser & Schatz, 1983; Pfaller et al., 1985).

b) Proteolytic processing of imported precursors

During or shortly after their translocation, many mitochondrial precursor proteins undergo some form of covalent modification. The most frequent modification is proteolysis. The occurrence of the processing protease in the mitochondrial matrix suggests that at least part of the precursor molecule must be transferred into the matrix before cleavage can take place. For those proteins of the matrix and inner membrane which are synthesised as larger precursors, cleavage occurs in one step and the resulting species are electrophoretically-indistinguishable from the mature proteins.

The matrix protease has been purified from yeast (McAda & Douglas, 1982; Böhni et al., 1983) and detected in rat (Conboy et al., 1982; Miura et al., 1982) and Neurospora (Teintze et al., 1982). The yeast enzyme is insensitive to serine protease inhibitors, sulphydryl-modifying reagents or small peptide protease inhibitors. It is insensitive to chelating agents including EDTA, GTP and 1,10-phenanthroline. The enzyme exhibits little species specificity.

Surprisingly, the transport of at least some proteins destined for the intermembrane space, which must only cross the outer membrane is rather complicated. These proteins are apparently processed by two successive proteolytic events, since an intermediate form between the original precursor and the mature form is transiently generated. Yeast and Neurospora cytochrome c_1 (Gasser et al., 1982; Ohashi et al., 1982; Teintze et al.,1982) and yeast cytochrome b_2 and cytochrome c peroxidase (Daum et al., 1982b; Reid et al., 1982) have been clearly shown to be processed in this fashion. The first cleavage requires the matrix protease described above, yielding the intermediate forms, which are subsequently cleaved into their mature forms by a second protease which is located on the outer face of the inner membrane. A two-step processing pathway has been proposed for the rat liver ornithine transcarbamylase (Mori et al., 1980; Kolansky et al., 1982). However, the conversion of the intermediate form into the mature form has not yet been conclusively demonstrated (Morita et al., 1981).

A hypothetical mechanism for the transfer of precursors into mitochondria by the various discussed pathways is presented in Fig. 1.5.

1.6.7 Assembly of imported mitochondrial proteins

Once imported into mitochondria, usually after being proteolytically matured, polypeptides must assume their active conformation. Individual monomers must first reach their proper submitochondrial compartment, then some of them may bind to ligands such as haem groups or FAD to become active. Finally, some proteins require the formation of homo-oligomers and/or association with other proteins to form hetero-oligomers with either products of the mitochondrial genome or with other imported polypeptides. This process is also expected to occur in a defined pathway. Assembly of various subunits of these complexes must also be a coordinated process since subunits are present

Fig. 1.5 Mechanisms involved in transfer of various precursors into mitochondria (Hypothetical sequence of events)

Step 1: Extramitochondrial precursors are recognised by specific receptors (R) at the mitochondrial surface.

Steps 2 and 3: The outermembrane (OM) and the inner membrane (IM) of the mitochondria come into contact at certain sites and form "fusion sites". The precursor-receptor complex reorients in this area, perhaps aided by an hypothetical "translocator" protein (T). Either the formation of these fusion sites or the reorientation of the complex in the fused areas (or both processes) depend on the electrochemical potential across the inner membrane.

Steps 4: A,B and C: Precursors are transiently or permanently inserted in the inner membrane, processed and allocated to their final destinations.

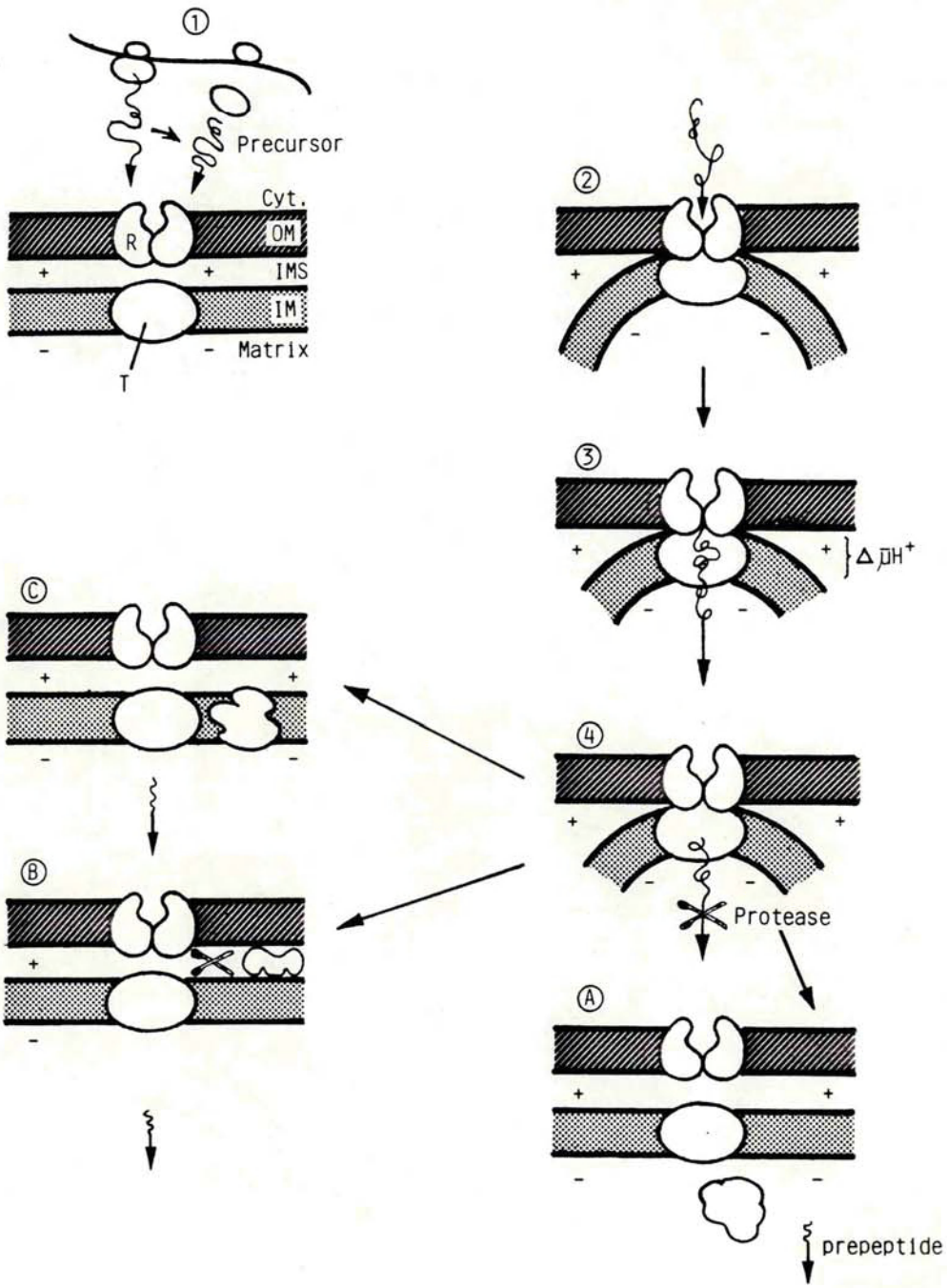
A: the precursor is attacked by a protease contained in the matrix. After removal of the presequences, the mature protein is discharged in the matrix.

B: the precursor is attacked by a protease in the matrix and relocated into the intermembrane space, a step which may involve a second proteolytic event (two-step processing pathway).

C: the proteolytically-processed precursor occupies its final destination in the inner membrane.

Polypeptides destined for the outer membrane and some destined for the inner membrane can be inserted without proteolytic processing (e.g. porin and the adenine nucleotide translocator) (not shown).

Modified from Hennig & Neupert (1983).


①
Precursor
Cyt.
R
OM
IMS
IM
Matrix
T
②
③
$\Delta\bar{\mu}H^+$
④
Protease
Ⓐ
prepeptide
Ⓑ
Ⓒ

in stoicheiometric amounts (Hennig & Neupert, 1983).

The techniques for detecting intermediate forms in the assembly of mitochondrial complexes have not yet been adequately developed due to the experimental difficulties of inhibiting assembly and distinguishing between assembled and non assembled polypeptides. In principle, it is possible to investigate this process by using yeast mutants in which synthesis of a specific subunit of a complex is deficient. It is also possible to isolate strains with mutations in components required for assembly.

The assembly of the H^+-translocating ATPase complex offers one interesting model to study the biosynthetic interactions between enzymes encoded in the mitochondria and in the nucleus. Using cell-free import systems, Lewin & Norman (1983) have shown that at least 3 of the newly-imported subunits of this complex are assembled inside the mitochondria with sufficient affinity to co-immunoprecipitate.

Evidence that polypeptides imported *in vitro* can be transported into their correct intramitochondrial locations and assembled into functionally-active units has been obtained (Gasser *et al.*, 1982a; Schleyer & Neupert, 1984). However, further experimentation is necessary to find a system capable of importing sufficient precursor to generate detectable enzyme activity.

1.6.8 The genetic approach

Many of the questions related to the structural features which allow precursor polypeptides to be recognised by mitochondria could be answered by a molecular analysis of these proteins. Such studies have been hindered by the low abundance of precursors. At the present, only the subunit of the yeast F_1-ATPase has been purified in relatively large amounts (Ohta & Schatz, 1984) by growing rho^- mutants in the presence of CCCP (Reid & Schatz, 1982a).

Recently developed methods for isolating and manipulating nuclear genes encoding mitochondrial proteins have provided a new tool to elucidate the molecular features of the import process. The sequences of about a dozen cloned genes have been determined. For those proteins synthesised as larger polypeptides, the amino acid sequence of the extra segment in the precursor (if the N-terminal sequence of the mature protein is known) can be obtained (Reid, 1985). Analysis of the N-terminal sequences showed no obvious homologies, but this is not surprising, considering that the N-terminal regions of different mitochondrial proteins vary in size, and the proteins so far investigated are transported into different submitochondrial compartments, so presumably have different addressing signals.

The N-terminal regions examined are predominantly basic. The signal sequences for secretory proteins are also basic and contain a stretch of uncharged amino acids which may form a transmembraneous segment during translocation. A similar feature has been found in the precursor to cytochrome c peroxidase, which is apparently imported in a two-step processing mechanism. The extra peptide of 68 amino acids includes a stretch of 23 uncharged amino acids in which 10 consecutive alanine residues are found. This hydrophobic region is assumed to form an α -helix to span the bilayer of the inner membrane when the precursor is imported, acting as a stop-transfer signal (Blobel, 1980; Kaput et al., 1982). The hydrophobic segment is flanked by basic residues, perhaps to anchor the protein in the membrane. The basic nature of the extra sequences and the hydrophobic segments are not sufficient to explain either intracellular or intramitochondrial sorting. In addition, information in the precursors must specify cleavage site(s) for processing enzymes. One approach that has been followed to delineate the targetting regions in the precursor molecules, which are

essential for processing or for correct localisation, has been the introduction of restricted deletions in different regions of the polypeptide chain and following the fate of the mutant protein (Hase et al., 1984; Mihara & Sato, 1985).

By combining deletions and gene fusion experiments, it has been shown that the signal regions of mitochondrial proteins (ornithine transcarbamylase, subunit IV of cytochrome c oxidase) can direct enzymes of non mitochondrial origin such as dihydrofolate reductase into mitochondria. (Hurt et al., 1984; Horwich et al., 1985). Fusions have also been constructed between proteins of the secretory pathway and signal sequences for mitochondrial proteins. In most studies, the information contained in the amino terminal region can direct the hybrid (or chimeric) proteins into the mitochondrion(Hurt et al., 1985).

Studies on molecular cloning of mitochondrial proteins have been successful in yeast (S. cerevisiae). This is due, among other reasons, to the possibility of transformation of these cells by plasmid vectors and the availability of genetically-well characterised strains. Mutants which have defective components in the mitochondrial import machinery; receptors, translocating components, proteases and other unknown molecules could be obtained. Yaffe and coworkers have characterised two of these mutants (Yaffe & Schatz, 1984a; Yaffe et al., 1985).

The import of proteins into chloroplasts shares many features with mitochondrial protein import (Chua & Schmidt, 1979); however, there must be clearly recognisable differences between the precursors destined for mitochondria and those for chloroplasts since the two import processes are able to coexist in the plant cell.

It is now thought that the selection of nuclear genes and their manipulation, in conjunction with well characterised and highly

efficient transcription-translation systems will make it feasible to reconstitute the import pathways *in vitro*. This will permit a more detailed analysis of the molecular events involved in the biogenesis of mitochondria.

Chapter 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals

The following reagents were obtained from Sigma Chemical Co., Poole, Dorset, U.K. : substrates and coenzymes for enzymatic assays, CoA esters, PMSF, TEMED, benzamidine-HCl, Coomassie Brilliant Blue types R250 and G, 1,10-phenanthroline, N-ethylmaleimide, Mops, DNP leupeptin, digitonin, deoxycholic acid (sodium salt), Tween 20, EGTA, DTNB.

Leuco 2',7' dichlorofluorescein diacetate was purchased from Eastman-Kodak Ltd., Lancs., U.K. Poly(ethylene glycol) 6000 was obtained from Serva, Heidelberg, W. Germany. Iodogen™ was from Pierce Labs, Rockford, Illinois, U.S.A. FCCP was a generous gift from Dr. P.G. Heytler, Du Pont De Nemours & Co., Delaware, U.S.A. DMSO, DTT, PPO and sucrose were purchased from Koch-Light Laboratories, Colnbrook, Berks, U.K. Sodium salicylate was obtained from Aldrich Chemical Co., Ltd., Gillingham, U.K.

All other chemicals including reagents for polyacrylamide gel electrophoresis were AnalaR grade from BDH Chemicals Ltd., Poole, U.K.

2.1.2 Tissue culture materials

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

Eagle's medium (minus L-methionine, minus L-glutamine), new born calf serum and foetal calf serum were purchased from GIBCO, Paisley, U.K.

Penicillin G and streptomycin sulphate were from Sigma Chemical Co., Poole, Dorset, U.K.

"Versene" solution (0.6 mM-EDTA, 0.17 M-NaCl, 10 mM-Na_2HPO_4, 2.4 mM-KH_2PO_4, 0.002% (w/v) phenol red, pH 7.8) and "Trypsin" solution (0.25% (w/v) trypsin,10 mM-NaCl, 1 mM-sodium citrate, 0.002% (w/v) phenol red, pH 7.8) were supplied as sterile solutions by the Tissue Culture Unit of our Department.

Disposable plasticware was obtained from Sterilin, Teddington, U.K. Minus methionine medium was prepared by mixing 100 ml Eagle's medium (minus L-methionine, minus L-glutamine medium) with 10 ml of calf or foetal serum plus 5 ml of non-essential amino acids and 5 ml of 0.2 M-glutamine. Before use, this solution was diluted 19:1 with normal growth medium (low methionine medium).

2.1.3 Radiochemicals

N-ethyl[2,3-^{14}C] maleimide (6 mCi/mmol), L-[^{35}S]methionine (1100-1600 Ci/mmol), [^{14}C]methylated protein mixture, Na^{125}I (carrier free), [2-^{14}C]pyruvate (10.4 Ci/mmol) were obtained from Amersham International, Bucks, U.K.

N-[Et-2-^{3}H]Ethylmaleimide (50 Ci/mmol) dissolved in pentane and Protosol were purchased from New England Nuclear, Ltd., Southampton, U.K.

[γ-^{32}P]ATP (100-130 Ci/mmol) was either purchased from Amersham International or synthesised according to Maxam & Gilbert (1980) by Mr. D.Mease in our Department.

2.1.4 Enzymes and proteins

The following commercial protein preparations were obtained from Sigma Chemical Co. : citrate synthase, type II (porcine heart), actin (rabbit muscle), cytochrome c, type III (horse heart), protein A (Staphylococcus aureus, Cowan I strain), hexokinase, fraction II (yeast), glucose 6-P dehydrogenase, type VIII (baker's yeast), fumarase (affinity purified from porcine heart), bovine serum albumin, immunoglobulin G,

horse-radish peroxidase, TPCK-treated trypsin, TLCK-treated α-chymotrypsin and elastase type II (porcine pancreas).

Lipoamide dehydrogenase (diaphorase) from pig heart was from Boehringer Corp., (London) Ltd., Sussex, U.K.

Staphylococcus aureus V8 protease was purchased from Miles Laboratories, Stoke Poges, Slough, U.K.

Marker proteins for M_r determinations were obtained from Bio-Rad Laboratories (low molecular weight kit), Watford, Herts, U.K.

Pyruvate dehydrogenase complex from Escherichia coli was a gift from Dr. John Coggins in our Department. Purified pyruvate dehydrogenase kinase (bovine kidney) was the generous gift of Dr. Lester Reed, University of Texas, Texas, U.S.A.

Subunits of the yeast pyruvate dehydrogenase complex were kindly provided by Dr. Hans Bisswanger, Universitat Tübingen, Physiologisch Chemisches Institut, W. Germany.

Purified branched-chain 2-oxo-acid dehydrogenase complex and antiserum to the E2 subunit of this complex were a gift from Mr. G. Clarkson; 2-oxoglutarate dehydrogenase complex and antiserum to pig heart fumarase were kindly provided by Miss A. Hunter and Mr. G. Gibb, respectively (Department of Biochemistry, University of Glasgow).

2.1.5 Chromatographic reagents

Sepharose CL-2B, CL-6B and 4B were the products of Pharmacia, London, U.K. Phosphocellulose was obtained from Whatman Biochemicals, Kent, U.K. CNBr-activated Sepharose 4B was obtained from Sigma Chemical Co., Poole, Dorset, U.K.

The TSK 3000 GSW column for high performance gel chromatography was obtained from Toya-Soda Corp., Japan.

2.1.6 Animals

New Zealand white rabbits (approx. 4-months old) and male rats (Albino Wistar strain) were obtained from the colony of the Departmental Animal House.

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

2.1.7 Miscellaneous

Nitrocellulose paper (0.45 μm pore size) was obtained from Schleicher and Schüll, Dassel, W. Germany.

Standardised Staphylococcus aureus cell suspension (Pansorbin) and Aquacide II (M_r 500,000) were obtained from Calbiochem-Behring Corp., Bishops Stortford, Herts, U.K.

Nalgene filters (0.2 μm pore size) were purchased from Nalge Co., Rochester, N.Y. U.S.A.

Freund's adjuvants were obtained from Difco, West Molesey, Sussex, U.K.

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

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

2.2 METHODS

2.2.1 Measurement of protein concentration

The concentration of protein was routinely determined by the method of Lowry et al. (1951) as modified by Markwell et al. (1976). The dye binding method of Bradford (1976), as modified by Spector (1978), was utilised when the samples contained reagents which interfered with the method of Lowry e.g. urea, 2-mercaptoethanol.

The method of Bradford is based in the quantitative binding of protein to Coomassie Brilliant Blue. The reagent was prepared as follows: 100 mg of Coomassie Brilliant Blue G 250 were dissolved in 50 ml of 95% (v/v) ethanol, then 100 ml of 85% (w/v) phosphoric acid were slowly added. The solution was diluted to a final volume of 1 litre with glass distilled water and filtered through Whatman No.1 filter paper. The reagent was stored at room temperature.

Aliquots of the protein solution, containing up to 40 µg of protein in 100 µl, were mixed with 2.5 ml of the reagent and the absorbance was measured at 595 nm in a spectrophotometer using disposable plastic cuvettes.

Standard curves were constructed with each set of assays using bovine serum albumin as a standard. The concentration of the BSA solution was calculated using $A_{280}^{1\%}$ 6.6.

Protein was also determined by scanning the Coomassie Brilliant Blue-stained bands of proteins resolved by SDS/polyacrylamide gel electrophoresis. Various amounts (1 to 10 µg) of BSA were electrophoresed in the same gel. The values of the integrated area under each peak (arbitrary units) were used to establish a standard curve.

2.2.2 Dialysis

Dialysis was carried out using Visking Tubing (Scientific Instrument Centre Plc., London, U.K.), which has been immersed in boiling 0.5-1% (w/v) EDTA for 15 min and washed thoroughly with distilled water to remove impurities.

2.2.3 Concentration of protein samples

Protein solutions were concentrated by freeze-drying or by treatment with poly(ethylene glycol) or Aquacide (trade name for carboxymethyl cellulose). For this procedure, samples were placed in small diameter dialysis bags prepared as described in section 2.2.2 and layed on a flat surface. The bags were sprinkled with dry Aquacide or poly(ethylene glycol) to cover them completely and left at 4°C until the sample had concentrated to the required volume.

Frequently, samples for SDS/polyacrylamide gel electrophoresis were previously concentrated by addition of 4-6 vol. acetone and stored at -20°C for several hours.

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

2.2.4 Determination of radioactivity

^{125}I was counted in a LKB Wallac 1275 mini Gamma counter.

The isotopes ^{3}H, ^{14}C, ^{35}S, ^{32}P were counted in a Beckman IS 6800 scintillation counter. Aqueous samples (200-500 µl) were mixed with 5 ml scintillation fluid containing 35% (v/v) Triton X-100 and 5 g/l PPO in toluene.

2.2.5 Measurement of the incorporation of radioactivity into proteins

a) Filter paper discs method

To estimate the incorporation of radioactivity into proteinaceous samples, aliquots (10-25 µl) were spotted onto Whatman No.1 filter paper discs (2.5 cm diam.) and dropped into ice-cold 10% (w/v) TCA (approx. 10 ml/disc). After 30 min at 4°C, the TCA solution was discarded. Filters were washed twice with a similar volume of 10% (w/v) TCA and once with absolute ethanol. Each wash was carried out at 4°C with constant stirring for 30 min. Finally, the discs were air-dried for several hours or dried under an infra red lamp, transferred to scintillation vials and counted with 5 ml of scintillation fluid. Background radioactivity was estimated using blank discs that were mixed with the sample discs during the first wash.

b) Determination of radioactivity incorporated into gel slices

Samples of radioactive proteins were separated by SDS/polyacrylamide gel electrophoresis. The gels were stained with Coomassie Blue (section 2.2.6 d). After destaining, the gel was dried under vacuum on Whatman No. 3 MM filter paper and the protein bands of interest were cut with scissors. The slices were dropped into glass scintillation vials and reswollen in 0.5 ml of water. After 10 min, the pieces of filter paper were removed as well as the remaining liquid and the slices were incubated overnight at 37°C with 1 ml of 90% (v/v) Protosol in capped scintillation vials.

The samples were then used for radioactivity measurement after adding 9 ml of scintillation fluid.

Pieces of gel of similar dimensions were cut from blank tracks and used to estimate background radioactivity.

2.2.6 Electrophoretic separation of proteins in SDS/polyacrylamide gels

a) Preparation of samples for electrophoresis

Protein solutions for analysis by SDS/polyacrylamide gel electrophoresis were mixed 1:1 (v/v) with Laemmli sample buffer and boiled for 3-5 min before electrophoresis.

Laemmli sample buffer contained 62.5 mM-Tris/HCl buffer, pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose or glycerol (v/v) and Pyronin Y as tracking dye. The solution was kept at room temperature. Before use, 2-mercaptoethanol or DTT were added to a final concentration of 5% (v/v) or 10 mM, respectively.

b) Analytical SDS/polyacrylamide gel electrophoresis

For analytical purposes, electrophoresis of proteins in polyacrylamide gels was carried out in the discontinuous Tris-glycine-buffered system of Laemmli (1970).

Gels were cast using our own equipment in slabs of 19 cm x 9.5 cm x 1.5 mm. Separating gels were polymerized from solutions that contained 0.375 M-Tris/HCl buffer pH 8.8, 0.1% (w/v) SDS, 0.03% (v/v) TEMED, 0.075% (w/v) ammonium persulphate and acrylamide to 10% (w/v). The ratio of acrylamide to NN' methylene(bis)acrylamide was 30:0.8. The stacking gel solution contained 0.12 M-Tris/HCl buffer, pH 6.8, 0.1% (w/v) SDS, 0.03% (v/v) TEMED, 0.2% (w/v) ammonium persulphate, 5% (w/v) acrylamide and 0.133% (w/v) NN' methylene(bis)acrylamide.

On occasions, 10% (w/v) polyacrylamide gel rods (12 cm x 0.5 cm) were polymerised in siliconised tubes using the Laemmli system or the phosphate-buffered system as described by Weber & Osborn (1969).

The electrophoresis buffer contained 0.024 M-Tris, 0.192 M-glycine, 0.1% (w/v) SDS (Laemmli system) or 0.05 M-sodium phosphate buffer, pH 7.0, 0.1% (w/v) SDS (phosphate-buffered system).

Electrophoresis was performed at constant current at 40-60 mA/slab gel and 6-8 mA/rod gel.

c) Preparative SDS/polyacrylamide gel electrophoresis

Preparative gels were polymerised as a large scale version of the analytical procedure described above by increasing the gel thickness to 3 mm. This permitted application of up to 3 mg protein, while maintaining most of the high resolution power obtained with the analytical system.

d) Staining and scanning of gels

Protein bands were visualised by immersing the gels in a solution containing 0.04% (w/v) Coomassie Brilliant Blue R in 25% (v/v) isopropanol, 10% (v/v) acetic acid for 8-12 h, and destaining in 10% (v/v) acetic acid.

Slab gels were scanned with a LKB 2202 Ultroscan Laser Densitometer (LKB, Uppsala, Sweden). Rod gels were subjected to densitometric analysis at 595 nm in a Gilford 2410-S spectrophotometer with a scanning attachment.

e) Fluorography

Gels to be fluorographed were previously stained or directly fixed overnight by immersing in 25% (v/v) isopropanol, 10% (v/v) acetic acid.

Fluorography was performed according to the procedure described by Bonner & Laskey (1974) with DMSO-PPO or by following the method described by Chamberlain (1979) employing sodium salicylate as fluor.

Slab gels were dried under vacuum and subjected to fluorography with Kodak X-Omat S or XAR-5 film at -80°C for a suitable length of time.

f) Autoradiography

Gels were stained or fixed as described above. Before drying, gels were soaked for 30 min in a solution containing 20% (v/v) methanol, 10% (v/v) acetic acid and 0.5% (v/v) glycerol to reduce the risk of cracking during drying.

g) Determination of M_r by SDS/polyacrylamide gel electrophoresis

M_r of the polypeptides resolved by SDS/polyacrylamide gel electrophoresis was determined by calibrating 10% (w/v) slab gels with a set of standard proteins. They included: phosphorylase b (M_r 92,000); BSA (M_r 68,000); ovalbumin (M_r 43,000); carbonic anhydrase (M_r 31,000); soybean trypsin inhibitor (M_r 20,100) and lysozyme (M_r 14,000).

Relative mobility (m) was calculated as the ratio:

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

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

2.2.7 Isolation of mitochondria and submitochondrial fractions

a) Isolation of rat liver mitochondria

Rat liver mitochondria were prepared by differential centrifugation as described by Chance & Hagihara (1963).

Isolation medium:

0.225 M-mannitol, 0.075 M-sucrose, 500 µM-EGTA, 2 mM-Mops. The solution was adjusted to pH 7.2 with NaOH and stirred until the EGTA was completely dissolved; then the pH was finally adjusted to 7.4.

Rats were starved overnight and killed by cervical dislocation. The livers were removed, blotted, chopped to small pieces with scissors and transferred into ice-cold isolation medium.

Livers from 6 rats were homogenised in approx. 30 ml isolation medium per liver with a tight-fitting Potter-Elvehjem homogenizer (0.004-0,006 in clearance, shaft rotation approx. 1000 rev/min). Between 6 and 10 passes were required for maximal cell disruption. After sedimentation of the nuclear fraction at 800 g for 7 min in a MSE-21 8x50 ml angle rotor at 4°C, mitochondria were sedimented from the supernatant fluid by centrifugation at 6500 g for 15 min. Pellets were washed by manual resuspension in the same buffer and centrifugation at 6500 g for 15 min. To remove residual contamination from nuclei, red blood cells and unbroken cells, mitochondrial pellets were resuspended in one-half the initial volume of isolation medium and centrifuged at 800 g for 7 min. The pellets were discarded and the supernatant fractions were carefully combined and centrifuged at 6500 g for 15 min. The final pellets contained mitochondria which were kept frozen at -20°C until required.

For most purposes, the mitochondrial preparation described above was found to be satisfactory. On occasions, however, as for the preparation of sub-mitochondrial fractions, it was convenient to further purify the organelles. This was accomplished after a sucrose-density gradient centrifugation as described by D' Souza & Lindsay (1981). The purified mitochondria were found in the interface between the 45% (w/w) and the 39% (w/w) sucrose solutions (density 1.19 g/ml). Approx. 60% of the protein of the crude mitochondrial preparation was recovered in this band. The purified mitochondria were pelleted by centrifugation at 6500 g for 15 min after diluting by slow addition of 3 vol. isolation medium.

Mitochondria were washed once with 20 mM-Mops buffer, pH 7.2, 0.12 M-KCl, in order to remove any proteins which were adventitiously bound to the outer membrane.

b) Preparation of submitochondrial fractions from rat liver

Highly-purified mitochondria were subfractionated by a modification of the procedure described by Sottocasa et al. (1967).

Separation was achieved by osmotically swelling the mitochondria, followed by shrinking the inner membrane compartment, which led to partial release of the outer membrane.

Fractions were collected with the aid of a shortened Pasteur pipette. The "outer membrane" and "heavy" fractions, collected from the gradients, contained a mixed population of vesicles derived from the outer and inner mitochondrial membranes; hence these fractions were pooled in a mixed membrane fraction (M).

Membrane fractions were washed in 10 ml of 20 mM-Mops, 0.12M-KCl, adjusted to pH 7.2 with NaOH, by manual homogenisation followed by centrifugation at 105,000 g for 1 h to remove soluble proteins. The soluble fraction was centrifuged twice at 105,000 g for 1 h to remove any particulate material.

Activities of the enzymes used as markers for the submitochondrial compartments (monoamine oxidase, adenylate kinase, fumarase and cytochrome c oxidase) were determined in the freshly-prepared fractions. The remaining material was stored at -20°C until required.

c) Preparation of ox heart mitochondria and submitochondrial fractions

Ox heart mitochondria were isolated as described by Smith (1967). Approx. 1.5-1.8 g of mitochondrial protein were obtained from 300 g tissue. Mitochondrial pellets were stored at -80°C until use. Under these conditions PDC activity was stable for less than one month.

The fractions of ox heart mitochondria utilised in this study were prepared by Miss A. Phelps in our laboratory.

For the fractionation of mitochondria, samples containing approx. 200 mg protein were manually resuspended in 5 ml of 20 mM-potassium phosphate buffer, pH 7.5, 0.1 mM-EDTA and subjected to sonic oscillation at 4°C for three 20 s intervals using the maximum setting of an MSE sonicator. Sonicated mitochondria were diluted to 10 ml with the same buffer before centrifugation at 105,000 g for 1 h in a Beckman Ti 50 rotor. The clear supernatant or 'soluble' fraction contained most of the mitochondrial matrix and intermembrane space proteins. The dark-brown pellets consisted principally of sub-mitochondrial particles (Lee, 1979). The membraneous fraction was washed twice by manual homogenisation in 5 ml of 20 mM-potassium phosphate buffer, pH 6.5, 0.05 M-NaCl, followed by centrifugation at 30,000 g for 30 min at 4°C. The procedure was repeated once more.

Both soluble and washing fractions were concentrated by osmotic treatment with Aquacide, as described in section 2.2.3, prior to dialysis against 2x500 ml vol. 20 mM-Tris/HCl buffer, pH 7.4, 0.01% (w/v) NaN_3 at 4°C.

d) Preparation of cell extracts and subcellular fractions from cultured mammalian cells

Cell extracts for immunoblotting analysis were prepared under conditions which minimised proteolysis. Cells were grown in large roller bottles (burlers) until they reached confluence. Monolayers were washed twice with ice-cold phosphate-buffered saline (20 mM-sodium phosphate buffer, pH 7.4, 0.15 M-NaCl). Cells were harvested in a small volume of the same buffer by scraping with a rubber policeman. Portions of the suspension were immediately dissociated by boiling in Laemmli sample buffer for 5 min. Any insoluble material was removed

by centrifugation. The remaining extract was fractionated by the method described by Attardi & Ching (1979) to obtain nuclear and mitochondrial fractions.

To obtain particulate and cytosolic fractions, cells were fractionated by the method of Zuurendonk & Tager (1974) as modified by Mori et al. (1981a). Aliquots (1 ml) of the cell suspensions ($5x10^6$ cells) were mixed at 0°C with an isomotic digitonin solution at final concentrations of 0.25 to 5 mg/ml. After standing at 0°C for 2 min, the samples were centrifuged for 1 min at 14,000 g in an MSE Micro-Centaur centrifuge. The supernatant (cytosolic) fractions were assayed for citrate synthase and fumarase, sections 2.2.8 d and 2.2.8 f, respectively, to establish the degree of mitochondrial disruption. Samples were then kept frozen at -20°C until required.

2.2.8 Enzymatic assays

a) 2-oxo-acid dehydrogenase complexes

The overall activity of pyruvate dehydrogenase complex (EC 1.2.4.1, EC 2.3.1.12 and EC 1.8.1.4.) was determined by measuring NADH formation at 340 nm at 25°C with a recording spectrophotometer according to Brown & Perham (1976). Assays were carried out in a final volume of 1 ml in 50 mM-potassium phosphate buffer, pH 8.0 containing 2 mM-TPP, 1 mM-$MgCl_2$, 2.5 mM-NAD^+, 0.13 mM-CoA, 2.6 mM cysteine-HCl and 2 mM-sodium pyruvate. The activity of 2-oxo-glutarate dehydrogenase complex (EC 1.2.4.2, EC 2.3.1.6 and EC 1.8.1.4) was measured in a similar reaction mixture containing 2 mM-sodium oxo-glutarate instead of pyruvate.

A unit of activity (Kat) was defined as that amount of enzyme which produced 1 mol of NADH/s at 25°C under the conditions of assay.

b) Pyruvate dehydrogenase kinase. (EC 2.7.1.99) was assayed as described by Hucho et al. (1972) by measuring the rate of inactivation of the complex in the presence of 0.2 mM-ATP at 30°C or by estimation of the rate of incorporation of ^{32}P-labelled phosphoryl groups from [γ-^{32}P]ATP into PDC according to Stepp et al. (1983). Incubation mixtures contained 20 mM-potassium phosphate buffer, pH 7.5, 1 mM-$MgCl_2$, 0.1 mM-EDTA, 2 mM-DTT and 20-50 µg of PDC in 200 µl of assay mix. After equilibration at 30°C for 1 min, the reaction was initiated by addition of 0.02 ml of 2.5 mM [γ-^{32}P]ATP (approx. 13,000 d.p.m./nmol). At the indicated times, aliquots (20 µl) were applied to filter paper discs. The discs were immediately dropped into 10% (w/v) TCA, washed, dried and utilised for radioactivity counting as described in section 2.2.5.a.

c) Pyruvate dehydrogenase phosphate phosphatase (EC 3.1.3.43) was assayed by the procedure of Hucho et al. (1972).

^{32}P-labelled PDC was used as a substrate. The phosphorylated enzyme was prepared by incubation of PDC (1 mg) at 30°C in the presence of [γ-^{32}P]ATP (0.2 mM; 110,000 d.p.m./nmol) as described above. After 30 min, aliquots (10 µl) were withdrawn to estimate protein-bound radioactivity. The remaining sample was diluted with 10 ml of 20 mM-potassium phosphate buffer, pH 7.0 containing 0.1 mM-DTT, 0.01% (w/v)NaN_3 and concentrated by centrifugation at 144,000 g for 2 h in a Beckman Ti 50 rotor. The pellet was dissolved in 500 µl of 20 mM-Tris/HCl buffer, pH 7.5, 10 mM-$MgCl_2$, 0.5 mM-$CaCl_2$, and used for determination of phosphatase activity as described in the legend to Fig. 3.5.

d) Citrate synthase. Citrate-oxalacetate-lyase (EC 4.1.3.7.) activity was measured as described by Srere (1969) by following the deacetylation of AcSCoA at 412 nm. The production of CoA was detected by the formation of a mercaptide with DTNB (Ellman's reagent) which exhibits strong absorbance at 412 nm (ε_{412} 13,000). The assay mix contained, in a final volume of 1 ml, 200 µl, 0.5 M-Tris/HCl buffer pH 8.0, 200 µl, 0.5 mM-DTNB (prepared fresh), 100 µl of 3 mM-AcSCoA, and variable amounts of enzyme and water to 0.9 ml. The absorption was followed for 1-2 min to measure any endogenous levels of thiol or deacylase activity before initiating the reaction by the addition of 100 µl of 5 mM-oxalacetate (prepared fresh and neutralized with diluted KOH).

Enzyme units were expressed as the amount of enzyme that produced 1 µmol of CoA/min at 25°C.

e) Monoamine oxidase (Monoamine: oxygen oxidoreductase, deaminating; EC 1.4.3.4.) activity was measured as described by Köchli & Wartburg (1978) by following the oxidation of leuco 2'7' dichlorofluorescein to 2'7' dichlorofluorescein at 502 nm coupled to the monoamine oxidase-dependent production of H_2O_2 in the presence of benzamidine. Each cuvette contained in a final volume of 3 ml: 600 µl of 0.25 mM-leuco 2'7' dichlorofluorescein diacetate (freshly prepared in 10 mM-NaOH), 1.8 ml of horse-radish peroxidase, prepared by dissolving 0.83 mg in 10 ml of 0.1 M-sodium phosphate buffer, pH 7.15 and variable amounts of enzyme and water to a final volume of 2.95 ml. The absorbance at 502 nm was monitored for 2-3 min until a base line was obtained. Reactions were started by the addition of 50 µl of 0.1 M-benzamidine. One unit was defined as the amount of enzyme that produced 1 µmol of 2'7' dichlorofluorescein/min at 25°C using ε_{502} 92,000.

Crude extracts can lead to slow oxidation of the reagent in the absence of amines. Reaction rates were corrected with these blanks.

f) Fumarase. (EC 4.2.1.4.) activity was measured spectrophotometrically by recording the increase in absorbance at 240 nm following the formation of fumarate from malate as described by Hill & Bradshaw (1969). Each cuvette contained in a final volume of 1 ml, 0.97 ml of an L-malate solution prepared by dissolving 336 mg L-malic acid in 50 ml 0.1 M-potassium phosphate. The solution was adjusted to pH 7.6 with 2 M-NaOH. Reactions were initiated by the addition of the enzyme solution (0.01-03 ml).

Enzyme units were expressed as the amount of enzyme which produced 1 µmol fumarate/min at 25°C (ε_{240} 2,440).

g) Adenylate kinase. (ATP:AMP phosphotransferase; EC 2.7.4.11) activity was determined spectrophotometrically by monitoring the reduction of NAD^+ at 340 nm in a reaction mixture of 1 ml containing 50 mM-Tris/HCl buffer, pH 7.5, 5 mM-ADP, 10 mM-glucose, 10 units of hexokinase, 10 units of glucose 6-P dehydrogenase, 5 mM-$MgSO_4$ and 0.2 mM-$NADP^+$ at 25°C. (Sottocasa et al., 1967).

Units of enzyme activity were expressed as the amount of enzyme which produced 1 µmol of NADPH/min at 25°C (ε_{340} 6,220).

h) Cytochrome c oxidase. (EC 1.9.3.1) activity was monitored by following the decrease in absorbance of cytochrome (α band) at 550 nm coupled to the oxidation of cytochrome c as described by Cooperstein & Lazarow (1951). Each cuvette contained in a final volume of 3 ml; 2.98 ml of reduced cytochrome c solution prepared by dissolving 1 mg of cytochrome c in 30 ml of 30 mM-sodium phosphate buffer, pH 7.4 and adding 100 µl of 1.2 M-sodium dithionite.

The solution was shaken vigorously for 2 min to remove excess dithionite before starting the reaction by the addition of the enzyme solution.

Enzyme units were expressed as the amount of enzyme that oxidised 1 μmol of cytochrome c/min at 25°C. (ε_{550} 28,500).

2.2.9 Purification of pyruvate dehydrogenase complex from ox heart

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

All operations were carried out at 4°C, starting with 250 or 500 g of fresh ox heart. The tissue cubes were blended and extracted twice in a 50 mM-Mops buffer containing 2.7 mM-EDTA, 3% (v/v) Triton X-100 and 0.1 mM-DTT, adjusted to pH 7.0 with NaOH. The residual material was discarded. The pooled extracts contained assayable amounts of 2-oxoglutarate dehydrogenase activity. However, the presence of lactate dehydrogenase interfered with the estimation of the pyruvate dehydrogenase complex activity at the early stages of purification.

The complexes were precipitated by the addition of 0.12 vol. of a 35% (w/v) poly(ethylene glycol) solution at pH 6.45. Pellets were resuspended in Mops buffer supplemented with 1% (v/v) Triton X-100, 2.7 mM-EDTA, 0.1 mM-DTT, 1.5 μM-leupeptin and 0.5% (v/v) rat serum, adjusted to pH 6.8 with NaOH, by homogenisation with a loose-fitting glass-teflon homogenizer, followed by sonication three times for 30 s each with a Dawes Sonicator (3A, setting 6). This extract was adjusted to 13 mM-$MgCl_2$ and 50 mM-sodium phosphate before the addition of poly(ethylene glycol) for the second precipitation. The activation step at 30°C outlined in the original procedure was omitted.

The 2-oxo-acid dehydrogenase complexes were separated by differential precipitation with poly(ethylene glycol). Usually 0.04-0.06 vol. 35% (w/v) poly(ethylene glycol) was required to precipitate 90% of the 2-oxoglutarate dehydrogenase activity. The resultant fraction contained approx. 90-95% of the PDC activity. This complex was concentrated by centrifugation for 2 h at 176,000 g in a Beckman Ti 60 rotor or by addition of 0.15 vol. of 35% (w/v) poly(ethylene glycol) followed by centrifugation at 30,000 g for 10 min.

Pellets containing the 2-oxoglutarate dehydrogenase and pyruvate dehydrogenase activities were dissolved and purified separately by gel filtration chromatography on a Sepharose CL-2B column (106 cm x 3.5 cm) at 4°C. The Sepharose CL-2B column was equilibrated and developed with 50 mM-sodium phosphate buffer, pH 7.0 containing 1% (v/v) Triton X-100, 2.7 mM-EDTA at a flow rate of 24 ml/h. Fractions of 12 ml were collected and assayed for pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase activities. Fractions were pooled in 4-5 portions which were concentrated by centrifugation at 176,000 g in a Beckman Ti 60 rotor for 2.5 h at 4°C.

The yellow pellets were resuspended by standing overnight in a small volume of 50 mM-Mops buffer containing 2.7 mM-EDTA, 1% (v/v) Triton X-100, 0.01% (w/v)NaN_3, 0.15 μM-leupeptin adjusted to pH 6.8 with NaOH. Solubilization was completed by manual homogenisation in the same buffer before storing the enzyme at 4-6 mg/ml at 4°C.

2.2.10 Resolution of the ox heart pyruvate dehydrogenase complex

a) Dissociation of the complex and resolution by gel filtration chromatography

Pyruvate dehydrogenase complex (25-50 mg) was dissociated under conditions of high salt and high pH, essentially as described by Linn et al. (1972). Before dissociation, the complex was concentrated

by centrifugation in a Beckman Ti 50 rotor for 2.5 h at 144,000 g. Pellets were resuspended in a small volume of 50 mM-sodium phosphate buffer, pH 7.5 containing 1 mM-DTT, 0.1 mM-EDTA. To this solution, sufficient 1 M-DTT, solid NaCl and 1 M-glycine buffer adjusted to pH 9.0 with NaOH were added to make the final concentration 1 M-NaCl, 10 mM-DTT and 0.1 M-glycine. The final pH was 9.0 and the protein concentration about 15 mg/ml.

The sample was allowed to stand for 30 min at room temperature before applying to a Sepharose CL-6B (or 4B) column (80 cm x 1.5 cm). This column was equilibrated and developed in 0.1 M-glycine buffer containing 1 M-NaCl, 2 mM-DTT, 1 mM-$MgCl_2$, 0.01 mM-EDTA, adjusted to pH 9.0 with NaOH. The flow rate was 8 ml/h. Fractions of 2 ml were collected and the A_{280} was measured. Protein was determined in the peak fractions by the method of Bradford (1976) as described in section 2.2.1.

Analysis of the polypeptide composition of the fractions was performed by SDS/polyacrylamide gel electrophoresis on 10% (w/v) slab gels. Samples containing approx. 15 µg protein were precipitated in 10% (w/v) TCA before electrophoresis as described in section 2.2.3.

PDC was also dissociated in 0.25 M-$MgCl_2$ as described by Kresze & Steber (1979). Samples were concentrated and redissolved as outlined above. Solid $MgCl_2$ was added to a final concentration of 0.25 M and the sample was dialysed overnight against 250 ml of 0.1 M-Tris/HCl buffer, pH 7.0, 0.25 M-$MgCl_2$.

To facilitate dissociation of the complex, the dialysed solution was frozen at -80°C for 16 h and thawed once; then it was left to stand at room temperature for 30 min before chromatography.

b) High performance gel filtration chromatography

PDC was dissociated with 0.25 M-$MgCl_2$ as described above. Aliquots of the solution containing the dissociated complex (1 mg) were injected onto a TSK 3000 GSW column (600 mm x 7.5 mm). Chromatography was performed at room temperature using a Gilson Model 303 high performance gel chromatography system. The flow rate was 0.5 ml/min and fractions of 0.5 ml were collected. A_{215} was monitored continuously with a Microm M3000 u.v. detector.

The polypeptide composition of the fractions was analysed by SDS/polyacrylamide gel electrophoresis. Protein was precipitated by addition of 2 ml of acetone and stored at -20°C for 16 h. Pellets were redissolved by boiling in 25 µl of Laemmli sample buffer before electrophoresis.

c) Fractionation of the dihydrolipoyl acetyltransferase-component X-kinase subcomplex

Peaks I and II, obtained after resolution of the PDC by gel filtration chromatography (see Fig. 3.6), were further resolved in order to isolate the individual constituents of the complex.

Peak I (called hereafter subcomplex I) contained the E2 subunit (transacetylase component), which forms the core of the complex and component X. Variable amounts of E1 and E3 remained associated with the core. PDC kinase can be purified from this fraction (Linn et al., 1972) after treatment with p-hydroxymercuriphenyl sulphonic acid.

Further dissociation of subcomplex I was attempted by following the procedure of Linn et al. (1972) as modified by Stepp et al. (1983)

Pooled fractions from this subcomplex were concentrated by treatment with Aquacide (section 2.2.3) to 5 mg protein/ml and dialysed for 24 h against 2x500 ml volume of 0.1 M-glycine containing 1 M-NaCl,

1 mM-$MgCl_2$, 2 mM-DTT, 0.01 mM-EDTA, adjusted to pH 9.0 with NaOH. Attempts to concentrate the samples by freeze-drying resulted in aggregation of the protein.

The dialysed solution was treated for 16 h at 0°C with p-hydroxymercuriphenyl sulphonic acid. This treatment caused reductive aggregation of the core, which was recovered after centrifugation at 150,000 g in a Beckman SW 60 rotor (acetyltransferase fraction). The transparent pellet was redissolved in a small volume of 50 mM-potassium phosphate buffer, pH 7.5 containing 30 mM-DTT, 0.1 mM-$MgCl_2$ 0.01 mM-EDTA and was stored at -20°C. The supernatant fluid was clarified twice by similar centrifugation steps to remove traces of the acetyltransferase. Finally, it was concentrated by treatment with Aquacide, dialysed overnight against 50 mM-potassium phosphate buffer, pH 7.5 containing 2 mM-DTT, 0.1 mM-$MgCl_2$, 0.01 mM-EDTA and stored at -20°C (kinase fraction).

d) Ammonium sulphate fractionation of Peak II

The second peak, emerging from the gel filtration chromatography columns of the dissociated complex, was subfractionated as follows: pyruvate dehydrogenase component (E1) was separated from the lipoamide dehydrogenase component (E3) by selective precipitation with ammonium sulphate as described by Linn et al. (1972). The E1 component was precipitated after the addition of solid ammonium sulphate to 30% (w/v) saturation at 0°C. The pellet was recovered by centrifugation at 8,000 g for 30 min. The supernatant fluid contained most of the E3 component.

Purified E1 was redissolved in a small volume of 50 mM-potassium phosphate buffer, pH 7.5 containing 1 mM-DTT, 0.1 mM-$MgCl_2$, 0.01 mM-EDTA before dialysis against 2x500 ml vol. of the same buffer.

Refractionation by ammonium sulphate precipitation was often found to be necessary to remove contaminating lipoamide dehydrogenase.

e) Separation of α and β chains of pyruvate dehydrogenase (E1)

E1 α and E1 β subunits were separated as described by Barrera et al. (1972). Approx. 5 mg protein were dissociated by dialysis at room temperature for 2 h against 200 ml 20 mM-imidazole containing 8 M-urea, 50 mM-2-mercaptoethanol, adjusted to pH 6.0 with HCl.

Phosphocellulose chromatography was carried out in a (10 cm x 0.9 cm) column equilibrated in the same buffer. After application of the sample, the column was washed with 10 ml buffer before the bound protein was eluted by increasing the concentration of LiCl in a stepwise manner up to 1 M.

Purified α and β subunits were subjected to SDS/polyacrylamide gel electrophoresis after measuring the protein concentration (Bradford, 1976). Fractions were dialysed against 50 mM-potassium phosphate buffer, pH 7.5 containing 0.1% (w/v) SDS and concentrated by treatment with Aquacide before storing at -20°C.

f) Elution of PDC subunits from polyacrylamide gels

Purified subunits of PDC were recovered from SDS/polyacrylamide gels basically as described by Tolan et al. (1980) with some modifications.

PDC (3 mg protein) was electrophoresed in 10% (w/v) preparative polyacrylamide gels (see section 2.2.6 c). Gels were stained for 4 h and destained with several changes of 10% (v/v) acetic acid, 20% (v/v) methanol. Stained protein bands were cut out of the gel and rinsed with distilled water for about 30 min. After extruding gel strips through the barrels of 2-ml disposable syringes to fragment the gels, pieces were transferred into glass scintillation vials and dried in a vacuum dessicator over NaOH pellets to remove the acid from

the destaining solution and to facilitate extraction. Dried gel pieces were swollen in a minimal volume (2-3 ml) of 50 mM-triethanolamine, 1% (w/v) SDS, 1 mM-DTT,adjusted to pH 8.0 with HCl (extraction buffer). The suspensions were heated for 5 min at 65-70°C and the gel slurry extracted by continuous stirring at room temperature for 16-20 h. Fragments of gel were removed by centrifugation at 1,000 g for 5 min to yield a clear blue-coloured extract. The gel slurry was extracted once more in a similar manner before pooling the extracts and concentrating them by freeze-drying.

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

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

The presence of SDS interfered with the estimation of proteins by the method of Bradford (1976), while traces of the dye interfered with the method of Lowry et al. (1951). The concentration of proteins was determined by quantitative scanning of Coomassie Blue stained bands of protein after resolution on polyacrylamide gels by comparison with BSA used as a standard (see Methods section 2.2.1).

As a convenient precaution against proteases,extraction buffer was either prepared fresh each time or kept frozen at -20°C. Before use, the solution was filtered through Nalgene filters.

2.2.11 Tissue culture methods

a) Growth conditions

All cell lines were maintained routinely as monolayers in glass or plastic Roux flasks. Eagle's medium (Glasgow modification) was used in all cultures. Penicillin (1000 U/ml), streptomycin (100 µg/ml) and non-essential amino acids were included in the standard media.

Buffalo rat liver cells (BRL) were maintained in the standard medium supplemented with 10% (v/v) calf serum. Bovine kidney (NBL-1) and pig kidney (PK-15) cells were cultured in the same medium supplemented with 10% or 5% (v/v) foetal calf serum, respectively.

b) Cell harvesting and counting

Cells were harvested in log phase and detached from their substrate by washing the monolayers with a solution of EDTA ("Versene" solution) followed by incubation for 5-10 min with a "Trypsin" solution (see section 2.1.2). Tryptic activity was inhibited by addition of 25 ml growth medium and cells were resuspended thoroughly by pipetting several times. The concentration of cells was determined by light microscopy using a haemocytometer.

Cells were subcultured by transferring one-fifth of this suspension into another Roux flask and adding approx. 50 ml of growth medium.

c) Incorporation of [^{35}S] methionine into cultured cells

Cells were harvested from log phase cultures and resuspended in medium at a concentration of 2-4 x 10^6 cells/ml. Portions containing 2-3 x 10^6 cells were transferred into plastic Petri dishes (10 cm diam.). Normal growth medium was added to a total volume of 10 ml and the cells were incubated in a 37°C incubator (Vindon Scientific Ltd), equilibrated with 5% (v/v) CO_2 and 95% (v/v) air.

Confluent monolayers were generally observed after 24-48 h.

On removal of normal medium, the monolayers were rinsed and then incubated with 4 ml low methionine medium (section 2.1.2) for 1-2 h to reduce endogenous levels of methionine.

Radiolabelling was initiated by the addition of [^{35}S] methionine to 100-200 µCi/dish. For short-term labelling, cells were incubated generally for 4 h, whereas for long-term labelling, cells were incubated for 16-20 h at 37°C in medium supplemented with 50-100 µCi of isotope/dish.

For accumulation of mitochondrial precursors, monolayers were incubated in low methionine medium for 1-2 h before adding either DNP or FCCP to final concentrations of 1-2 mM or 5-10 µM, respectively. Stock solutions of DNP (100 mM) and FCCP (1 mM) in ethanol, were prepared fresh on each occasion. After the addition of uncoupler, dishes were returned to the 37°C incubator for another 5-10 min before adding [^{35}S] methionine (200 µCi/dish).

d) Pulse-chase experiments

Monolayers were labelled as described in section 2.2.11 c, in the presence of uncouplers for 4 h at 37°C. The radioactive medium was removed and substituted by 10 ml of normal growth medium (warmed at 37°C) with or without uncoupler as required. Cells were harvested as described below after the indicated chase periods.

e) Preparation of cell lysates for immunoprecipitation

Solutions required:

PBS: 20 mM-potassium phosphate buffer, pH 7.4, 0.15 M-NaCl

Triton-TKM buffer: 0.1 M-Tris/HCl buffer, pH 8.2, 1% (v/v) Triton X-100, 0.1 M-KCl, 5 mM-$MgCl_2$.

3-D lysis buffer: 0.1 M-Tris/HCl buffer, pH 8.2,1% (v/v) Triton X-100, 1% (w/v) SDS, 2% (w/v) sodium deoxycholate, 0.1 M-KCl, 5 mM-$MgCl_2$.

Solutions were sterilized by filtration through Nalgene filters (0.22 μm pore size) type S (115 ml) and kept at room temperature.

The method of Mosmann et al. (1979) as described by Cushley (1981) was used to prepare the cell extracts used for the specific immunoprecipitation of radiolabelled polypeptides.

After the labelling or chase periods, medium was removed and monolayers were rapidly rinsed 3-4 times with ice-cold PBS. One ml of cold Triton-TKM buffer containing 4 mM-1,10-phenanthroline, 0.4 mM-PMSF and 0.3 μM-leupeptin was added to each dish. Cells were scraped into the solution with the aid of a small rubber stopper and the resulting lysate was transferred into a conical centrifuge tube. Unbroken cells and nuclei were removed by centrifugation at 1100 g for 5 min at 4°C, in a Beckman Model TJ 6 bench centrifuge. The concentration of deoxycholate was adjusted to 1% (w/v) and SDS to 0.5% (w/v) by mixing the samples with an equal volume of 3-D lysis buffer.

Any insoluble material or aggregates were removed by centrifugation at 30,000 g for 30 min at 4°C. The clarified extracts were used immediately for immunoprecipitation or stored at -80°C until required.

2.2.12 Immunological methods

a) Preparation of antibodies to native PDC

Production of high titre, monospecific, polyclonal antibodies against ox heart PDC required highly-purified preparation of antigen. Samples of PDC (20-30 μg) were electrophoresed in 10% (w/v) SDS/ polyacrylamide gels and their purity was estimated after densitometric scanning of the Coomassie Brilliant Blue-stained bands. Samples from the Sepharose C1-2B column were screened and those fractions found to

contain between 96-98% pure PDC, as judged by this criterion, were used to raise antibodies against the whole complex.

PDC (1 mg protein), dissolved in 50 mM-Mops buffer containing 2 mM-EDTA, 0.1 mM-DTT adjusted to pH 7.0 with NaOH was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites in the neck and back of a 4-months old New Zealand white rabbit. Similar amounts of enzyme were administered at 2-3 week intervals thereafter. Ten to fourteen days after the fourth injection, blood was obtained from a marginal ear vein. Additional booster injections were administered at approx. one-month intervals with 0.5 mg of protein mixed with incomplete Freund's adjuvant.

Blood (approximately 40 ml) was collected in Universal bottles and left to clot at 4°C for 24 h. The serum was collected, clarified by centrifugation and stored frozen in 1.0 ml aliquots at -20°C.

b) Preparation of antibodies to PDC subunits

For the production of subunit-specific antisera, samples of PDC (approx. 2.5 mg) were resolved on preparative slab gels as described in section 2.2.6.c. After staining with Coomassie Blue, protein bands were excised from the gels with a scalpel, diced and rinsed twice with distilled water to remove solvents. The pieces were ground finely in a mortar and pestle whilst kept frozen by adding small amounts of liquid nitrogen. The frozen powder was weighed and stored at -20°C until use. This technique yielded between 100-300 µg protein per gram of gel (Mihara & Blobel, 1980).

Before injection into rabbits, 1 g gel was homogenized with 0.5 ml 0.9% (w/v) NaCl with a tight-fitting glass teflon homogenizer. The gel slurry was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into a rabbit. The rest of the

protocol followed was similar to that described above for the production of antiserum to native PDC.

Antisera to subunit E3 and citrate synthase were obtained after injecting commercially purified pig heart lipoamide dehydrogenase and citrate synthase. Protocols for immunisation were similar to that for native PDC (section 2.2.12.a).

c) Studies on the effects of several antisera on PDC activity

Samples of PDC (approx. 20 µg protein) were incubated with varying amounts of antisera (0-100 µl) in a final volume of 250 µl in 0.1 M-sodium phosphate buffer, pH 7.0, 0.15 M-NaCl. After incubation at room temperature for 90 min, aliquots of 10-100 µl were assayed in duplicate for overall PDC activity as indicated in section 2.2.8.a. Control experiments showed that PDC activity was stable in this buffer for at least 90 min.

d) Studies on the effects of several antisera on pyruvate dehydrogenase kinase activity

The effects of several antisera on PDC kinase were studied by either measuring the rate of inactivation of PDC in the presence of 0.2 mM-ATP or the rate of incorporation of [γ -^{32}P] ATP into TCA-precipitable material. For the former experiments, samples of PDC (66 µg) were incubated for 30 min at room temperature (20°C) with 100 µl of the indicated antiserum. The incubation mixtures contained in a final volume of 200 µl, 20 mM-potassium phosphate buffer, pH 7.5, 2 mM-$MgCl_2$, 0.01 mM-EDTA, 1 mM-DTT. Inactivation of PDC was followed at 30°C after the addition of ATP to a final concentration of 0.2 mM. Aliquots of 10-100 µl were withdrawn at the times stated in the Figure legends for measurement of PDC activity.

For the latter experiments, samples of PDC (50 μg) were incubated as described above with 100 μl antiserum. Phosphorylation was started at 30°C by adding 25 μl of a mix containing 3 μCi [γ-^{32}P] ATP, 50 nmol ATP and 2 nmol $MgCl_2$. Aliquots of 20 μl were removed after various times and spotted onto Whatman No.1 filter paper discs to estimate protein-bound radioactivity (see section 2.2.5.a).

e) Analysis of antibody specificity by immunoblotting techniques

Relevant information on the specificity and relative titre of the antibodies raised against native PDC and its constituent polypeptides was obtained by immunoblotting techniques.

Proteins were resolved on SDS/polyacrylamide gels prior to electrophoretic transfer onto nitrocellulose sheets. After incubation with specific antisera, formation of immunocomplexes was detected by the binding of ^{125}I-labelled protein A.

One dimensional SDS/polyacrylamide gel electrophoresis was performed in slab gels until the tracking dye had moved to 1-2 cm from the bottom of the resolving gel. Usually, samples of purified PDC and ^{125}I-labelled M_r standards (approx. 30,000 c.p.m.) were included to facilitate identification of the individual proteins by their characteristic electrophoretic mobility.

Electrophoretic transfer of proteins onto nitrocellulose sheets was performed essentially as described by Towbin et al. (1979), except that 0.02% (w/v) SDS was included in the transfer buffer.

Pyronin Y binds tightly to nitrocellulose and can therefore serve as a readily visible indicator of transfer. It also allows the identification of individual tracks on the transfers (blots) so they can be excised and treated separately.

The blots were subjected to incubation with gentle shaking at room temperature in blotting buffer (20 mM-Tris/HCl buffer, pH 7.2 containing 0.15 M-NaCl, 0.5% (v/v) Tween 20) for at least 1 h and then with the particular antiserum diluted 1:50-1:200 in blotting buffer for 90 min. Inactivated horse or goat serum (5% (v/v)) was included as carrier at this stage. Excess antiserum was removed by washing with 5 changes of blotting buffer (approx. 50 ml each) over a period of 30 min. Incubation with ^{125}I-labelled protein A (approx. 0.8-1.0 x 10^5 c.p.m/ml) in blotting buffer for 1 h, was followed by another 5 washes (5-10 min each) with blotting buffer, to remove excess unbound radiolabel. Finally, the blots were air-dried and exposed for 24-48 h to Kodak X-Omat S film with intensifying screens at -80^{o}C.

The inclusion of Tween 20 to 0.5% (v/v) in all steps as suggested by Batteiger et al.(1982) was found to reduce substantially the background due to non-specific binding.

To identify unambiguously some immunoreactive proteins or to assess the quality of the transfer, nitrocellulose sheets were stained for 5 min with 0.1% (w/v) Amido Black 10-B dissolved in 45% (v/v) methanol, 10% (v/v) acetic acid for 2-5 min and destained with 50% (v/v) methanol, 10% (v/v) acetic acid for another 5 min.

Immunoblots can be sequentially treated with another different antiserum without the removal of the preceding antibodies.

^{125}I-labelled protein A and M_r standards were prepared by the Iodogen method as described in section 2.2.14.a.

f) Immunoprecipitation methodology

This method permits the isolation and identification of small amounts of a particular protein in a complex mixture by means of its specific interaction with an antibody. Immunoprecipitation of antigens is usually performed in two stages. First, specific antibody is

added to a detergent cell lysate containing the radiolabelled antigen. Next, the immune complexes are bound to a second agent that provides sufficient mass to facilitate precipitation. This second agent can be an immunoglobulin directed against the first antibody (double antibody precipitation system) or chemically-fixed protein A-bearing strains of Staphyloccus aureus bacteria. The isolated antigen is resolved by SDS/polyacrylamide gel electrophoresis and detected by fluorography.

The procedure was applied to purified PDC labelled with N-[^{3}H]ethylmaleimide (section 2.2.13) and to [^{35}S]methionine-labelled cellular extracts (section 2.2.11.e). Immune complexes were precipitated by binding to formalinised S. aureus bacteria (Pansorbin).

Immunoprecipitation of ^{3}H-labelled PDC

Immunoprecipitations were carried out in a final volume of 100 µl containing 1 µg (approx. 140,000 c.p.m.) of tritiated complex in Triton-buffer (0.01 M-Tris/HCl buffer, pH 7.4, 1% (v/v) Triton X-100, 0.3 M-NaCl, 5 mM-EDTA) or in 3-D TKM buffer (0.1 M-Tris/HCl buffer, pH 8.2, 0.1M-KCl, 1% (v/v) Triton X-100, 1% (v/v) sodium deoxycholate, 0.5% (w/v) SDS, 5 mM-$MgCl_2$.

PDC was immunoprecipitated by adding 10-50 µl of antiserum. Appropriate controls were included by replacing the specific antiserum with similar amounts of preimmune serum.

After incubation with the antiserum for 1 h at room temperature, samples were incubated overnight (16-20 h) at 4°C. Immunocomplexes were precipitated by addition of 40-50 µl of a 10% (w/v) suspension of standardized S. aureus cells (Cowan I strain) previously washed and resuspended in immunoprecipitation buffer. Incubations were extended for another hour at room temperature with occasional mixing.

Pellets were collected by centrifugation at 14,000 g for 5 min in an MSE Micro-Centaur centrifuge, washed 3 x with 1 ml of immunoprecipitation buffer and finally with 1 ml of 20 mM-Tris/HCl buffer, pH 7.4 in order to remove detergents. Immunocomplexes were dissociated by boiling with 40-50 µl of Laemmli sample buffer. Aliquots of 5 µl were counted and the remaining sample was loaded onto polyacrylamide slab gels. Immunoprecipitated proteins were detected after fluorography (section 2.2.6.e).

Immunoprecipitation of cellular extracts

Aliquots of cell lysates containing 10-20 x 10^6 c.p.m. ^{35}S-label were incubated with 10-20 µl of antisera prior to adsorption on S. aureus cells. Sterile 3-D TKM buffer was employed for the washing steps. Immunoprecipitation was carried out essentially as described above for the ^3H-labelled antigen.

g) Immune-mapping techniques

This procedure premits the characterisation of antigens by immunoblotting of proteolytic fragments of these proteins resolved by SDS/polyacrylamide gel electrophoresis.

Pyruvate dehydrogenase complex (1 mg) was incubated with elastase (4.5 µg/ml) or trypsin (13.5 µg/ml) in 0.1 M-potassium phosphate buffer, pH 7.0 at 25°C. Aliquots (30 µl) were withdrawn at appropriate time intervals (0-60 min). Reactions were terminated by boiling for 5 min with 30 µl of Laemmli sample buffer. Samples were loaded onto polyacrylamide gels that were either stained with Coomassie Brilliant Blue or used for immunoblotting analysis as described in section 2.2.12.e.

h) Immunoadsorption

Immunoadsorption was utilised to remove unwanted antibody activity (anti-E2) from the antiserum raised against E1 β subunit of PDC.

Highly-purified subunit E2 (100 µg) isolated from polyacrylamide gel slices (section 2.2.10.f) was coupled to 1 ml of CNBr-activated Sepharose 4B according to the manufacturer's instructions. Beads were washed with 30 ml PBS (20 mM-potassium phosphate buffer, pH 7.4 containing 0.15 M-NaCl, 0.01% (w/v) NaN_3) and dispensed into three 1.5 ml Eppendorf tubes. The beads were sedimented by centrifugation and the supernatant fluid was discarded. One ml of antiserum to E1 β was placed into one of the tubes and incubated for 30 min at 37°C in an end-over-end mixer. The serum was separated from the beads and the immunoadsorption repeated twice with the beads contained in the second and third tubes.

This procedure was found to be more efficient than using a column and also minimized dilution of the antiserum with PBS. Efficiency of immunoadsorption was checked by immunoblotting using PDC as antigen.

The used beads were regenerated by washing with 3 ml 3 M-KSCN in 0.5 M-NH_4OH followed by washing with 30 ml PBS. The beads were stored at 4°C in PBS until required.

i) Preparation of affinity-purified IgG

Affinity chromatography on protein A-Sepharose columns was used to obtain small amounts of highly-purified IgG from rabbit serum.

Protein A-Sepharose columns were prepared by coupling 2 mg protein A to 3 ml of swollen CNBr-activated Sepharose 4B, following the manufacturer's instructions. One ml columns were packed in the barrels of 2 ml disposable syringes, plugged with glass wool and washed with PBS (20 ml), followed by 3 ml of 3 M-KSCN and 30 ml PBS. One ml serum was applied to each column at approximately 6 ml/h at room temperature. The eluant was recirculated for 2 h. The non-bound material was discarded and the columns were washed with PBS until the A_{280} was <0.02. Then, the IgG bound was eluted with 3 ml of 3 M-KSCN.

Fig. 2.1 Affinity-purified IgG fractions

Immunoglobulins were purified by affinity chromatography on protein A- Sepharose columns as described in section 2.2.12.i.

Samples of each fraction were mixed with Laemmli sample buffer and boiled for 5 min, before electrophoresis on a 10% (w/v) SDS/ polyacrylamide gel. Protein bands were visualized after staining with Coomassie Blue.

Lane 1, M_r standards; lane 2, anti-X IgG fraction, 12 µg protein; lane 3, IgG control, 14 µg; lane 4, commercially purified IgG, 10 µg.


10^{-3} x M_r
92
68
43
31
20
14
1
2
3
4

Fractions of 1 ml were collected and the A_{280} measured. Peaks of u.v. absorbing material were pooled and dialysed against 4 x 250 ml of PBS for 48 h. The yield of IgG was determined from the A_{280} assuming that it was mostly IgG ($A^{1\%}_{280}$ 13.5) (Fig. 2.1).

j) Sucrose-density gradient centrifugation of PDC incubated with purified IgG

Anti-X and control IgG were purified from rabbit serum (section 2.2.12.i) and radioiodinated with ^{125}I by the Iodogen method as indicated in section 2.2.14.b.

Native PDC (1.9 mg; 100 nKat/mg)dissolved in 200 µl of 20 mM-potassium phosphate buffer, pH 7.2, 20 µM-TPP, 1 mM-$MgCl_2$, 0.1 M-KCl, 0.15 µM leupeptin was incubated with anti-X or control ^{125}I-labelled IgG (2 x 10^6 c.p.m. each). After 1 h at room temperature, samples were loaded on the top of linear 10-30% (w/v) sucrose-density gradients formed in tubes of the Spinco SW 27 rotor. Sucrose solutions were prepared in the same buffer. A cushion of 2 ml 50% (w/v) sucrose was placed on the bottom of the tubes. Centrifugation was performed at 96,300 g at 4°C for 4 h.

Fractions of 1 ml were collected manually with the aid of a peristaltic pump. Aliquots of 200 µl were used to estimate the distribution of ^{125}I in the gradients with a Gamma counter. PDC activity was assayed spectrophotometrically as described in section 2.2.8.a.

2.2.13 Preparation of native and denatured N-[^3H]ethylmaleimide-labelled PDC

Thiol groups of dissociated PDC were labelled with N-[^3H] ethylmaleimide as follows: a 100 µl portion of sample containing 100 µg protein was precipitated with 80% (v/v) acetone by storing

at -20°C for several hours. The pellets were air-dried and re-dissolved in 200 µl of 20 mM Tris/HCl buffer, pH 7.4, 2% (w/v) SDS before incubation with 50 µCi N-[^{3}H]ethylmaleimide at room temperature for 30 min. Reactions were terminated by addition of 10 µl 2-mercapto-ethanol and 4 vol. acetone. Pellets were collected as before, washed once more with acetone and dissolved by boiling in the same buffer. Before use, samples were diluted 5-fold with Triton buffer (see section 2.2.12.f).

Incorporation of radioactivity into protein was determined as described in section 2.2.5.a. Approx. 140,000 c.p.m. ^{3}H were incorporated per µg protein. ^{3}H labelled denatured PDC is the standard used in Ch.8.

Native PDC (200 µg) was labelled with 50 µCi N-[^{3}H]ethylmaleimide after incubation at room temperature for 30 min in 200 µl of 20 mM-potassium phosphate buffer, pH 7.2, 0.5 mM-$MgCl_2$, 0.25 mM-EDTA in the absence of pyruvate. Reactions were terminated by the addition of DTT to 10 mM and the samples were dialysed extensively against 4 x 500 ml of 20 mM-Tris/HCl buffer, pH 7.4, 0.1 mM-DTT, 0.01% (w/v) NaN_3. Approx. 56,000 c.p.m. ^{3}H were incorporated/µg protein and 96% of the counts were TCA-precipitable.

2.2.14 Radioiodination of proteins by the Iodogen method

Iodogen is a mild solid-phase reagent that efficiently reacts with aqueous mixtures of I^- and proteins to produce iodinated proteins (Fraker & Speck, 1978).

a) Radioiodination of protein A and M_r standards

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

Proteins for iodination (1 mg protein A or 578 µg low M_r value standards) were dissolved in 0.5 ml 0.1 M-Tris/HCl buffer pH 7.2, 0.15 M-NaCl and transferred into the container with the Iodogen. After addition of 200-400 µCi [^{125}I] NaI, incubations were carried out for 15 min at room temperature with occasional mixing. Reactions were terminated by removing the samples from the containers and applying to Sephadex G-25 or G-50 columns packed in 10-ml disposable syringes and equilibrated with the same buffer. Fractions (1 ml) were collected in disposable plastic tubes. The iodinated protein was generally found in fractions 4-7; this was confirmed with a gamma counter. The peak fractions were pooled and stored at -20°C. Protein A for immunoblotting analysis was dispensed in 0.05-0.1 ml aliquots containing approx. 3 x 10^6 c.p.m. each.

b) Radiolabelling of IgG

Samples containing IgG (approx. 20 µg) were iodinated with 200 µCi of [^{125}I] NaI in test tubes (13 cm x 1.0 cm) containing 25 µg Iodogen. Incubations were performed for 15 min at room temperature in 50 µl of 0.25 M-potassium phosphate buffer, pH 7.5. Proteins were applied to 5 ml columns of Sephadex G-50, previously washed with 10 mg of BSA and equilibrated with PBS. The rest of the procedure was similar to that described for radiolabelling of protein A.

c) Radioiodination of subunits E2 and X isolated from SDS/ polyacrylamide gels

Purified acetyltransferase subunit (E2) and component X, obtained as described in section 2.2.10.f, were iodinated in (7.5 cm x 1 cm) test tubes containing 50 µg of Iodogen essentially as described by Tolan et al. (1980).

Samples containing 16 µg of E2 protein and 18 µg of component X in 50 µl of 1% (w/v) SDS were mixed with 20 µl of 100 mM-Tris/HCl buffer, pH 7.4 at 0°C. Reactions were started by addition of 20 µl 5 mM-KI containing 100 µCi ^{125}I and allowed to proceed at 0°C for 15 min, with occasional mixing. Mixtures were transferred into Eppendorf tubes containing 10 µl 2-mercaptoethanol before precipitation of protein with 4 vol. acetone and 100 µg PDC, which was used as a carrier. Pellets were recovered after storage at -20°C for 16 h, washed 3x with 1 ml acetone to remove free ^{125}I, air-dried and dissolved by boiling in 500 µl 125 mM-Tris/HCl buffer, pH 6.8, 0.5% (w/v) SDS and 10% (v/v) glycerol.

Aliquots of 5 µl were withdrawn to estimate radioactivity in a gamma counter. Approx. 1 x 10^5 c.p.m. ^{125}I were incorporated/10 µl sample.

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

a) Acetylation assay

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

Standard mixtures (200 µl) contained 50 mM-potassium phosphate buffer, pH 7.2, 0.2 mM-TPP, 0.5 mM-$MgCl_2$, 0.25 mM-EDTA and 200 µg PDC. Catalysis was initiated after a 1 min preincubation at 30°C by addition of 0.10 mM-[2-^{14}C] pyruvate (10.4 mCi/mmol).

Samples (10 µl) were withdrawn at the indicated times, spotted onto Whatman No.1 filter paper discs and processed for measurement of TCA-insoluble radioactivity (section 2.2.5.a).

In some experiments NEM was added to a final concentration of 0.5 mM before preincubation. Stock 10 mM-NEM solution was prepared fresh each time.

b) Pretreatment of PDC with NEM

PDC (1 mg) was preincubated at 0°C or at room temperature for 30 min in the presence of 0.5 mM-NEM in 20mM-potassium phosphate buffer, pH 7.5. The sample was then diluted with 5 ml of the same buffer and concentrated by centrifugation at 144,000 g for 2.5 h in a Beckman SW 60 rotor. The pellet of PDC was resuspended in a small volume of the same buffer. Protein concentration was determined by the procedure of Markwell et al. (1976) and aliquots containing 200 µg protein were included in the standard acetylation assay described in section 2.2.15.a.

c) Pretreatment of PDC with pyruvate

PDC (1 mg) was acetylated in the presence of 2 mM-pyruvate after incubation with 20 mM-potassium phosphate buffer, pH 7.2 containing 0.2 mM-TPP, 0.5 mM-$MgCl_2$, 0.25 mM-EDTA as described above.

Samples were concentrated by centrifugation at 144,000 g for 2.5 h in a Beckman SW 60 rotor, essentially as described for pre-treatment with NEM. Acetylated PDC was used as control in the acetylation experiments.

d) Acetylation of phosphorylated PDC

PDC (500 µg) was incubated for 30 min at 30°C in the presence of 0.2 mM-ATP (see section 2.2.8.b). Under these conditions, the activity of the complex declined to less than 5% of its original value after a 35 min incubation. Aliquots (200 µg) were used for standard acetylation assays. Control samples incubated as above in the absence of ATP were also included.

e) Deacetylation assay

PDC was incubated as described in section 2.2.15.a for 30-40 min to allow the complete acetylation of the complex. CoA was added to a final concentration of 1 mM and the deacetylation reaction was followed

at 30°C by removing 10 µl aliquots of reaction mixtures, spotting onto filter paper discs and counting the TCA-insoluble radioactivity.

The effects of several compounds on the deacetylation reaction were tested in similar fashion. The following compounds were included with CoA at the indicated final concentration: NADH (1 mM), NAD^+ (0.5 mM).

The following compounds were added instead of CoA at a final concentration of 1 mM: AcSoA, malonyl CoA, propionyl CoA, acetoacetate, acetoacetyl CoA, n-butyryl CoA, palmitoyl CoA, carnitine.

To study the effects of NEM on the deacetylation reaction, NEM (0.5 mM) was added at the indicated times.

f) Estimation of moles of acetyl group incorporated/mol complex

The concentration of the [2-^{14}C] pyruvate solution was estimated using the NAD^+ reduction assay for PDC (section 2.2.8.a). The concentration of stock solution was found to be 3.86 mM. Its specific activity was assumed to be identical to the value given by the manufacturer.

Efficiency of counting for ^{14}C by the filter paper discs was estimated as 56% using ^{14}C-labelled proteins as a standard. Samples of the stock solution of ^{14}C-labelled proteins (5 µCi/ml) were mixed with 50 µg BSA and spotted into Whatman No.1 filter paper discs. The filters were washed and counted as done with samples of PDC for the acetylation assays.

M_r for the ox heart PDC was assumed to be 8.5×10^6. (Reed & Pettit, 1981)

2.2.16 Limited proteolysis and peptide mapping of subunits E2 and X

a) Radioiodinated proteins

Peptide analysis of proteins was performed as described by Cleveland et al. (1977) in 15% (w/v) SDS/polyacrylamide gels with

purified subunits E2 and X extracted from gel strips and radioiodinated as described in sections 2.2.10.f and 2.2.14.c.

Aliquots of 20 µl containing approx. 200,000 c.p.m. ^{125}I-labelled protein were mixed with 20 µl of distilled water and 50 µl 125mM-Tris/HCl buffer, pH 7.4, 1% (v/v) Triton X-100, reducing the SDS concentration to 0.1% (w/v). Proteolysis was started by the addition of 10 µl of the protease solution. Samples were incubated at 35°C for 0-60 min. After the indicated times, 10 µl aliquots were removed with 10 µl Laemmli sample buffer and boiled for 5 min. Samples were loaded on a 15% (w/v) polyacrylamide slab gel and run under standard conditions. The gels were dried under vacuum and autoradiographed for 14 days at room temperature.

Protease solutions were prepared as follows:

Elastase: Stock solution of 50 µg/ml was prepared in 0.1 M-potassium phosphate buffer pH 7.0 and stored at -20°C in small aliquots. Before use, the enzyme was diluted in 0.1 M-Tris/HCl buffer, pH 7.4 to a final concentration of 3 µg/ml.

Chymotrypsin: A solution (6 µg/ml) was prepared fresh each time in 0.1 M-Tris/HCl buffer, pH 7.4. Samples of TLCK-treated α-chymotrypsin were weighed on a Mettler ME22 microbalance.

Protease V8, S. aureus: Stock solution (1 mg/ml) was prepared in 0.01 M-ammonium bicarbonate and stored at -20°C in small aliquots (for up to one month). Before use, the enzyme stock was diluted to 15 µg/ml in 0.1 M-Tris/HCl buffer, pH 7.4.

The final ratios protease: protein (w/w) were estimated as: 0.75% for elastase, 1.5% for chymotrypsin and 3.75% for protease V8. These ratios were selected after preliminary studies designed to determine the optimal values and most suitable incubation times to produce high-quality maps.

b) ^{14}C-labelled proteins

Peptide maps using ^{14}C-labelled subunits E2 and X were carried out as follows: Native PDC (2 mg) was incubated for 30 min with 0.1 mM-[2-^{14}C] pyruvate under standard conditions (see section 2.2.15a). The complex was concentrated by precipitation in the presence of 5% (w/v) poly(ethylene glycol) and 10 mM-$MgCl_2$ at 0°C for 1 h. The yellow pellet was recovered after centrifugation in a microfuge at 14,000 g for 5 min. The acetylated enzyme was dissociated in SDS by re-suspending the pellet in 2% (w/v) SDS by manual homogenisation followed by incubation at 4°C for 16 h.

Attempts to concentrate the enzyme by freeze-drying or by extraction from gels using the procedure described in section 2.2.10.f resulted in heavy losses of radioactivity and/or protein. Boiling in SDS was also avoided owing to the instability of the S-acetyl dihydrolipoamide. Recovery of radioactivity at this stage was 90% as estimated by the filter paper discs method. The sample was resolved on a 10% (w/v) SDS/polyacrylamide preparative gel slab and the bands of protein were visualized after staining with Coomassie Blue. Gel strips containing the subunits E2 and X were washed with distilled water and cut into smaller pieces with a scalpel to fit into the wells of a second polyacrylamide gel. Gel slices were equilibrated by washing in SDS-containing buffer as described by Cleveland et al. (1977). Slices were placed on the second gel and overlayed with various amounts of elastase. Digestions were allowed to proceed directly in a 4.5 cm long stacking gel made up with 4% (w/v) polyacrylamide in a Bethesda Res. Lab. Model V-16 Slab Apparatus (16 cm x 17 cm).

Electrophoresis was carried out at 20 mA until the tracking dye (Pyronin Y) reached the bottom of the stacking gel. The current was turned off and the digestions were allowed to take place for 30 min. Then,electrophoresis was resumed until the tracking dye reached 1 cm from the bottom of the 15% (w/v) SDS/polyacrylamide resolving gel. Slabs were stained with Coomassie Brilliant Blue and subjected to fluorography as described in section 2.2.6.e.

Peptide maps were also obtained with the SDS-denatured subunits E2 and X labelled with ^{14}C as described below.

PDC (6 mg), dissolved in 600 µl 50 mM-potassium phosphate buffer, pH 7.5, 0.25% (v/v) Triton X-100, 1 mM-NAD^+, 5 mM-$MgCl_2$, 2 mM-TPP, 0.5 mM-NEM, was incubated for 24 h at 4°C. Unreacted reagents were removed by gel filtration in 1 ml columns of Sephadex G-25-F equilibrated in 50 mM-potassium phosphate buffer, pH 7.5, 1% (v/v) Triton X-100, 1 mM-NAD^+, 5 mM-$MgCl_2$, 2 mM-TPP according to Penefsky (1977). This procedure blocked all NEM-sensitive sites available in the complex in the absence of pyruvate (J.A. Hodgson, unpublished results).

To this sample, 5 µCi of N-ethyl[2, 3-^{14}C] maleimide in n-pentane were added to a final concentration of 1.44 mM. After removal of the solvent by a gentle stream of N_2, 5 µCi [2-^{14}C] pyruvate were added immediately, to a final concentration of 0.8 mM. After 15 min incubation at room temperature, the reaction was terminated by the addition of 2-mercaptoethanol to 17.5 mM. Approx. 130 c.p.m. ^{14}C-label were incorporated/µg protein.

The ^{14}C-labelled complex was mixed with the same volume of Laemmli sample buffer (no sulphydryl reagents added) and the sample was loaded onto two 10% (w/v) SDS/polyacrylamide preparative slab gels. Gel slices corresponding to subunits E2 and X were extracted as described in section 2.2.10.f. Samples containing approx. 6,000 c.p.m. of each protein were incubated with elastase as described in the legend to Fig. 7.10, before SDS/polyacrylamide gel electrophoresis. Proteolytic fragments were detected by Coomassie Blue staining followed by fluorography.

RESULTS AND DISCUSSION

Chapter 3: PURIFICATION, CHARACTERISATION AND RESOLUTION OF THE PYRUVATE DEHYDROGENASE COMPLEX FROM OX HEART

3.1 Purification of PDC from ox heart

The mammalian pyruvate dehydrogenase complex has been purified from a variety of tissues: bovine kidney and heart (Linn et al., 1972) porcine liver (Roche & Cate, 1977), porcine heart (Hayakawa et al., 1969) pigeon breast muscle (Severin & Glemža, 1964) and rat heart (Matuda et al., 1983). Electron microscopy and sedimentation equilibrium analysis indicate that the complexes isolated from these sources are similar in size and appearance (icosahedral design) and have M_r values in the range 7-8.5 x 10^6.

Most procedures for the purification of the complex rely on an initial large scale isolation of mitochondria. Conventional preparations of liver and kidney mitochondria tend to contain lysosomes and PDC is particularly sensitive to proteolytic cleavage by lysosomal proteases. Wieland (1975) has shown that extracts from rat liver mitochondria are capable of inactivating PDC owing to the presence of a thiol protease or "inactivase", which has been partially purified and characterised (Lynen et al., 1978). Proteolytic attack leads to dissociation of the complex, accompanied by loss of activity (Kresze & Steber, 1979). Mitochondrial extracts from ox heart and kidney contain at least 5 times more PDC activity than extracts from liver. On this basis, ox heart seems to be the most suitable source of mammalian PDC.

More recently, a procedure which allows the purification of the ox heart 2-oxo-acid dehydrogenase complexes from whole tissue by homogenisation in the presence of Triton X-100 has been described (Stanley & Perham, 1980). This method eliminates the tedious procedures involved in the purification of mitochondria and the freeze-thaw treatment required for efficient release of the multienzyme complexes. In addition, it employs relatively few steps in producing purified complexes in good yields.

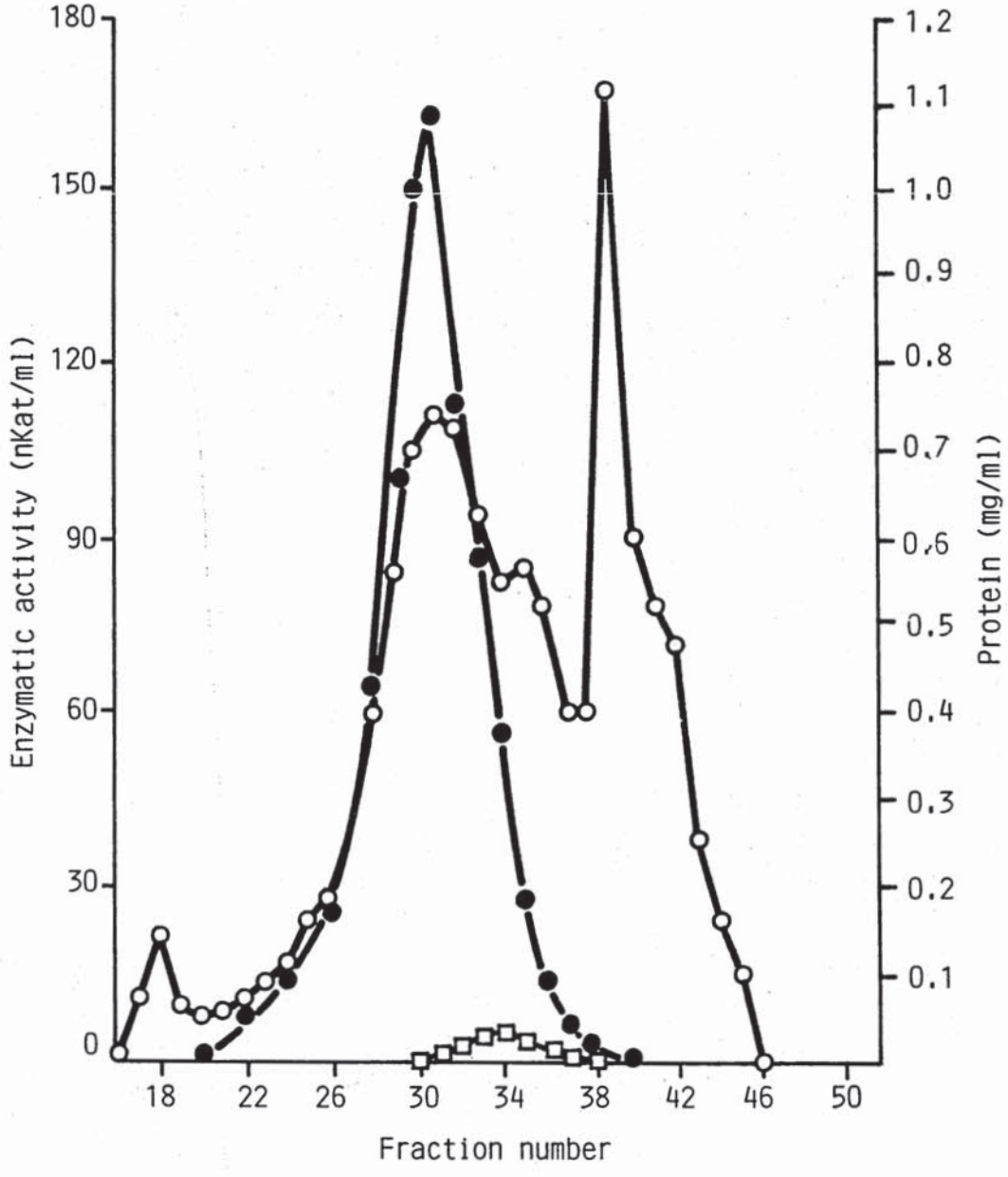
Most schemes for purification of 2-oxo-acid dehydrogenase complexes are centred on selective precipitation with poly(ethylene glycol), originally described by Linn et al. (1972). Poly(ethylene glycol) precipitation is a relatively mild procedure not detrimental to enzymatic activity. Quantitative precipitation occurs at very low concentrations (around 5% w/v) of the polymer (M_r 6000) at pH values near the isoelectric points of the complexes.

The purification scheme developed by Stanley & Perham was utilised with some minor modifications, introduced after experiencing difficulties in purifying the 2-oxoglutarate dehydrogenase complex.

Inclusion of leupeptin (0.15 μM) and rat serum (0.5% (v/v)) in all buffered solutions after the first precipitation with poly(ethylene glycol) stabilised the activity of the 2-oxoglutarate dehydrogenase complex. These compounds are potent inhibitors of the lysosomal protease which specifically inactivates 2-oxo-acid dehydrogenase multienzyme complexes (Lynen et al., 1978). Alternatively, it is possible that the components of this complex are more susceptible to dissociation or proteolysis than the pyruvate dehydrogenase complex. It was observed that the activation step described in the original procedure did not cause any appreciable increase in PDC activity

Fig. 3.1 Purification of ox heart PDC by gel filtration chromatography on Sepharose CL-2B

Partially purified PDC (approx. 100 mg) was chromatographed on a column (106 cm x 3.5 cm) of Sepharose CL-2B equilibrated and developed in 50 mM-sodium phosphate buffer, pH 7.0, containing 2.7 mM-EDTA and 1% (v/v) Triton X-100. Overall PDC activity (—●—) and 2-oxoglutarate dehydrogenase activity (—□—) were determined spectrophotometrically as described in section 2.2.8.a. Protein concentration (—○—) was determined by the procedure described by Markwell et al. (1976).


Enzymatic activity (nKat/ml)
Protein (mg/ml)
Fraction number
180
150
120
90
60
30
0
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
18
22
26
30
34
38
42
46
50

while the omission of 13 mM-$MgCl_2$ at this stage led to lower yields of the complex. Pellets from the second poly(ethylene glycol) precipitation step were usually extracted twice in 50 mM-Mops buffer, pH 6.8 containing 2.7 mM-EDTA, 0.1 mM-DTT and 1% (v/v) Triton X-100 plus the above indicated inhibitors. This was necessary to achieve maximal solubilisation of the enzymic activities.

After separation of the 2-oxoglutarate dehydrogenase activity by differential precipitation with poly(ethylene glycol), the fraction containing partially-purified PDC was loaded onto a Sepharose CL-2B column to remove traces of the contaminating 2-oxoglutarate dehydrogenase complex. This enzyme was also purified and utilised for a parallel study by Miss A. Hunter in our laboratory.

Fig. 3.1 shows a typical separation of the pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase complexes by gel filtration chromatography on Sepharose CL-2B. A large peak of dark-brown material eluting after the peak of 2-oxoglutarate dehydrogenase activity was found consistently. The nature of this contaminant is not known but it may be derived from membranes since it also sedimented after centrifugation at 150,000 g for 2 h. Peak fractions of PDC activity were analysed separately to establish their specific activity and polypeptide composition.

3.2 Characterisation of purified ox heart PDC

Table 3.1 shows how a typical purification of PDC, starting with 250 g fresh ox heart, gave highly-purified enzyme with specific activities 16-fold higher than the initial poly(ethylene glycol) precipitate.

The range of specific activities found for 8 different preparations was 100-240 nKat/mg. These values compare favourably with those reported by Stanley & Perham (1980) using the same procedure and with

Table 3.1 PURIFICATION OF PYRUVATE DEHYDROGENASE COMPLEX FROM OX HEART

Stage in purification	Total protein (mg)	PDC activity (nkat)	Specific activity (nkat/mg)	Purification factor	Yield (%)
First poly(ethylene glycol) precipitate	3,042	20,869	6,86	1.0	100
Second poly(ethylene glycol) precipitate	921.5	18,297	19.86	2.89	87.7
Extracted precipitate	748.0	16,365	21.87	3.19	78.4
2-oxoglutarate dehydrogenase	287.5	1,471	5.11	---	---
Pyruvate dehydrogenase [a]	115.5	13,241	114.64	16.7	63.4
Pyruvate dehydrogenase complex after gel filtration on Sepharose CL-2B	55.8	9,387	115.6-215.7 [b]	16.8-31.4	45.9

All values refer to a preparation of the complex from about 250 g of ox heart.
a Enzymic activities determined after removal of 2-oxoglutarate dehydrogenase complex.
b Range of values found after analysis of various fractions from the gel filtration step.

values of 150-280 nKat/mg reported by Linn et al. (1972).

Overall recoveries ranged from 45-65% of the activity observed in extracts after the first poly(ethylene glycol) precipitation. Average yields of PDC were approx. 40 μKat/Kg heart as compared with 73 μKat/Kg heart reported by Stanley & Perham (1980). The reasons for this discrepancy are not known, but it may reflect losses by proteolysis or dissociation of the complex during purification.

Purified PDC fractions were substantially free of 2-oxoglutarate dehydrogenase activity. The degree of cross-contamination was estimated as 0.5-1.5% on the basis of activity.

Enzymic activity was found to be stable for up to 3 months at 4°C in 20 mM-sodium phosphate buffer, pH 7.2 containing 1% (v/v) Triton X-100, 2.7 mM-EDTA, 0.1 mM-DTT, 0.01% (w/v) NaN_3, 0.15 μM-leupeptin. Freezing in the same buffer or omission of Triton X-100 caused appreciable loss of enzymic activity. (approx. 50% in one month at -20°C).

3.2.1 Polypeptide composition

Pooled fractions from the gel filtration step, as well as samples from earlier stages of the purification, were analysed by SDS/polyacrylamide gel electrophoresis. Fig. 3.2 shows a typical result from one of the routine preparations of the complex.

Highly-purified PDC showed a characteristic protein pattern similar to that described previously for the ox heart enzyme (Linn et al., 1972). Four main bands were evident, corresponding to the dihydrolipoyl acetyltransferase subunit (E2), the lipoamide dehydrogenase subunit (E3) and the α and β subunits of the pyruvate dehydrogenase (E1). An additional band of approx. M_r 50,000, migrating more rapidly than the E3 component, was also observed.

Fig. 3.2 SDS/polyacrylamide gel analysis of samples at successive stages in the purification of PDC from ox heart

PDC was purified from ox heart as indicated in section 2.2.9. Portions of the samples obtained at different stages of the purification (20-50 µg protein) were electrophoresed on a 10% (w/v) SDS/polyacrylamide slab gel. Protein bands were visualised after staining with Coomassie Blue.

Lane a) First poly(ethylene glycol) pellet after sonication.

b) Second poly(ethylene glycol) pellet after sonication.

c) First extract of the second poly(ethylene glycol) pellet.

d) Second extract of the second poly(ethylene glycol) pellet.

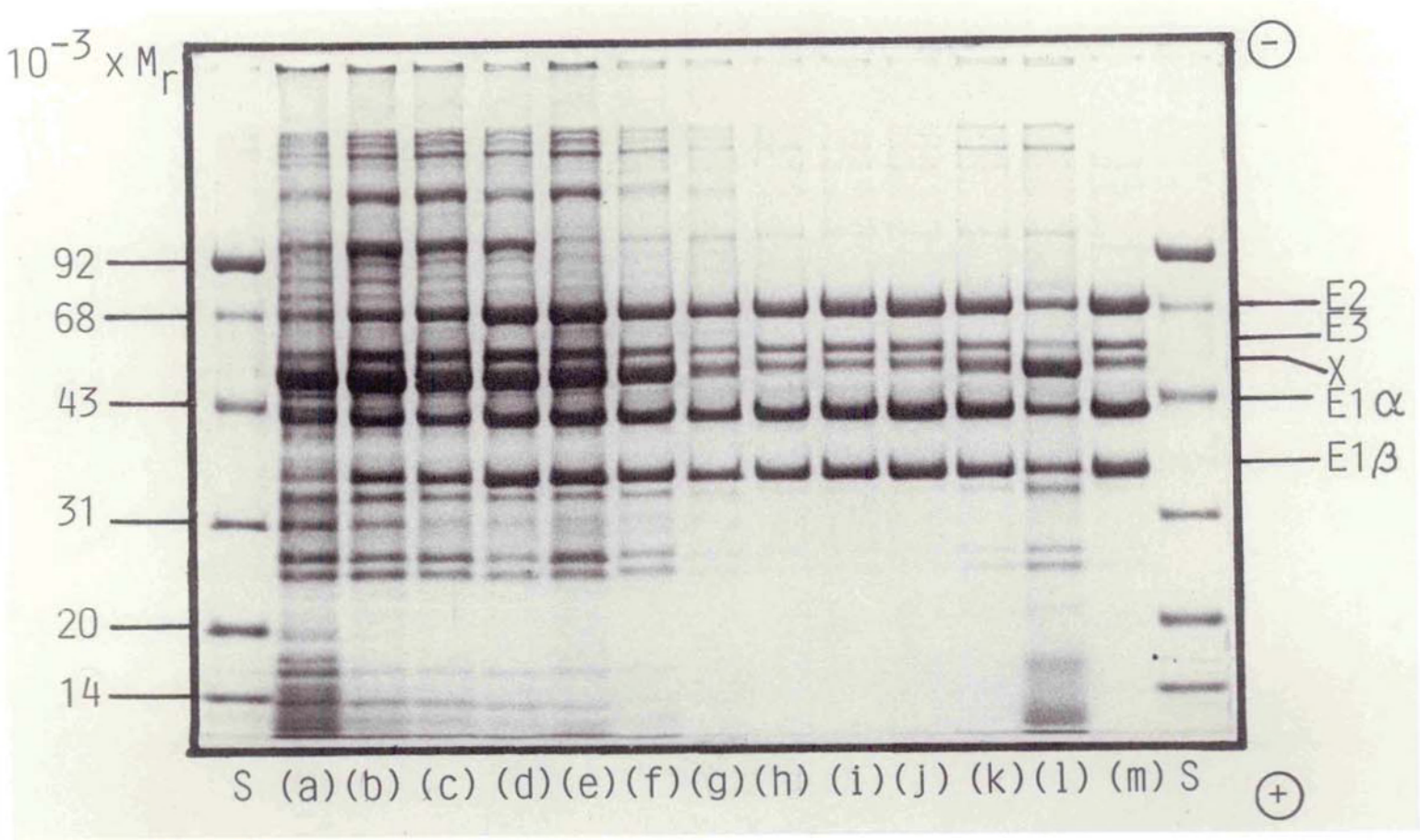
e) Pooled extracts.

f) PDC fraction after separation of the 2-oxoglutarate dehydrogenase complex.

g)- 1) Peak fractions from the gel filtration chromatography step.

m) Purified PDC fraction utilised to raise antibodies.

S, M_r standards.


10⁻³ x Mr
92
68
43
31
20
14
S (a) (b) (c) (d) (e) (f) (g) (h) (i) (j) (k) (l) (m) S
E2
E3
X
E1α
E1β

Additional minor bands of $M_r \geq 100,000$ and in the region of 29,000 were detected occasionally. Similar contaminants have been described before (Roche & Cate, 1977; Sugden & Randle,1978; Kresze & Steber, 1979).

In some instances, a sucrose-density gradient was included as a final step in the purification of the complex (section 2.2.12.j). These contaminant polypeptides were removed by this procedure, as well as traces of the brown material eluted from the column, which contained a main component with subunit M_r 48,000. In contrast, the polypeptide of M_r 50,000, termed by us component X,was always present in highly-purified PDC preparations in reproducible amounts (see also Chapter 6, Table 6.1).

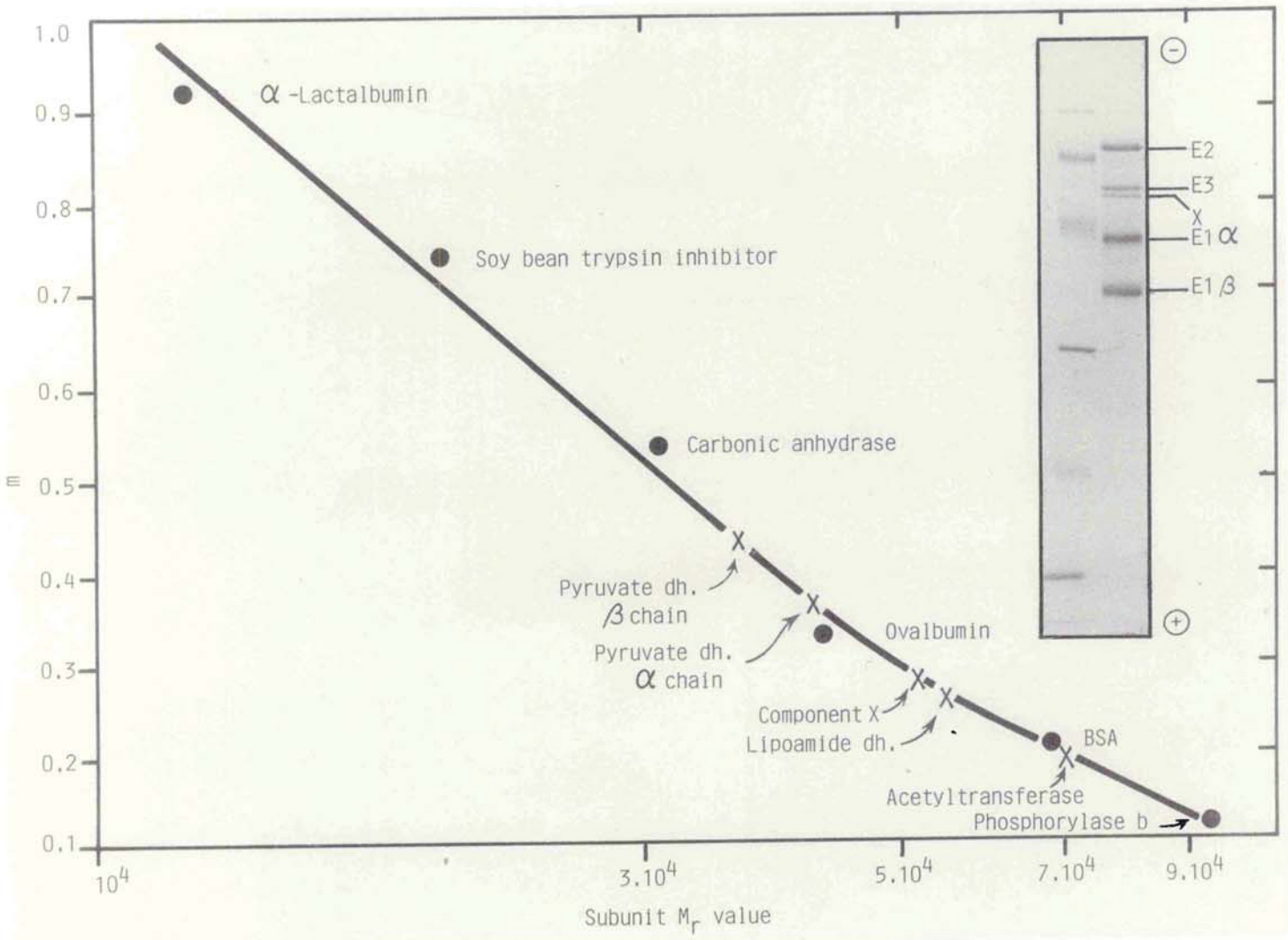
M_r values calculated for the subunits of PDC after resolution on 10% (w/v) SDS/polyacrylamide gels, were similar to those previously reported for the ox heart complex (Fig. 3.3). The M_r for component X was estimated by this method as 51,000 ± 1,000.

The degree of purity of the various fractions, obtained after purification of PDC by gel chromatography, was estimated by densitometric scanning of the Coomassie Blue-stained gels from each preparation. Areas under the peaks corresponding to the five major bands were quantitatively evaluated with a densitometer (section 2.2.6.d). Peak fractions e.g. as shown in Fig. 3.2 (lane m) were apparently homogeneous ($\geq$ 98% purity). This fraction met the standard requirement for use as an immunogen.

The protocols for preparation of antibodies against native PDC and for characterisation of the antiserum are discussed in Chapter 4.

Fig. 3.3 Determination of the apparent M_r of PDC subunits by SDS/polyacrylamide gel electrophoresis

A standard curve of relative electrophoretic mobility (m) against log subunit M_r was established after scanning a 10% (w/v) SDS/polyacrylamide slab gel in which M_r standard proteins and purified PDC (15 µg) had been co-electrophoresed. Electrophoresis was performed in the Laemmli buffer system and the gel was stained with Coomassie Brilliant Blue (insert).


1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
E
α -Lactalbumin
Soy bean trypsin inhibitor
Carbonic anhydrase
Pyruvate dh.
β chain
Pyruvate dh.
α chain
Ovalbumin
Component X
Lipoamide dh.
BSA
Acetyltransferase
Phosphorylase b
10^4
3.10^4
5.10^4
7.10^4
9.10^4
Subunit M_r value
E2
E3
X
E1 α
E1 β
⊖
⊕

No bands at 47,000-45,000 corresponding to the subunits of PDC kinase (Stepp et al., 1983) were detected suggesting that the kinase constituted a minor component of the purified complex (< 1%). However, preparations of PDC used in this study did contain a very active kinase.

3.2.2. Presence of pyruvate dehydrogenase kinase and absence of phosphatase in the purified PDC

Assays of pyruvate dehydrogenase kinase were performed by monitoring the initial rate of inactivation of the complex in the presence of 0.2 mM-ATP (Fig. 3.4) or by measuring the incorporation of ^{32}P from [γ -^{32}P] ATP into protein-bound radioactivity (Fig. 3.5). Pyruvate dehydrogenase phosphatase activity was not detected when assayed as the change (increase) in overall PDC activity following incubation of ATP-inactivated PDC in a medium containing 10 mM-$MgCl_2$, 0.5 mM-$CaCl_2$ (Fig. 3.4), or by measuring the difference (decrease) in protein-bound radioactivity from ^{32}P-labelled PDC after incubation under similar conditions (Fig. 3.5). Controls in which no $MgCl_2$ or $CaCl_2$ were added, were also included (not shown). Fig. 3.5(B) demonstrates that phosphorylation of PDC is confined to the α chain of pyruvate dehydrogenase (E1).

Similar analysis has been conducted previously to test for the presence of pyruvate dehydrogenase phosphatase in purified preparations of the complex (Kresze & Steber, 1979). However, it has also been reported that inorganic phosphate slightly inhibits phosphatase activity (Hucho et al., 1972) in the presence of 10 mM-$MgCl_2$. Low levels of phosphatase could be masked if pyruvate dehydrogenase kinase was not fully inhibited at high Mg^{2+} concentrations. These results were confirmed as depicted in Fig. 3.5. In this case, incubations were

Fig. 3.4 Assay of pyruvate dehydrogenase kinase and phosphatase by monitoring changes in the overall activity of the complex

PDC (20-50 µg) was incubated at 30°C in 20 mM-potassium phosphate buffer, pH 7.5 containing 0.5 mM-$MgCl_2$, 2 mM-DTT in the presence (——●——) or absence (——○——) of 0.2 mM-ATP. Samples were removed for spectrophotometric assay of PDC activity as described in section 2.2.8.a. At the time indicated by the vertical arrow, $MgCl_2$ and $CaCl_2$ were added to give a final concentration of 10 mM and 0.5 mM, respectively. Activity measurements were continued for a further 60 min. Results are means of duplicate determinations which varied by less than ± 5%.

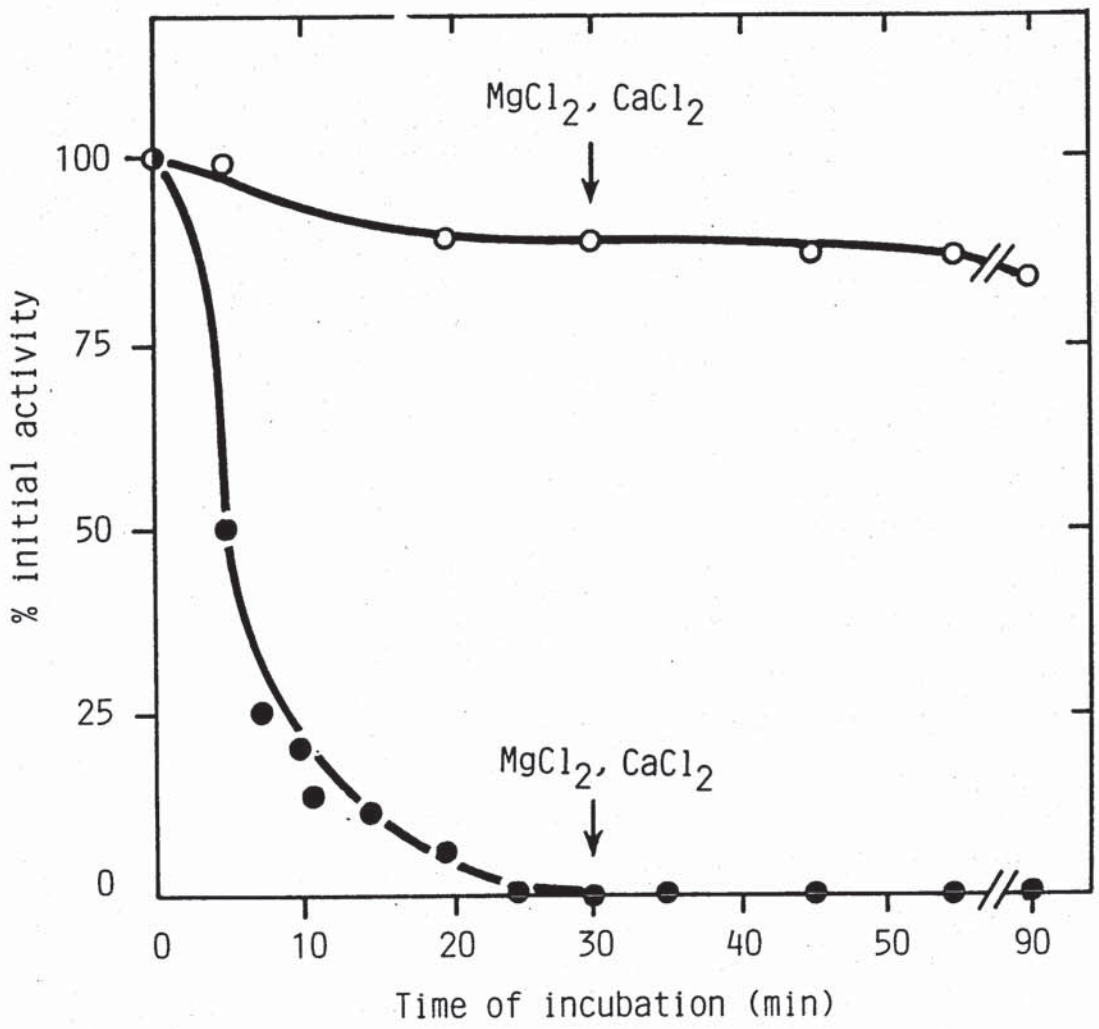
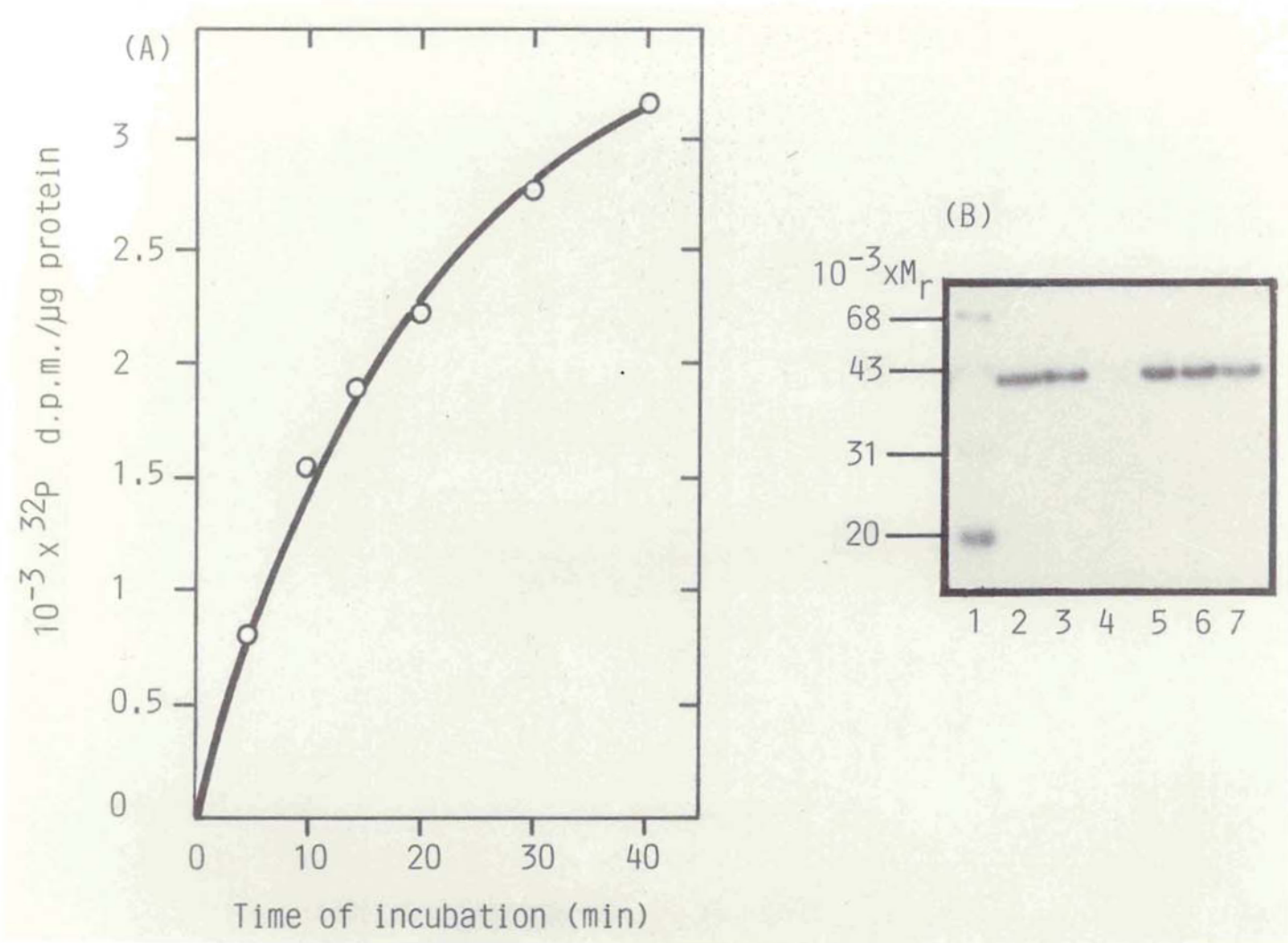

MgCl2, CaCl2
MgCl2, CaCl2
100
75
50
25
0
% initial activity
0
10
20
30
40
50
90
Time of incubation (min)

Fig. 3.5 Assay of pyruvate dehydrogenase kinase and phosphatase by measuring changes in the amount of ^{32}P-labelled PDC

For the kinase assays, PDC (50 µg) was incubated at 30°C in 20mM-potassium phosphate buffer, pH 7.5 containing 0.5 mM-$MgCl_2$, 2 mM-DTT. Reactions were initiated by the addition of [γ -^{32}P] ATP (0.2 mM; 100,000 d.p.m./ nmol). Aliquots of 10 µl were applied to filter papers to estimate the time course of incorporation into TCA-precipitable radioactivity (A).

For the phosphatase assays, ^{32}P-labelled PDC (50 µg), prepared as described in section 2.2.8.c (approx. 240,000 d.p.m./mg), was incubated in 20 mM-Tris/HCl buffer, pH 7.5 containing 10 mM-$MgCl_2$, 0.5 mM-$CaCl_2$ for 60 min at 30°C in the presence of various amounts of native PDC. Aliquots (20 µl) were removed to estimate radioactivity. The remaining sample was solubilised in Laemmli sample buffer and electrophoresed on a 10% (w/v) SDS/polyacrylamide gel. After staining with Coomassie Blue, the gel was dried and autoradiographed (B).

Lane 1, ^{125}I-labelled M_r standards; lane 2, ^{32}P-labelled PDC before incubation; lane 3, ^{32}P-labelled PDC incubated with 50 µg heat-inactivated PDC; lane 4, PDC 10 µg, used as a marker; lanes 5, 6 and 7 ^{32}P-labelled PDC incubated with 50 µg, 75 µg and 120 µg PDC, respectively.


(A)
10^{-3} x ^{32}P d.p.m./µg protein
3
2.5
2
1.5
1
0.5
0
0
10
20
30
40
Time of incubation (min)
(B)
10^{-3}xM$_r$
68
43
31
20
1 2 3 4 5 6 7

performed in the absence of inorganic phosphate and [γ ^{32}P] ATP. The amount of radioactivity associated with ^{32}P-labelled PDC remained constant during the period of incubation. It can be concluded that no dephosphorylation or proteolysis of E1 α took place under these experimental conditions.

It has been previously established that pyruvate dehydrogenase phosphatase is less tightly-bound to the complex than the kinase and can be separated from the remaining components of the complex by ultracentrifugation. (Linn et al., 1972). Pyruvate dehydrogenase phosphatase becomes associated with the dihydrolipoyl acetyltransferase core of the complex in a Ca^{2+}-dependent process (Pettit et al., 1972). This property has afforded a basis for its purification from bovine kidney and heart (Pratt et al., 1982; Pettit et al., 1982) and from pig heart mitochondria (Seiss & Wieland, 1972).

In contrast, the other regulatory enzyme of the complex, pyruvate dehydrogenase kinase, is retained as an integral component of the complex during purification (Linn et al., 1972; Stepp et al., 1983).

Whereas phosphatase activity requires a divalent cation (Mg^{2+} or Ca^{2+}) for activity, either ion, if present in sufficient concentration, will cause inhibition of the kinase activity. In particular, Ca^{2+} inhibits the kinase at very low concentrations, whereas Mg^{2+} inhibits it at higher levels (> 10 mM) (Wieland et al., 1975).

3.3 Resolution of ox heart PDC

This procedure for the purification of PDC gave relatively large amounts (50-60 mg) purified complex from 250 g tissue. One of the approaches adopted in this study for production of subunit-specific antisera was to achieve resolution of the complex by standard published procedures and employ the individual activities for immunisation.

Most of the classical procedures for resolution of the 2-oxo-acid dehydrogenase complexes were developed initially by Linn et al. (1972) and Barrera et al. (1972). These methods allow the isolation of the dihydrolipoyl acetyltransferase, lipoamide dehydrogenase and pyruvate dehydrogenase components as well as partially-purified pyruvate dehydrogenase kinase and phosphatase.

3.3.1 Gel filtration chromatography

PDC was resolved into its constituent enzymes after dissociation of the complex at alkaline pH and high salt before chromatography on Sepharose CL-6B or Sepharose 4B columns.

Fig. 3.6 shows a typical profile obtained with 50 mg complex. Similar patterns were obtained when the chromatographic separation was performed in 0.25 M-$MgCl_2$ at neutral pH (Kresze & Steber,1979). Analysis of the fractions obtained from the two peaks, showed that Peak I contained the dihydrolipoyl acetyltransferase core and component X along with smaller amounts of E1 and traces of E3. Peak II contained most of the E1 and E3 present in the original sample. Owing to the small difference in size between the pyruvate dehydrogenase component, M_r 154,000 and E3, M_r 110,000, they were not clearly separated from each other.

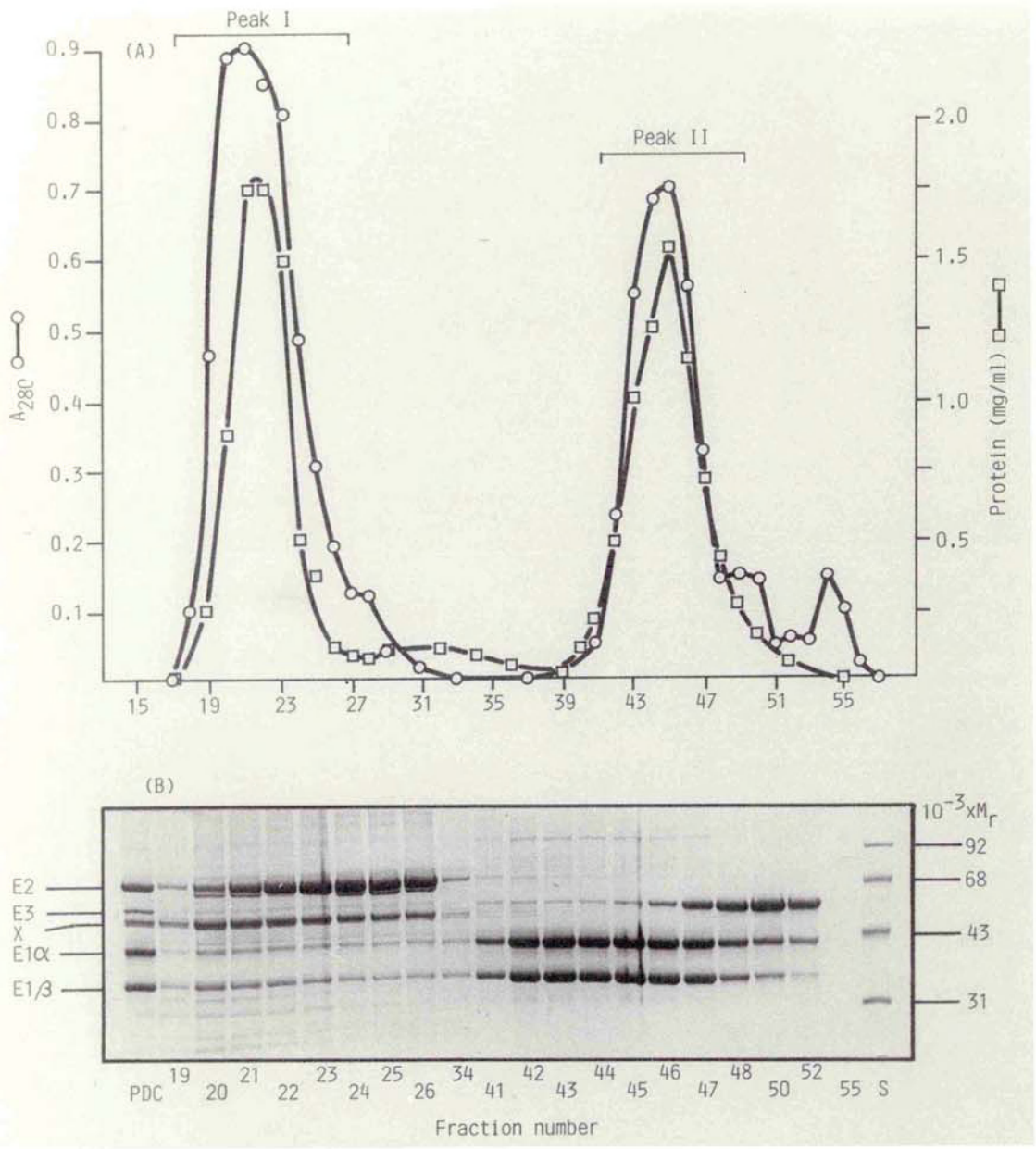
Attempts to remove the E1 component from Peak I by prolonged incubation of the complex or by repeated freezing and thawing failed to increase substantially the degree of dissociation of E1 from the core. This may reflect strong interactions between the components, orpartial reassociation of the complex during chromatography, or both.

The profile obtained by high pressure gel permeation chromatography of the complex in 0.1 M-Tris/HCl pH 7.0, 0.25 M-$MgCl_2$ (Fig. 3.7) is very similar to Fig. 3.6, except for the appearance of a third peak containing most of the E1 α subunit. The reasons for this difference

Fig. 3.6 Elution profile of constituent enzymes of ox heart PDC on Sepharose CL-6B

Purified PDC (50 mg) was dissociated in 0.1 M-glycine, 1 M-NaCl, 1 mM-$MgCl_2$, 2 mM-DTT, 0.1 mM-EDTA, adjusted to pH 9.0 with 1 M-NaOH and chromatographed on a column of Sepharose CL-6B (80 cm x 1.5 cm) equilibrated and developed with the same buffer as described in section 2.2.10.a. A_{280} (—○—) and protein concentration (—□—) were measured in the fractions. Samples containing 15 µg protein were precipitated in 10% (w/v) TCA in order to reduce the salt concentration. Pellets were dissolved in Laemmli sample buffer and applied on a 10% (w/v) SDS/polyacrylamide slab gel. (A) Elution profile, (B) Coomassie Blue stained gel.

A small peak of u.v.absorbing material was consistently observed eluting at the total column volume (fractions 53-56).


Peak I
Peak II
(A)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A_{280}
2.0
1.5
1.0
0.5
Protein (mg/ml)
15
19
23
27
31
35
39
43
47
51
55
(B)
E2
E3
X
E1α
E1β
$10^{-3}xM_r$
92
68
43
31
PDC
19
20
21
22
23
24
25
26
34
41
42
43
44
45
46
47
48
50
52
55
S
Fraction number

are not known, but it is possible that subunit E1 α was retarded by interacting with the matrix of the column. This result is interesting since it may provide a mild method for isolating native E1 α subunit. Dissociation of E1 has so far only been achieved under strong denaturing conditions (Barrera et al., 1972).

3.3.2 Separation of pyruvate dehydrogenase (E1) α and β chains

Peak II from the gel filtration step was further fractionated by ammonium sulphate precipitation to yield pyruvate dehydrogenase, collected at 30% (w/v) saturation, and a supernatant fraction containing most of the E3 polypeptide.

Samples of pyruvate dehydrogenase were dissociated before chromatography on a phosphocellulose column in the presence of 8 M-urea, 50 mM-2-mercaptoethanol at pH 6.0. The elution pattern and the polypeptide composition of the fractions obtained are shown in Fig. 3.8 (A) and (B), respectively.

Bands were identified from their respective apparent M_r of 36,000 for the β subunit and 42,000 for the α subunit (Barrera et al., 1972). At very low LiCl concentration, the eluted fraction contained the β chain. Subunit α was bound to the column and was eluted at 0.05 M-LiCl. A third peak was obtained when the ionic strength was increased to 0.075M-LiCl.

Examination of the polypeptide pattern in the eluted fractions revealed that subunit E1 β was purified to a large extent (approx. 95%), whereas subunit E1 α was contaminated with E3 present in the original E1 sample. (Fig. 3.8, lane 2). Therefore, it is possible to obtain highly-purified subunits E1 α and E1 β providing that all traces of contaminating E3 component are removed from E1 fractions by repeated ammonium sulphate fractionation before chromatography on phosphocellulose columns.

Fig. 3.7 Subfractionation of dissociated PDC by high pressure gel permeation chromatography

Purified complex (1 mg) was dissociated 0.1 M Tris/HCl, pH 7.0, containing 0.25 M-$MgCl_2$ as described by Kresze & Ronft (1979) and resolved on a TSK G3000 SW column equilibrated and developed with the same buffer.

Absorbance at 215 nm was monitored continuously (A). Samples from the peak fractions were subjected to SDS/polyacrylamide gel electrophoresis to determine their polypeptide composition, after staining the gel with Coomassie Blue (B).

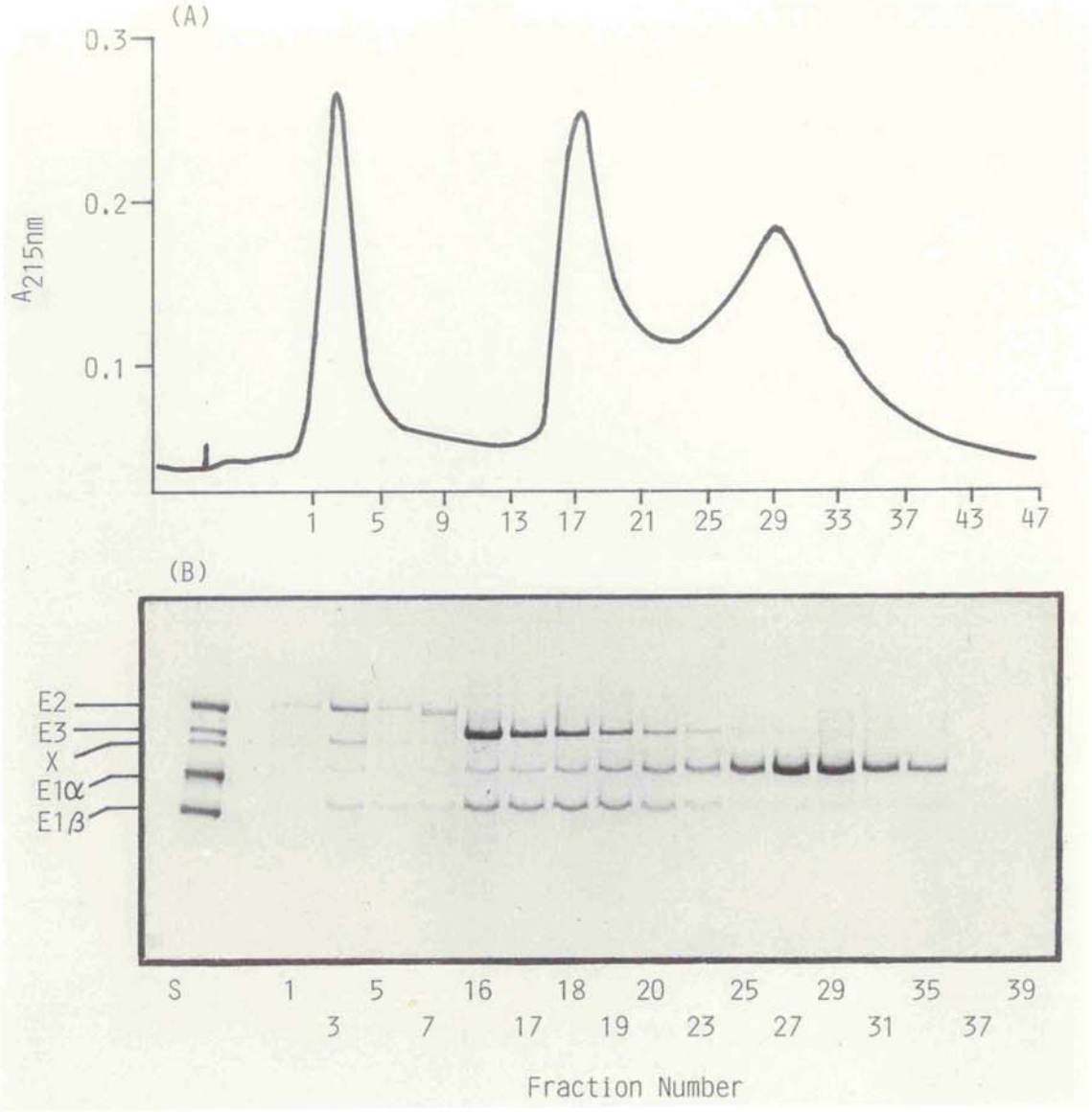
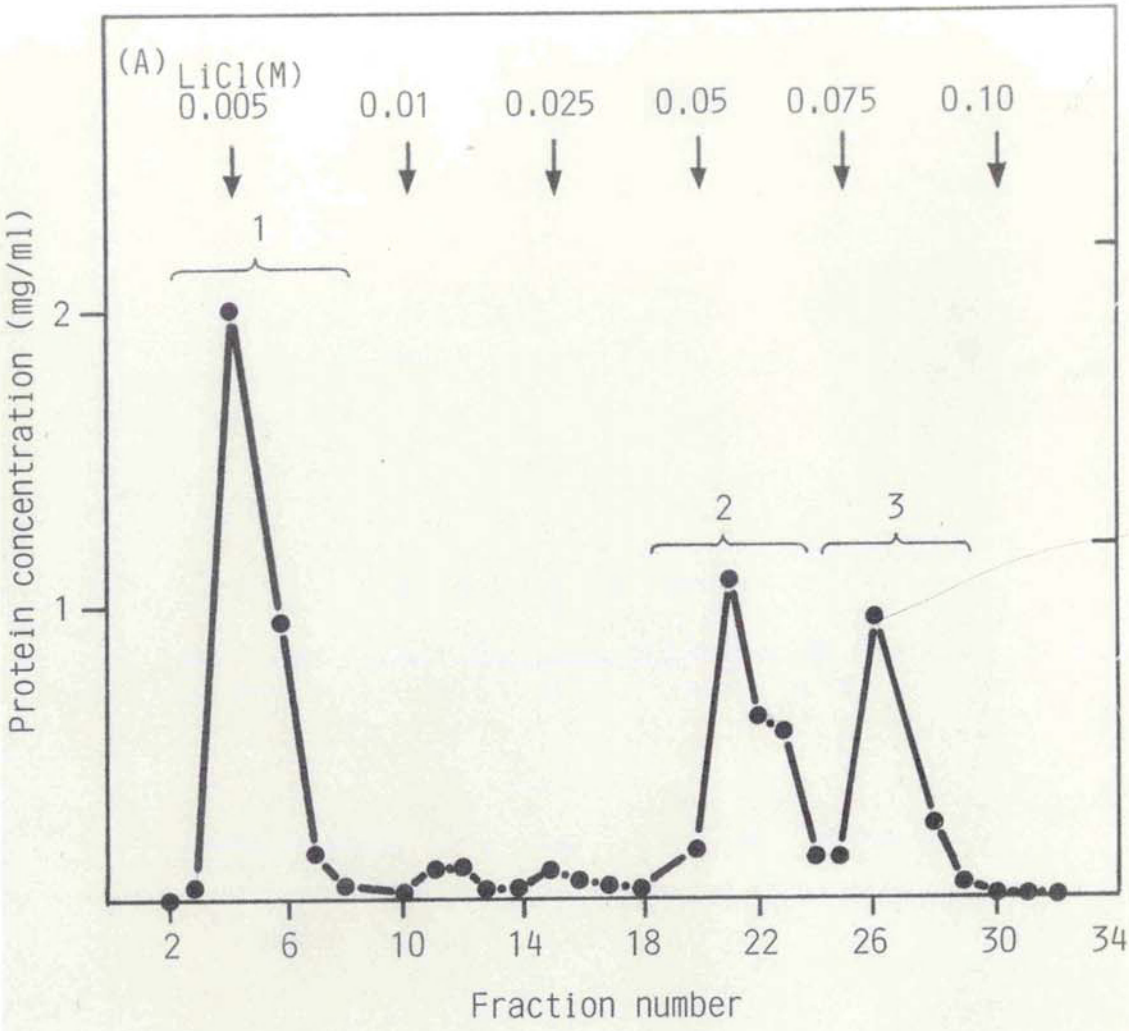

(A)
0.3
0.2
0.1
A_{215nm}
1 5 9 13 17 21 25 29 33 37 43 47
(B)
E2
E3
X
E1α
E1β
S 1 5 16 18 20 25 29 35 39
3 7 17 19 23 27 31 37
Fraction Number

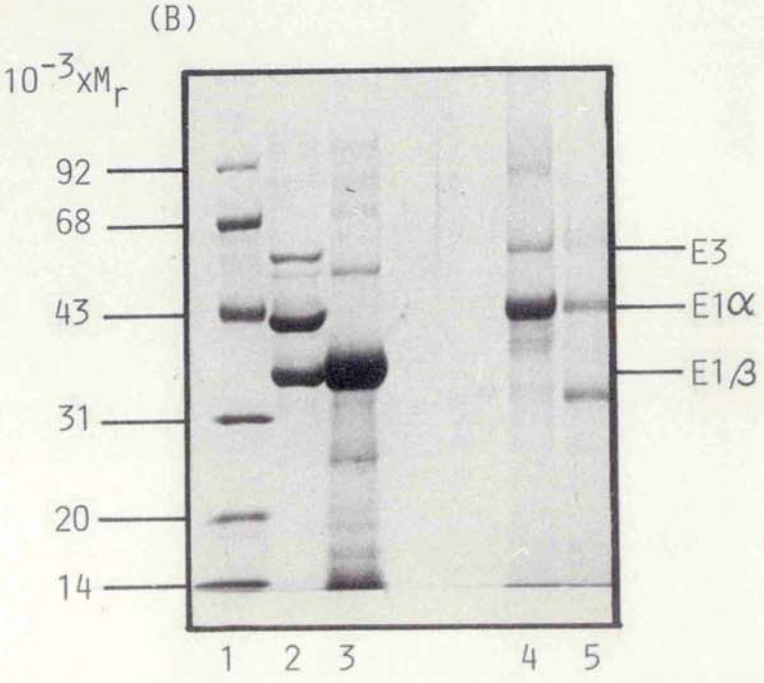
Fig. 3.8 Chromatographic separation of α and β chains of ox heart pyruvate dehydrogenase (E1)

Pyruvate dehydrogenase (approx. 5 mg) was chromatographed on a phosphocellulose column (13 cm x 1 cm) equilibrated in 0.02 M-imidazole buffer containing 8 M-urea, 0.05 M-2-mercaptoethanol, adjusted to pH 6.0 with HCl. The column was developed by stepwise increases in the LiCl concentration. Flow rate was 24 ml/h and fractions of 2 ml were collected.

Protein was determined in the eluted fractions by the method of Bradford (1976). Aliquots containing approx. 10 µg protein were applied onto a 10% (w/v) SDS/polyacrylamide gel. Protein bands were visualised after staining with Coomassie Blue.

(A) Elution profile, (B) SDS/polyacrylamide gel of the pooled fractions. Lane 1, M_r standards; lane 2, pyruvate dehydrogenase sample before chromatography; lane 3, peak 1; lane 4, peak 2; lane 5, peak 3.


(A)
LiCl(M)
0.005
0.01
0.025
0.05
0.075
0.10
1
2
3
Protein concentration (mg/ml)
1
2
2
6
10
14
18
22
26
30
34
Fraction number


(B)
10^{-3}xM$_r$
92
68
43
31
20
14
E3
E1α
E1β
1
2
3
4
5

3.3.3 Treatment of subcomplex E2-X with p-chloromercuriphenyl sulphonic acid

Attempts to further resolve Peak I from gel filtration chromatography of the dissociated complex, by treatment with the reagent p-chloromercuriphenyl sulphonic acid at pH 9.0, resulted in a dihydrolipoyl acetyltransferase fraction substantially free of E1 and E3. However, component X was still associated with the enzyme (see Chapter 6, Fig. 6.7, lane 2).

It has been shown that p-chloromercuriphenyl sulphonic acid treatment induces aggregation and precipitation of the core enzyme and promotes the release of pyruvate dehydrogenase kinase (Stepp et al., 1983). However, this procedure failed to dissociate the subcomplex E2-X (Fig. 6.7, lane 3).

3.3.4 Purification of the constituent polypeptides of PDC by extraction from SDS/polyacrylamide gels

At this stage, it became obvious that this approach for isolation of PDC subunits in a highly-purified state (> 95% purity), involved the preparation of large amounts of purified complex and tedious and time-consuming chromatographic procedures, often giving low yields at $\leq$90% purity.

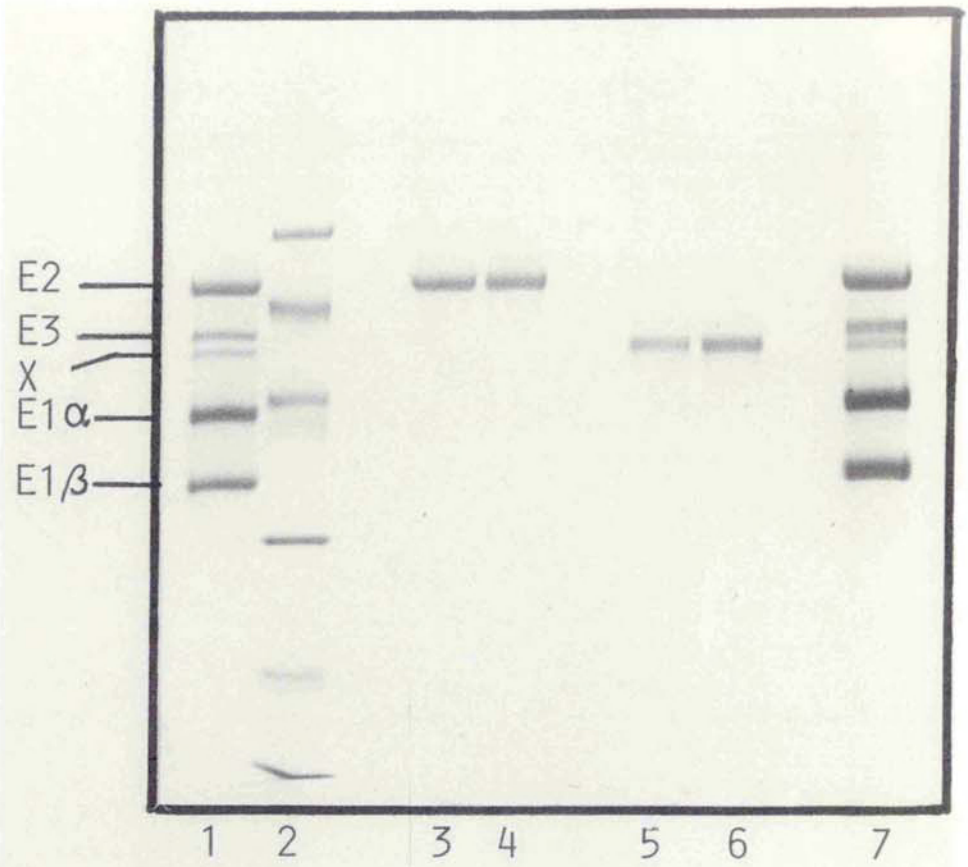
To circumvent these difficulties, the complex was dissociated with SDS, and resolved on 10% (w/v) SDS/polyacrylamide preparative gels. Polypeptides corresponding to the subunits E2, X, E1 α and E1 β were identified by their electrophoretic mobilities and prepared for immunisation as described in section 2.2.12.b. Studies on the characterisation of the antisera are discussed in Chapter 4.

Fig. 3.9 illustrates the use of preparative electrophoresis to obtain purified SDS-denatured PDC subunits. Proteins were extracted from 10% (w/v) SDS/polyacrylamide gels. After concentration and protein

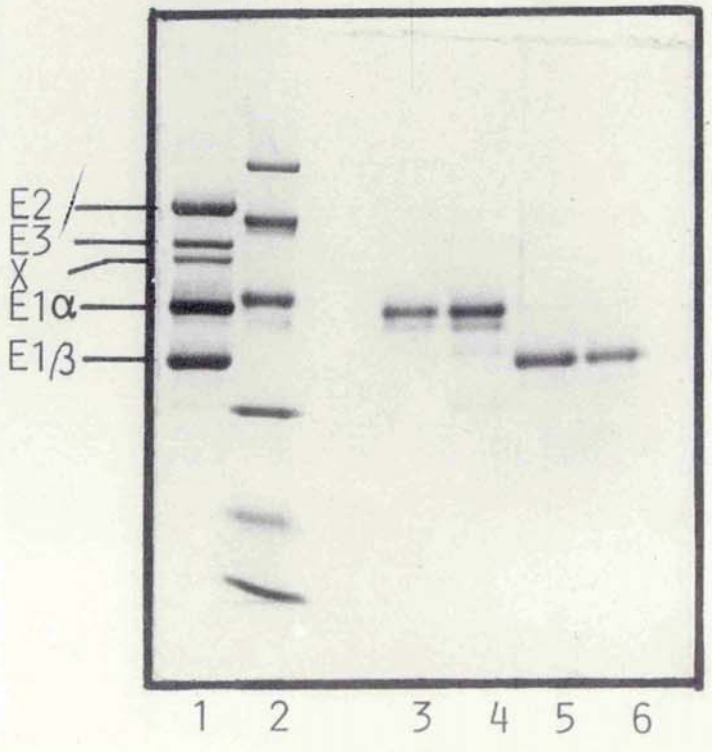
Fig. 3.9 SDS/polyacrylamide gel electrophoresis of PDC components purified by extraction from preparative SDS/polyacrylamide gels

PDC (approx. 3 mg) was resolved into its individual components in a 10% (w/v) SDS/polyacrylamide slab gel. After brief staining, gel strips were extracted in 50 mM-triethanolamine containing 1% (w/v) SDS, 1 mM-DTT adjusted to pH 8.0 with HCl, as described in section 2.2.10.f. Portions containing 2-5 μg of the extracted proteins were loaded onto a 10% (w/v) SDS/polyacrylamide gel.

Gel (A): lanes 1 and 7, PDC 12 μg each; lane 2, M_r markers; lanes 3 and 4, acetyltransferase fraction 3 μg and 4 μg, respectively; lanes 5 and 6, component X, 2 μg and 3 μg. Gel (B): lane 1, PDC 12 μg protein; lane 2, M_r markers; lanes 3 and 4, E1 α subunit 3 μg and 5 μg, respectively; lanes 5 and 6, E1 β subunit 3 μg and 2 μg respectively.



(B)



determination, samples were re-electrophoresed on a 10% (w/v) analytical gel.

Preparative gels could be loaded with a maxiumum of 3 mg of PDC protein to ensure complete resolution of component X from E3 subunit. In purifying proteins by the method of Tolan et al. (1980), recoveries were improved by concentrating the extracts by freeze-drying before acetone precipitation and by omitting precipitation with potassium acetate.

Total yields ranged from 45-65% as estimated by the recovery of ^{32}P-labelled subunit E1 α . Between 80-90% of the protein extracted was recovered in the final samples.

It was also observed that subunit E1 α was particularly sensitive to degradation (Fig. 3.9, gel B). This problem can be overcome by performing the extractions in the presence of protease inhibitors such as PMSF.

Chapter 4: IMMUNOLOGICAL STUDIES ON THE MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX

4.1 Production of antisera to native PDC and its individual subunits

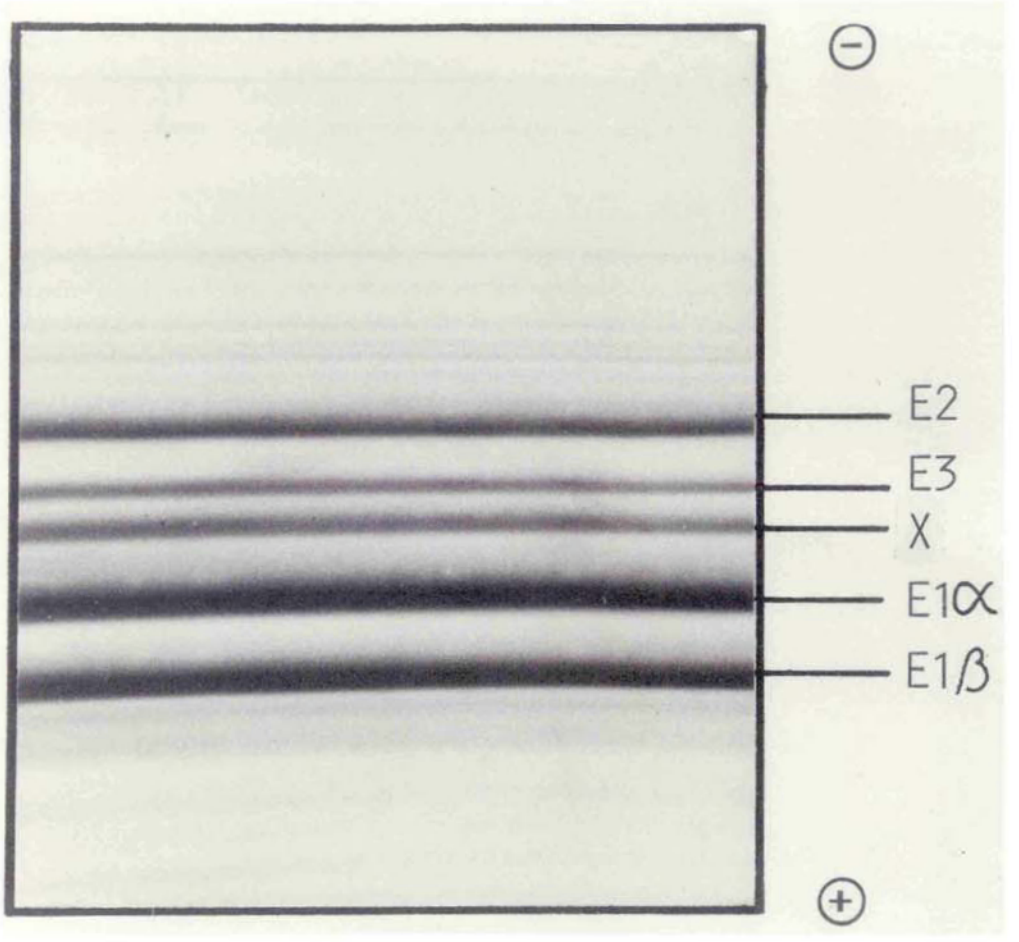
Knowledge about the biogenesis of mitochondria and other organelles has increased considerably during the last ten years. Part of this success is related to the development of a variety of immunological techniques with wide applications in this field.

In most studies on biogenesis of mitochondria, radioactively-labelled cellular or mitochondrial extracts are employed as the source of newly-synthesised mitochondrial proteins. Antibodies are used to detect and isolate the protein under study, which is identified after electrophoretic analysis of the dissociated immunocomplexes. The specificity of the reaction depends largely on the properties of the antiserum. Therefore, a prerequisite to ensure the experimental value of this methodology is the availability of specific antibodies that recognise only a defined protein in the extracts.

The production of specific antibodies requires the availability of a highly-purified antigen. Some proteins are weak immunogens and the presence of small amounts of a more antigenic contaminant leads to decreased specificity of the resulting serum which limits its usefulness. The protein(s) is generally considered pure enough to be used as an antigen if it has at least 95% purity when examined by SDS/polyacrylamide gel electrophoresis. However, this may not always be the case and may be necessary to resort to other fractionation procedures e.g. isoelectric focusing or two-dimensional gel electrophoresis.

Fig. 4.1 Preparative SDS/polyacrylamide gel electrophoresis of PDC

Partially purified PDC (approx. 90% purity; 2.2 mg) was resolved into its constituent polypeptides on a 10% (w/v) SDS/polyacrylamide preparative slab gel. After staining with Coomassie Blue, the protein bands were excised from the gel and employed for the production of subunit-specific antisera as outlined in section 2.2.12.b.


⊖
E2
E3
X
E1α
E1β
⊕

The approach adopted in this study for obtaining samples of the individual components of the complex, free of cross-contamination for the production of subunit-specific antisera, has been to employ preparative SDS/polyacrylamide gel electrophoresis. As shown in Fig. 4.1, PDC was resolved into its constituent polypeptides which were later identified by their characteristic electrophoretic mobilities.

Bands corresponding to the subunits E2, X, E1 α and E1 β were excised from the gels and the protein-polyacrylamide mixtures were used for immunisation of rabbits as described in section 2.2.12.b. In this way it has been possible to obtain monospecific antisera to these components without the necessity for isolating the individual enzymes (Chapter 3, section 3.3). In dealing with limited amounts of valuable proteins, techniques which enhance the immune response should be used. The best responses are elicited with the proper use of adjuvants. Adjuvants increase the persistence of antigen in the host and protect the antigen from degradation by proteolytic enzymes allowing more antibody-producing cells to be exposed to the antigen. Commonly used adjuvants are inorganic suspensions of alumina cream and aluminium sulphate. Particulate materials such as charcoal and polyacrylamide gels are very immunogenic and when mixed with the protein elicit an enhanced immune response.

The most popular and successful adjuvants are the water/light mineral oil emulsions developed by Freund. These reagents are emulsified with either solutions or suspensions of the immunogen (incomplete Freund's adjuvant). The addition of heat-killed mycobacteria (Mycobacterium butyricum, Mycobacterium tuberculosis) in small amounts to the suspension (complete Freund's adjuvant) leads to further enhancement of the immune response. This has been attributed to the increased local inflammatory response.

For priming immunisation, complete Freund's adjuvant is most suitable. If the subcutaneous (intradermal or intramuscular) route is chosen, multiple injections in various sites are better than a single large injection. Further booster (secondary) immunisations are usually given in complete Freund's adjuvant, since further immune responses against the mycobacteria can be detrimental to the host and lead to enhanced inflammatory responses.

In general, high affinity antibodies are produced after immunisation with low doses of antigen and the best sera are obtained 3-5 months after immunisation.

For this study, rabbits were chosen as they produce a higher titre of antibody than guinea pigs and mice. Also, there were animal house facilities for these animals and reasonable amounts of blood (approx. 60 ml) could be collected, by bleeding of a marginal ear vein.

Test bleedings were usually made after the fourth injection and treatment repeated after approx. 4 week intervals. In most cases antisera exhibited low titre after the first two bleedings and it was beneficial to apply additional booster injections.

Purification of the antibodies was not required for most purposes and unfractionated serum was used throughout.

4.2 Effects of several antisera on PDC activity

It has been recognised for some time that antibodies can inhibit enzyme activity. Some enzymes are completely inhibited, others are partially inhibited and some are not (for a review, see Cinader, 1977). To some extent, the inhibitory characteristics of an antibody depends on the spatial relation between the antigenic determinants and the catalytic site. Inhibition may result from steric hindrance of substrate or ligand sites owing to binding of antibody near the active site. On the other hand, there are differences between the antisera raised to native or

denatured protein antigens. Denaturation of proteins often exposes new antigenic determinants which may be inaccessible in the native structure. Denaturation of protein antigens has been extensively investigated, and decreased immunogenicity is often observed in comparison with the native form. Studies on native and chemically-modified human BSA (Jacobsen et al., 1972) as well as many similar studies have led to the conclusion that protein antigens may contain two types of antigenic determinants, namely, sequential and conformational. Sequential determinants are those occurring in a linear sequence as in the unfolded form of an SDS/denatured protein. Conformational determinants are those that are recognised by their homologous antibodies when they occur in a particular conformation. This latter case includes determinants formed from amino acids that are located at distant points in the peptide chain, but are in close proximity in the native molecule. It seems also likely that proteins displaying quaternary structure have unique conformational determinants.

Other factors which may also be important in contributing to differences in the immune response to native and denatured antigens are the accessibility of determinants and the M_r value and/or state of aggregation of the protein. In general, the greater the M_r, the greater the response that can be expected.

In summary, there are many variables implicated in influencing the multifaceted and complex sequence of events of the immune response, beginning with the particular species and the physiological state of the animal, the presentation of the antigen and the physicochemical characteristics of the protein in question (Maurer & Callahan, 1980).

Antisera against the whole complex and the individual subunits were characterised regarding their ability to inhibit the overall activity of the complex.

Fig. 4.2 shows that antisera to PDC and subunit E3 were capable of inhibiting totally or partially the activity of the complex, respectively, whereas antisera to subunits E2, X, E1 α and E1 β did not inhibit at all. Antiserum to PDC exhibits a very high titre and approx. 1 μl serum was capable of inactivating 0.02-0.04 units of enzymic activity (nKat) or approx. 0.2 μg protein.

Effects of several antisera on pyruvate dehydrogenase kinase activity were also determined (Chapter 6, section 6.5,Fig. 6.5 and 6.6).

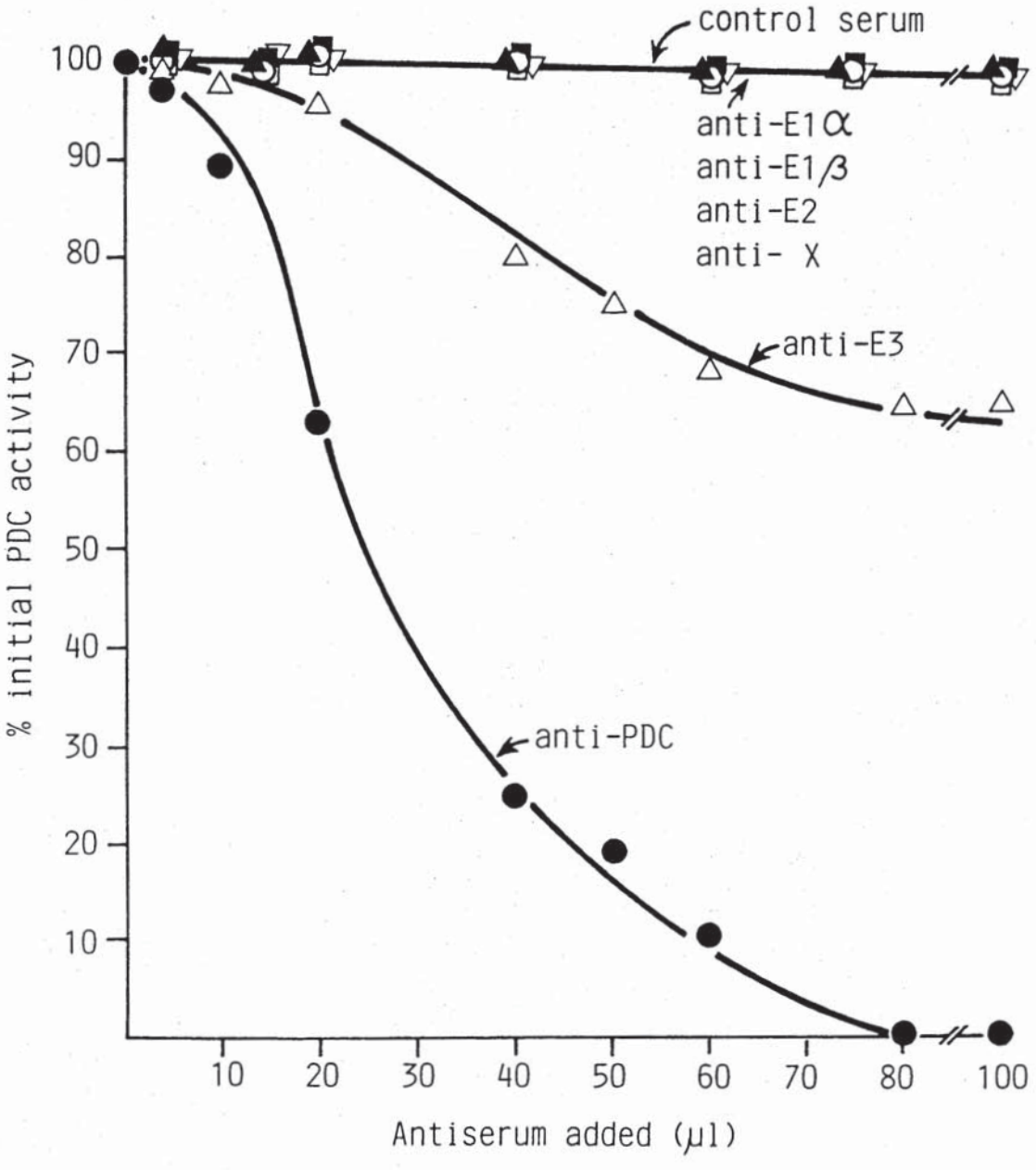
Analysis of the effects of antisera on enzymic activity provides information which has little relevance for studies on biogenesis. Most studies involve exposure of proteins to detergents before immunological reaction. These detergents are often strong denaturants e.g. SDS and deoxycholate. Detergent treatment may cause more less pronounced conformational changes in the protein that may affect the binding of antibodies. Hence, before using the antisera, relevant information on their specificity and titre must be obtained.

Binding of antigen and antibody can be detected by allowing the proteins to diffuse through agar or agarose gels (immunodiffusion) or electrophoretically driving the antigen against the antibody (immunoelectrophoresis). A precipitate will form at the point at which they reach an optimal ratio (equivalence point). These are the simplest methods to detect precipitating antibodies and can be made quantitative. Many modifications of the immunoelectrophoresis technique had been developed (Johnston & Thorpe,1982). The limitations of these methods are basically imposed by their low sensitivity and poor electrophoretic resolution of complex antigenic mixtures on agarose gels as compared with polyacrylamide gels. Other difficulties arise in connection with performing immunological analysis in the presence of detergents e.g. SDS.

Fig. 4.2 Effects of various antisera on the activity of PDC

PDC (16.8 μg) was incubated with varying amounts of antiserum in 0.1 M-sodium phosphate buffer, pH 7.0, 0.15 M-NaCl. The incubations were carried out at room temperature for 90 min. Aliquots of 5-100 μl were assayed for PDC activity. Results are means of two determinations, differing by less than ± 5%.

(—●—) anti-PDC serum; (—△—) anti-E3 serum; (—○—) control serum; (—□—) anti-E2 serum; (—■—) anti-E1 α serum; (—▲—) anti-E1 β serum; (—▽—) anti-X serum.


control serum
anti-E1α
anti-E1β
anti-E2
anti- X
anti-E3
anti-PDC
% initial PDC activity
100
90
80
70
60
50
40
30
20
10
10
20
30
40
50
60
70
80
100
Antiserum added (μl)

Recently, a simple but powerful immunological technique has been developed by Towbin et al. (1979) which allows the detection of antibodies even in sera of low titre. The technique offers several advantages as compared with other immunoassay methods, in particular it gives information about the capacity of antibodies to bind SDS-denatured antigens.

4.3 Characterization of antisera by immunoblotting technique

The immunoblotting (immunoreplica) technique has provided a simple but extremely sensitive experimental tool to assess both the specificity and titre of an antiserum. The combination of SDS/polyacrylamide gel electrophoresis and radioimmunoassay is the basis for its sensitivity which enables the detection of very small amounts of individual proteins in complex mixtures. The method depends on the electrophoretic transfer of proteins and their immobilisation in such a way that a faithful replica of the original polyacrylamide gel pattern is generated on nitrocellulose sheets.

The only prerequisite is that the protein is not altered during the adsorption process and the binding sites remain accessible to the antibody. The technique is applicable to the study of other types of interactions e.g. hormone-receptor and protein-nucleic acid.

Several recent review articles deal with the parameters affecting the efficiency of transference of proteins from SDS/polyacrylamide gels to nitrocellulose paper. (Gershoni & Palade, 1983). Basically, transfer efficiencies depend on the elution rate of the individual proteins from the gel and their ability to adsorb onto nitrocellulose. For most proteins the rate of transfer is inversely related to their M_r value. Some membrane proteins are transferred very slowly because of their hydrophobicity. However, virtually all proteins are transferred

if 0.02% (w/v) SDS is added to the transfer buffer. This modification does not interfere with adsorption or with immunodetection.

The immunoblotting technique has the advantage over alternative immunological methods that the M_r values of all polypeptides reacting with the antiserum are clearly indicated. Information on the titre can be obtained with fixed amounts of antigen by testing various dilutions of the antiserum. Monospecificity of an antiserum is best demonstrated by showing that it reacts only with a single component when challenged with a cell extract.

4.4. Characterization of anti-PDC serum by immunoblotting analysis

In this study, cell extracts from various cultured cell lines were tested with the various antisera to assess the specificity and reactivity of the antibodies with the individual components of the complex. These cell lines were later used for studies on the biosynthesis of PDC (Chapter 8).

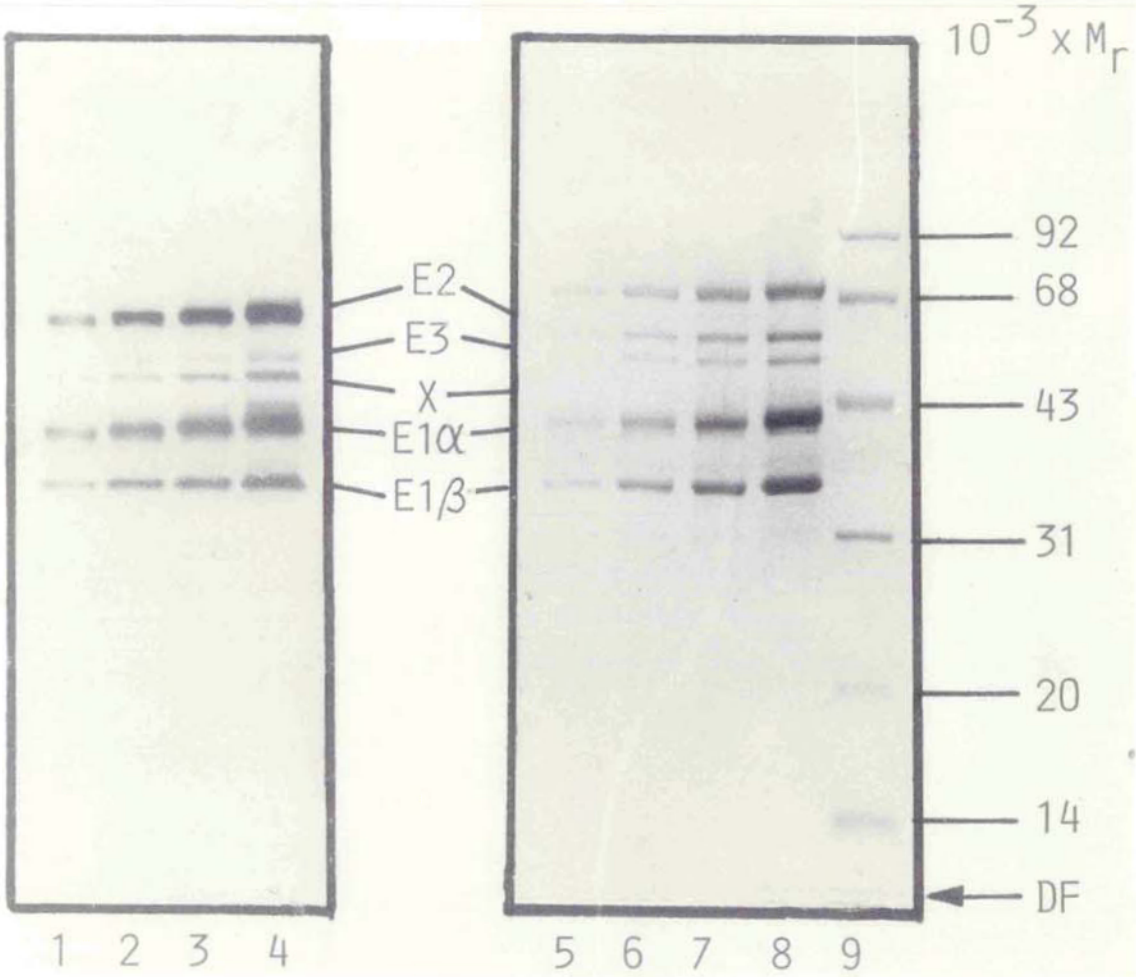
Information concerning the specificity and relative reactivities of the antiserum raised to the whole complex against the individual components was initially obtained by immunoblotting analysis of samples of purified PDC.

As shown in Fig. 4.3, anti-PDC serum exhibits high reactivity against all the components of the complex except the lipoamide dehydrogenase (E3). When low amounts of PDC were loaded on the gel (lanes 1 and 2), no reaction with E3 subunit could be detected whereas the bands for the other components were clearly visible. It was determined that the minimal amount of complex required to detect the E3 component was about 0.75-1.0 μg protein, whereas the other components were seen when 0.01-0.03 μg protein were present. This indicated that the levels required to detect E3 were about 20-50 times higher than those for detection of the remaining components of the complex.

Fig. 4.3 Reactivity of antiserum to ox heart PDC with the constituent polypeptides of the complex

Varying amounts of purified PDC were subjected to electrophoresis on 10% (w/v) SDS/polyacrylamide slab gels. Half of the gel was used for transferring the resolved proteins onto nitrocellulose paper (lanes 1-4); the duplicate half was stained with Coomassie Blue (lanes 5-8). After incubation with the antiserum, the immune complexes were "decorated" with ^{125}I-labelled protein A before autoradiography (see details in section 2.2.12.e.).

Lanes 1-4, 0.3 μg, 0.75 μg, 1.5 μg and 3 μg protein, respectively. Lanes 5-8 contained 3.3 times more enzyme (1 μg, 2 μg, 5 μg, and 10 μg) than their counterparts in lanes 1-4 to facilitate direct visible staining with Coomassie Blue. Lane 9, M_r markers.


10^{-3} x M_r
92
68
43
31
20
14
DF
E2
E3
X
E1α
E1β
1 2 3 4
5 6 7 8 9

In an analogous way, antiserum raised against the 2-oxoglutarate dehydrogenase complex was not capable of reaction with the E3 subunit whereas it detected small amounts of the E1 and E2 components of this complex (De Marcucci et al., 1985a).

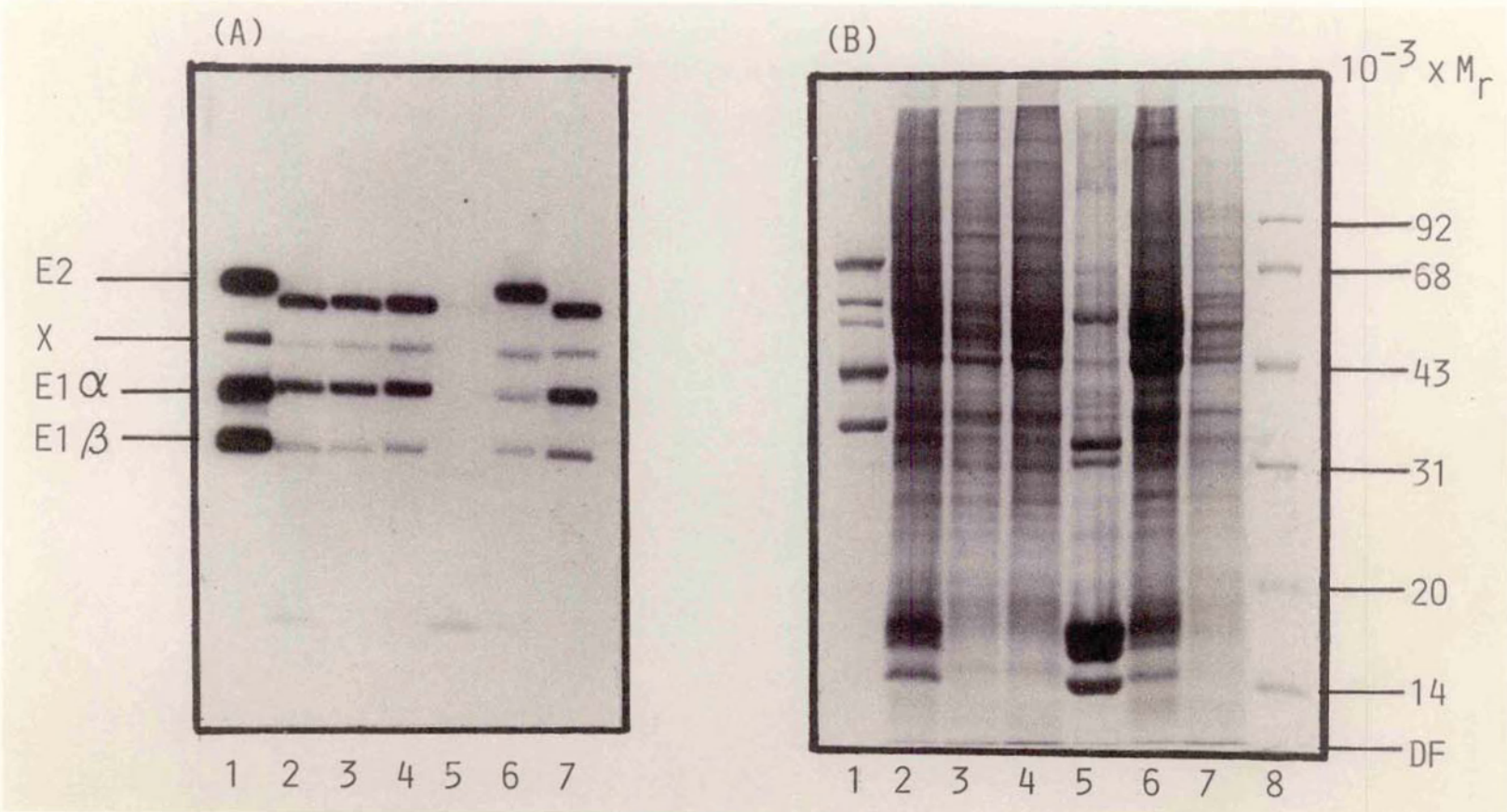
Lipoamide dehydrogenase is the only component common to the three types of 2-oxo-acid dehydrogenase complex. The poor immunological response elicited by this polypeptide relative to the other components of the complex cannot be explained on the basis of this component not being exposed on the surface of the assembly, perhaps rendering it inaccessible to the immune system. Current ideas on the structure of 2-oxo-acid dehydrogenase complexes indicate that E3 is located on the surface of the complexes since it can be released without disrupting the organisation of the core (Bleile et al., 1981). Moreover, it is not possible to attribute these results to a low content of E3 in the preparation of PDC used as antigen. The proportion of E3 estimated by densitometric analysis of PDC after SDS/polyacrylamide gel electrophoresis was approx. 10%, whereas the percentage of component X was approx. 6% (Chapter 6, table 6.1). However, the titre for component X was at least 20 times higher than that for the lipoamide dehydrogenase.

Fig. 4.4 shows that anti-PDC serum was capable of detecting the components of the complex present in various cell extracts with high specificity and sensitivity. The antigenic proteins were detectable in SDS-extracts of cultured rat liver (BRL) or bovine kidney cells (NBL-1). The antiserum did not detect the components of the complex in the nuclear fraction but exclusively in mitochondria (Fig. 4.4, lane 7). In this immune replica analysis there were several interesting features. The most obvious was the absence of the E3 subunit. The

Fig. 4.4 Immunodetection of PDC polypeptides in whole cell extracts and cellular subfractions

Samples of purified PDC and various cell extracts were electrophoresed on 10% (w/v) SDS/polyacrylamide gels. Half of the slab gel was employed for transference of the polypeptides onto nitrocellulose paper and subsequent immunoblotting analysis (A); the other half was stained with Coomassie Blue (B).

(A and B) Lane 1, PDC, 0.4 µg and 10 µg protein, respectively; lane 2, SDS-extract of BRL cells, 80 µg; lane 3 and 4, post-nuclear supernatant fraction, 20 µg and 40 µg; lane 5, BRL nuclei, 50 µg; lane 6, SDS-extract of NBL-1 cells, 100 µg; lane 7, BRL mitochondria, 40 µg; lane 8, M_r markers.


(A)
E2
X
E1α
E1β
1 2 3 4 5 6 7
(B)
10⁻³ x Mr
92
68
43
31
20
14
DF
1 2 3 4 5 6 7 8

sizes of the transacetylase components of bovine and rat tissues were quite clearly different. M_r values for the E2 subunit have been estimated as 70,000-74,000 for the bovine heart and kidney complex but only 68,000 for the rat heart enzyme (Matuda et al., 1983). A further important finding was that component X was located in the mitochondrial fraction. This location argued against the possibility that the protein became adventitiously bound to the complex during its isolation. (see also Chapter 6, section 6.4).

Immunological studies using antiserum to native PDC have been employed previously. Matuda et al. (1983) produced antiserum to rat heart PDC and component enzymes. The antisera were characterized by immunodiffusion and immunoprecipitation techniques. The immunoprecipitation patterns were difficult to analyse because the heavy chain of IgG (M_r 50,000) interfered with the detection of E3 and X components in the Coomassie Blue-stained gels.

Sheu & Kim (1984) have examined PDC from rat and bovine brain using antibodies raised against the bovine kidney enzyme. In a later report, Sheu et al. (1985) have characterised this antiserum by immunoblotting and immunoprecipitation. Immunoblotting patterns reported by these authors showed only three bands, identified as the E2, E3 and E1 α subunits. Subunits E1 β and X, although present in the antigen preparation, were not detectable on immunological analysis.

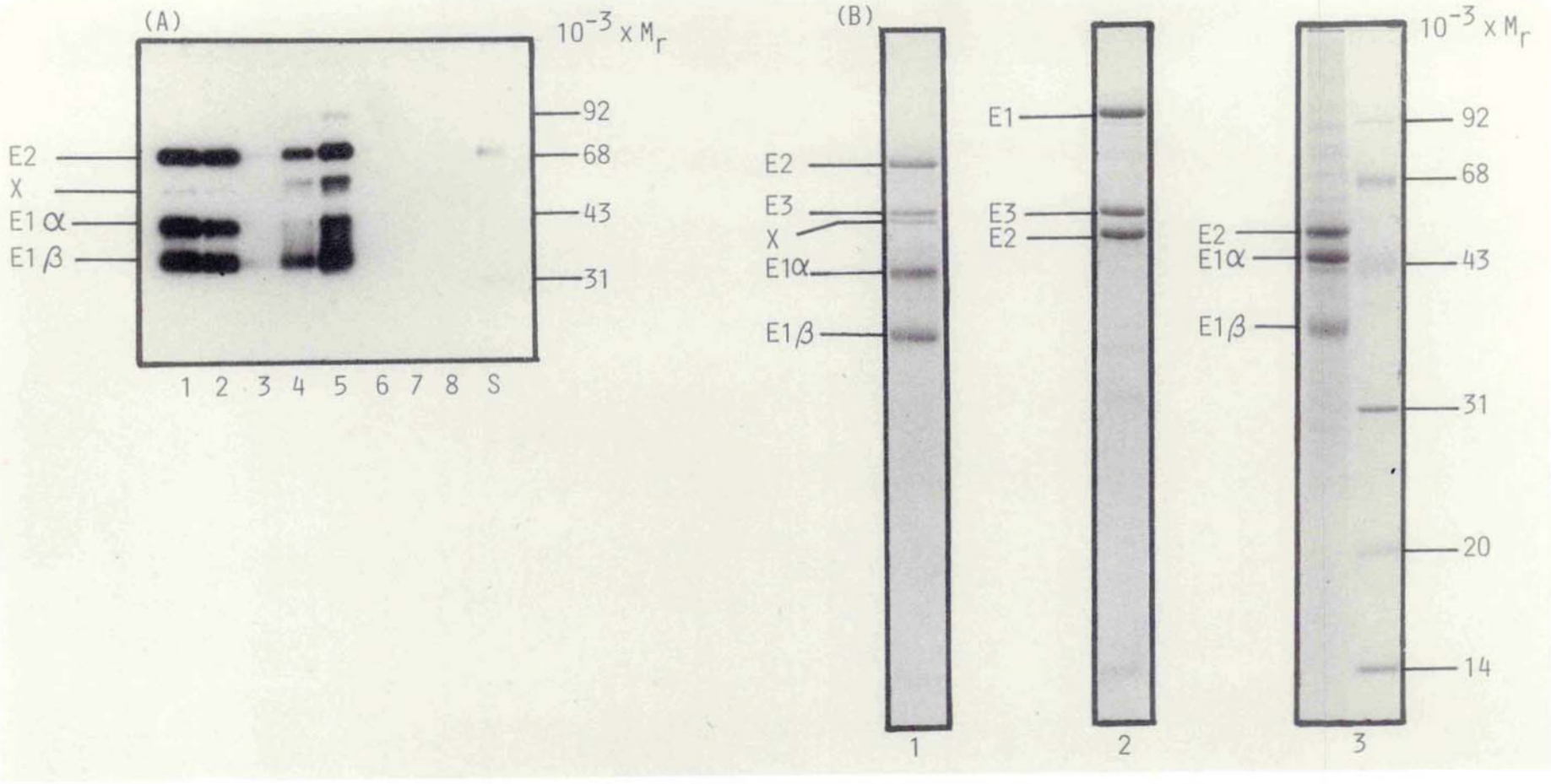
The striking differences between these results and the ones described in this study may be attributable to the various factors that affect the immune response (see section 4.2).

As shown in Fig. 4.5, anti-PDC serum does not cross-react with components E1 and E2 of the 2-oxoglutarate and branched-chain 2-oxo-acid dehydrogenase complexes.

Fig. 4.5 Cross-reactivity of antiserum to ox heart PDC with the bovine 2-oxoglutarate dehydrogenase and branched-chain 2-oxo-acid dehydrogenase complexes

Varying amounts of purified pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and branched-chain 2-oxo-acid dehydrogenase complexes were electrophoresed onto 10% (w/v) polyacrylamide slab gels and employed for immunological analysis with anti-PDC serum (A). A similar gel was stained with Coomassie Blue (B).

(A) Lanes 1 and 2, PDC 0.25 μg and 0.4 μg, respectively; lanes 3, 4 and 5, 2-oxoglutarate complex, 5 μg, 10 μg and 20 μg; lanes 6, 7 and 8, branched-chain 2-oxo-acid dehydrogenase complex, 5 μg, 10 μg and 20 μg, respectively; lane S, ^{125}I-labelled M_r markers. In (B) lane 1, PDC, 15 μg; lane 2, 2-oxoglutarate dehydrogenase complex, 12 μg; lane 3, branched-chain 2-oxo-acid dehydrogenase complex, 15 μg.


(A)
10^{-3} x M_r
E2
X
E1α
E1β
92
68
43
31
1 2 3 4 5 6 7 8 S
(B)
10^{-3} x M_r
E2
E3
X
E1α
E1β
E1
E3
E2
E2
E1α
E1β
92
68
43
31
20
14
1
2
3

When relatively high amounts of 2-oxoglutarate dehydrogenase complex were present in the immune replica (lane 4), bands corresponding to the components of PDC were clearly detected as well as the cross-reacting component E3. At higher levels of protein (lane 5) a weak cross-reaction with the 2-oxoglutarate decarboxylase component (E1) was observed. The lipoyl succinyltransferase component (E2), the structural core of the 2-oxoglutarate dehydrogenase complex (E2) was not detectable. Also, no cross-reaction was observed with the components of the bovine kidney branched-chain 2-oxo-acid dehydrogenase complex under these conditions.

It is currently accepted that component E3 is common to the branched-chain, pyruvate and 2-oxoglutarate dehydrogenase complexes (Guest, 1978). E3 is only loosely-associated with the branched-chain 2-oxo-acid dehydrogenase complex (Pettit et al.,1978) and is absent in most purified preparations of this complex (Fig. 4.5 (B), lane 3).

The detection of small contaminating amounts of PDC in the 2-oxoglutarate dehydrogenase complex preparation reveals the high sensitivity of the immunoblotting technique. Cross-contamination with pyruvate dehydrogenase complex was estimated as approx. 1.5%, very close to the value of 1% obtained from activity measurements.

Matuda et al,(1983) arrived at similar conclusions regarding the specificity of anti-PDC serum raised against the rat heart enzyme. They observed no immunological cross-reactions between the three 2-oxo-acid dehydrogenase complexes when examining the effects of anti-PDC serum on the enzymic activities of the complexes present in rat mitochondrial extracts.

4.5 Characterisation of subunit-specific antisera by immunoblotting analysis

Immunological detection of the dihydrolipoyl acetyltransferase subunit of PDC by using its subunit-specific antisera is shown in Fig. 4.6.

The antiserum showed a high specificity for subunit E2 and cross-reacted with the enzyme present in various tissues. It was also apparent that the rat enzyme exhibited a lower M_r value in comparison with the enzymes from bovine and porcine tissues. The variability in the M_r value for subunit E2 was previously observed with the antiserum to PDC (Fig. 4.4).

The antiserum showed a very high titre since only 10 ng of the complex were sufficient to detect the E2 component with 1:200 dilutions of the antiserum, under standard conditions.

No other protein in the cell extracts was recognised by these antibodies, which demonstrated the monospecificity of the antiserum to subunit E2. A similar analysis was performed by immunoblotting with the antiserum to subunit E1 α . The protein band detected in all the mammalian cells tested had an M_r value corresponding to the E1 α subunit. (Fig. 4.7). It was also observed that the antiserum appeared to react more strongly with the E1α subunit from rat cells than with the bovine cell line. Similar results were also obtained after immunoprecipitation of cell extracts from these cell lines (Chapter 8).

Fig. 4.8 (B) and (C) reveals that antisera to subunits E1 β and E3 possess high titre and specificity. The specificity for subunit E1 β was greatly increased after pre-adsorption of the antiserum to CNBr-activated Sepharose 4B to which subunit E2 had been bound covalently. The reasons for the presence of contaminating anti-E2 antibodies in

Fig. 4.6 Immunoblotting analysis of dihydrolipoyl acetyltransferase (E2) in various cell extracts

Purified PDC and various cell extracts were resolved by electrophoresis on a 10% (w/v) SDS/polyacrylamide slab gel. The gel was used for immunoblotting analysis with antiserum raised against subunit E2 (A). A parallel gel was stained to Coomassie Blue (B).

Lanes 1 and 5, PDC 0.1 μg protein in (A) and 12 μg protein in (B); (A and B); lane 2, SDS-extract of BRL cells, 80 μg and 60 μg; lane 3, BRL mitochondria, 40 μg; lane 4, rat liver mitochondria, 40 μg; lane 6, ox heart mitochondria, 15 μg and 50 μg; lane 7, SDS-extract of NBL-1 cells, 80 μg and 50 μg; lane 8, SDS-extract of PK-15 cells, 80 μg and 50 μg; lane 9, PK-15 mitochondria, 20 μg; lane 10, PK-15 cytosol, 60 μg; lane 11, ^{125}I-labelled M_r markers.

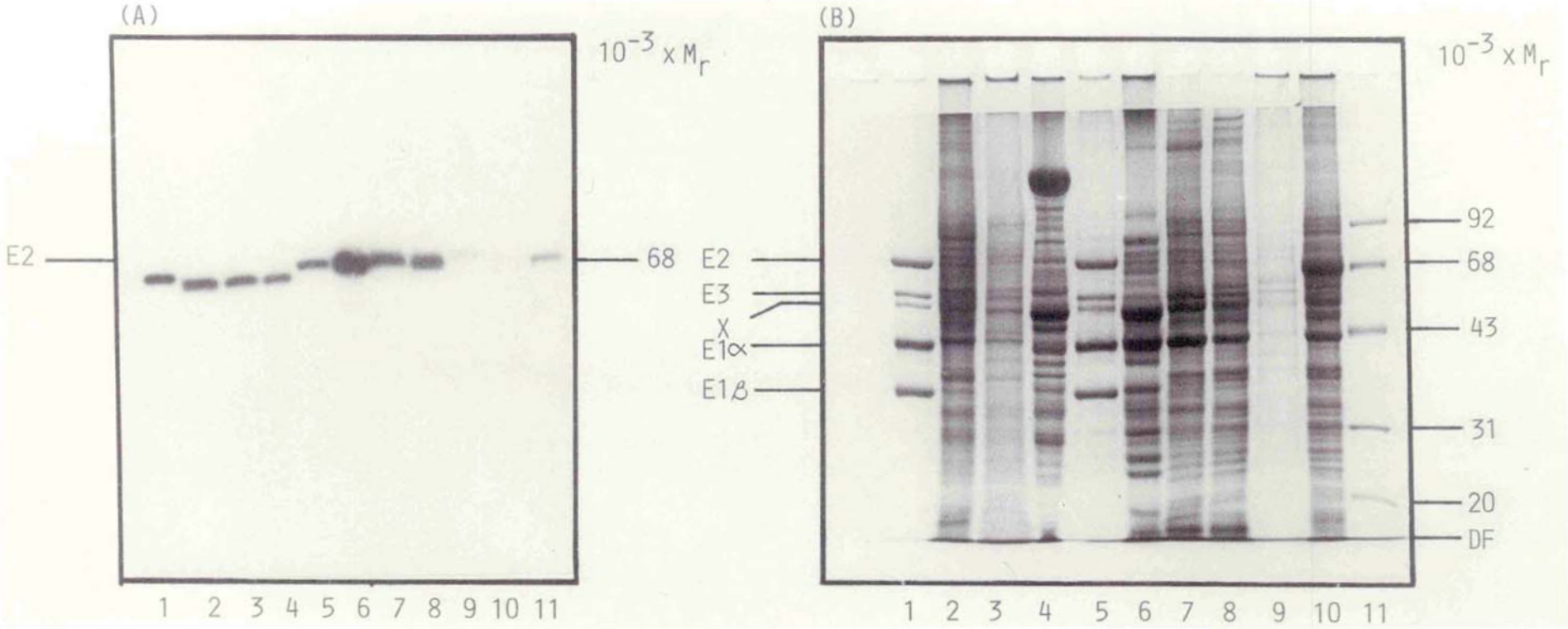
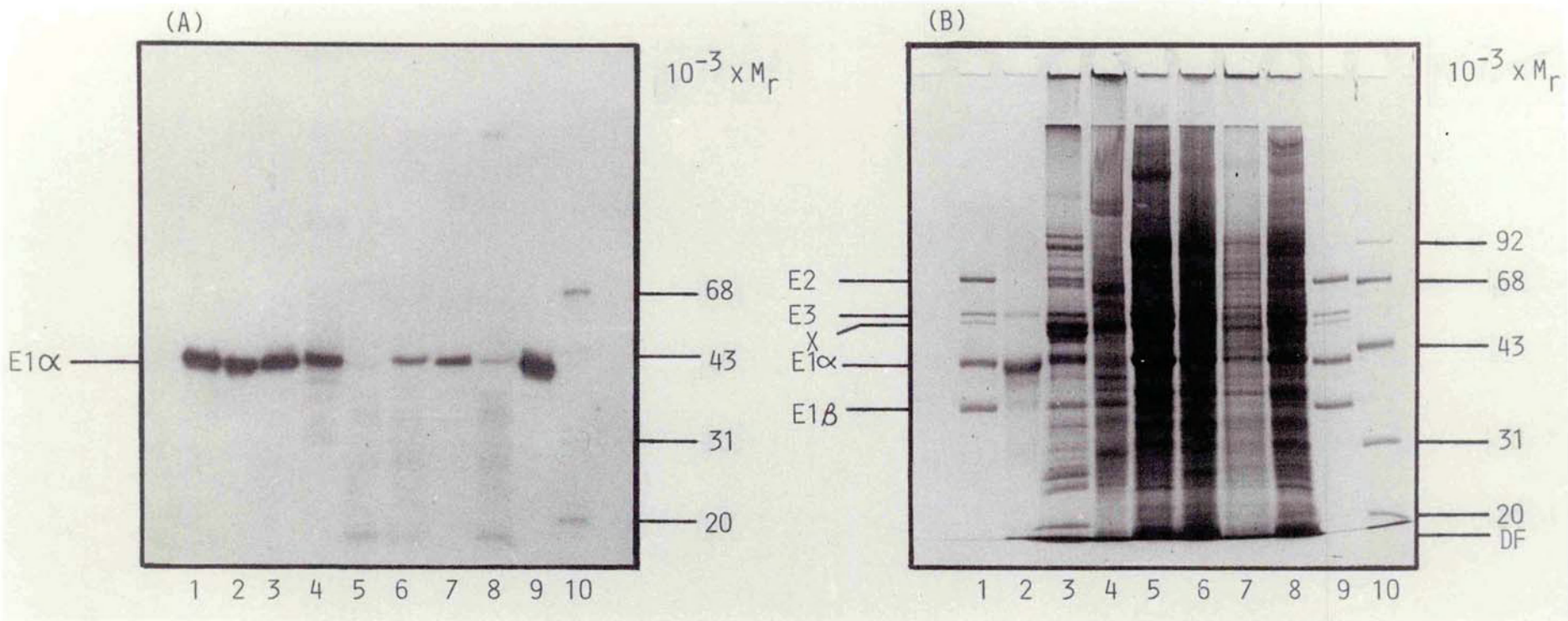

(A)
10^{-3} x M_r
E2
68
1 2 3 4 5 6 7 8 9 10 11
(B)
10^{-3} x M_r
E2
E3
X
E1α
E1β
92
68
43
31
20
DF
1 2 3 4 5 6 7 8 9 10 11

Fig. 4.7 Immunological detection of pyruvate dehydrogenase (E1) subunit α in various cell extracts

Samples of purified PDC and various cell extracts were electrophoresed on 10% (w/v) SDS/polyacrylamide slab gels. One half was utilized for the transfer of polypeptides onto nitrocellulose paper and subsequent immunoblotting analysis using antiserum raised against the subunit E1 α (A). The other half was stained with Coomassie Blue (B).

Lanes 1 and 9, PDC 1 μg protein in (A) and 12 μg in (B); (A and B), lane 2, partially purified subunit E1 α , 0.2 μg and 3 μg; lane 3, ox heart mitochondria, 40 μg; lane 4, rat liver mitochondria, 80 μg and 50 μg; lane 5, SDS-extract of NBL-1 cells, 90 μg and 70 μg; lane 6, BRL cells, 80 μg each; lane 7, BRL mitochondria, 60 μg; lane 8, SDS-extract of PK-15 cells, 120 μg and 80 μg; lane 10, ^{125}I-labelled M_r markers.


(A)
10^{-3} x M_r
E1α
68
43
31
20
1 2 3 4 5 6 7 8 9 10
(B)
10^{-3} x M_r
E2
E3
X
E1α
E1β
92
68
43
31
20
DF
1 2 3 4 5 6 7 8 9 10

this antiserum could only be explained by the presence of small amounts of an E2 fragment in the SDS-denatured antigen. As discussed by Suissa & Reid (1983), the use of gel electrophoresis for purifying antigenic proteins has some disadvantages. Although a single band is observed after electrophoresis on polyacrylamide gels, a serum raised against this preparation may react with a higher M_r protein. This can result from the presence of fragments derived from the larger polypeptide which co-migrate with the band of protein of interest.

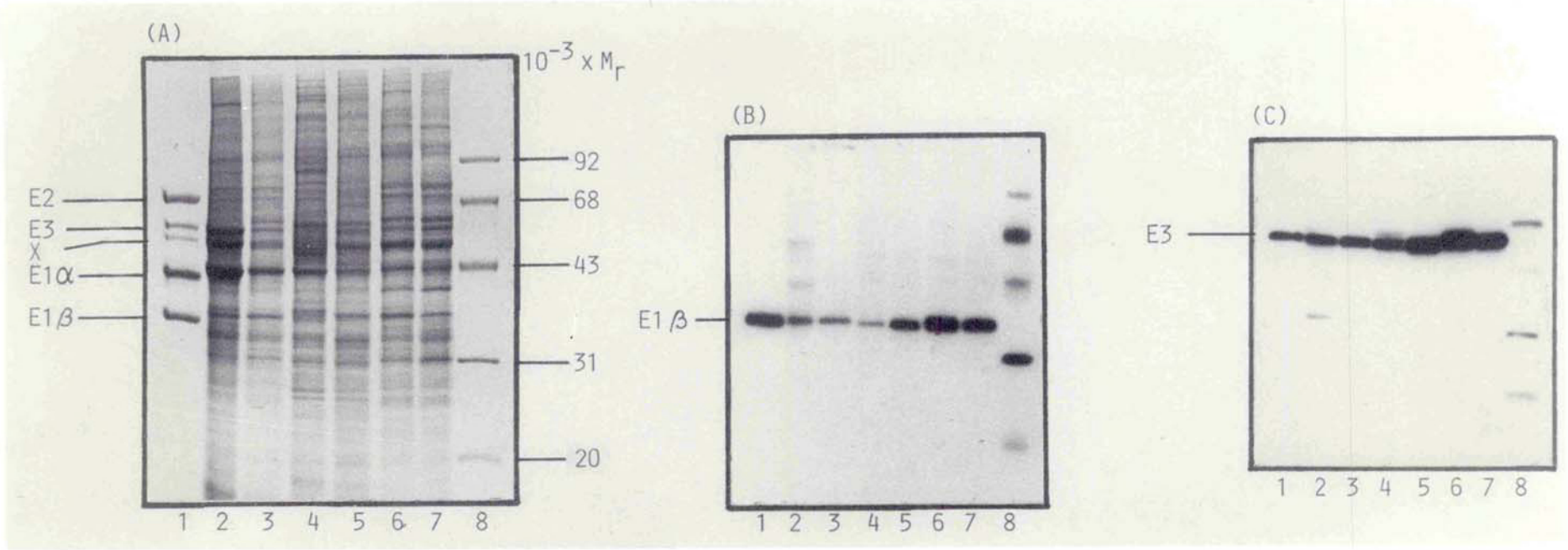
Studies on the structure of bovine heart dihydrolipoyl acetyltransferase indicated that the enzyme can be cleaved by trypsin-like proteases into fragments of apparent M_r 36,000 and 26,000. The former fragments contains the lipoyl domain and the latter, the subunit-binding domain. The lipoyl domain is acidic and contains an unusually high proportion of prolyl residues (Kresze & Ronft, 1980; Bleile et al., 1981). This peptide co-migrates with the E1 β subunit and, as discussed in Chapter 7 (section 7.6) seems very immunogenic. Therefore, it is probable that small amounts of a fragment of similar M_r value to the E1 β subunit derived from the E2 polypeptide were present in the preparation used for immunisation against the E1 β subunit. This contamination would explain the presence of unwanted anti-E2 specificity in the antiserum. In spite of this difficulty the application of the immunoadsorption technique helped to remove the contaminating anti-E2 antibodies and rendered the antiserum monospecific for subunit E1 β . Experimental details are provided in section 2.2.12.h.

Fig. 4.8 (B) shows that only a single band was detected in the cell extracts from various cell types by immunoblotting analysis with the antiserum to E1 β , with a M_r value identical to subunit E1 β in all cells. In Fig. 4.8 (C), it is clear that small differences in M_r exist between the E3 subunit from various mammalian cells. However,

Fig. 4.8 Immunological detection of PDC subunits E1 β and E3 in various cell extracts

Samples of purified PDC and cell extracts from cultured cells were subjected to electrophoresis on a 10% (w/v) SDS/polyacrylamide gel. Proteins resolved were transferred onto nitrocellulose paper and employed for immunoblotting analysis with antisera to the E1 β subunit (B) or to the E3 subunit (C). A similar gel was stained with Coomassie Blue (A).

(A,B and C) Lane 1, PDC, 12 μg, 0.25 μg and 1 μg protein, respectively; lane 2, SDS-extract of NBL-1 cells, 60 μg, 40 μg and 40 μg; lane 3, NBL-1 mitochondria, 50 μg, 40 μg and 40 μg; lane 4, SDS-extract of BRL cells, 60 μg each; lane 5, BRL mitochondria, 50 μg each; lane 6, SDS-extract of PK-15 cells, 60 μg each; lane 7, PK-15 mitochondria, 50 μg each; lane 8, ^{125}I-labelled M_r standards.


(A)
10^{-3} x M_r
E2
E3
X
E1α
E1β
92
68
43
31
20
1 2 3 4 5 6 7 8
(B)
E1β
1 2 3 4 5 6 7 8
(C)
E3
1 2 3 4 5 6 7 8

there was no evidence for the existence of multiple forms of this enzyme. Sakurai et al. (1970) suggested that the multiple forms of lipoamide dehydrogenase detected by some authors on DEAE-cellulose chromatography could be explained as a result of proteolytic modification of the enzyme and not as physiological isoenzymes. Alternatively, it is also possible that the antiserum cross-reacted with only one form of the enzyme (see also Chapter 1, section 1.3.6).

4.6. Cross-reactivity of antisera to ox heart PDC with the complex from yeast (S. cerevisiae).

PDC has been purified from the yeasts Saccharomyces cerevisiae (Kresze & Ronft, 1981a, 1981b) and S. carlbergensis (Keha et al., 1982).

The baker's yeast complex is similar in size, S_{20w} 77S, to the mammalian enzyme (pig heart 67.5S, Koike & Koike, 1976; bovine heart 90S, Linn et al., 1972). This indicates an M_r of $7\text{-}8 \times 10^6$ (Kresze & Ronft, 1981b). The complex possesses icosahedral symmetry suggesting a core structure of 60 subunits as in the mammalian complex. The yeast complex has been purified to homogeneity and the polypeptide pattern examined after SDS/polyacrylamide gel electrophoresis. M_r values have been assigned as: 58,000 (E2), 53,000 (E3), 45,000 (E1 α) and 35,000 (E1 β). A minor band, M_r 50,000, was also found. This polypeptide behaved in a analogous manner to the minor component (X) detected in purified PDC from mammalian tissues (Kresze & Ronft, 1981b).

The yeast complex differs from the complexes from gram-negative bacteria such as Escherichia coli, which contain one large E1 chain instead of two subunits and resembles gram-positive bacteria such as Bacillus stearothermophilus and B. subtilis (Henderson & Perham, 1980). However, there is no evidence for regulation by covalent modification (phosphorylation) of the E1 α subunit as occurs in the mammalian and N. crassa enzymes (Kresze & Ronft, 1981a).

Fig. 4.9 illustrates the peptide pattern of the isolated yeast PDC components, obtained as a gift from Dr. H. Bisswanger (Universität Tübingen, W. Germany). E2 showed a main band at 58,000 as well as many fragments of smaller size, presumably resulting from proteolysis of the protein during isolation or transit to the U.K., since most of them cross-react with the anti-PDC serum (lanes 2 and 3). Subunit E3 was similar in size to the mammalian enzyme (lane 10), although it also reacted poorly with this antiserum (lanes 4 and 5). Component E1 showed extensive degradation of the E1 α subunit, since an approx. 1:1 ratio between the α and β chains should be expected in the Coomassie Blue pattern. A strong cross-reaction was found with the E1 β subunit. No cross-reaction with the E1 α subunit was detected, even when relatively large amounts of protein were present in the immune replica (lane 7).

The low cross reactivity between the E1 α subunit from yeast and mammalian complex was confirmed by loading up to 10 μg of E1 protein on the gels and carrying out the immunoblotting analysis with anti-E1 α serum (results not shown).

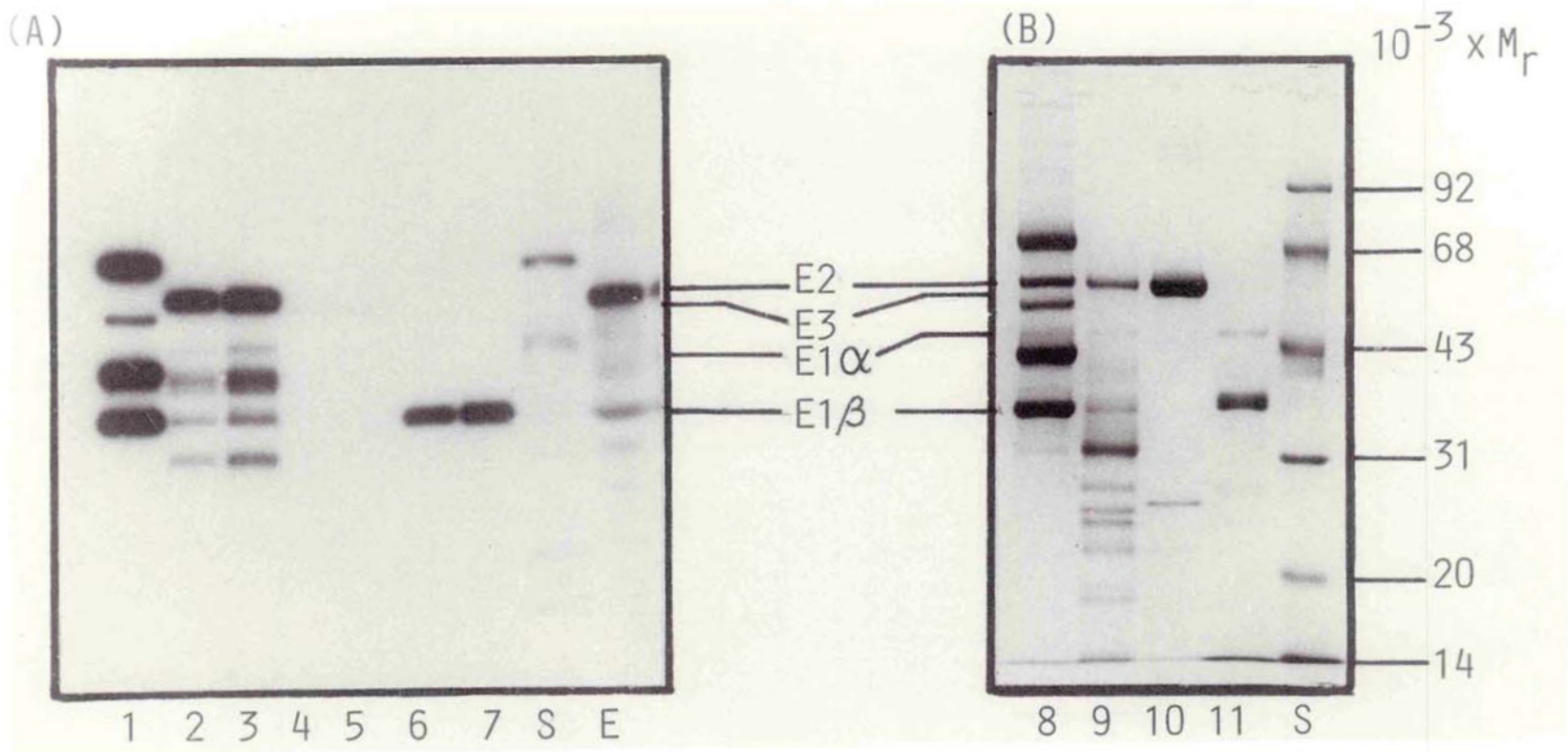
Fig. 4.10 (A) lane E indicates that only subunits E2 and E1 were detected in whole yeast cell extracts. Since the titre against subunit E3 in the anti-PDC serum was very low (this Chapter, section 4.2), the cross-reactivity of the E3 polypeptide was also evaluated by immunoblotting analysis using the subunit-specific antiserum. The immunoblots indicated strong cross-reaction with the purified lipoamide dehydrogenase from yeast and with the enzyme present in yeast mitochondria (results not shown).

Fig. 4.9 Immunological cross-reaction of antiserum to ox heart PDC with the isolated components of the yeast complex

Samples of purified ox PDC and components of the S. cerevisiae complex were electrophoresed onto 10% (w/v) SDS/polyacrylamide slab gels. One half was employed for immunological detection with anti-PDC serum (A). Bound antibodes were detected by autoradiography after incubation with ^{125}I-labelled protein A as described in section 2.2.12.e.. The parallel gel was stained with Coomassie Blue (B).

Lanes 1 and 8, ox heart PDC 0.2 μg and 12 μg, respectively, lanes 2, 3 and 9, yeast dihydrolipoyl acetyltransferase component (E2), 1 μg, 2 μg and 3 μg, respectively; lanes 4, 5 and 10, yeast lipoamide dehydrogenase (E3), 1 μg, 2 μg and 2.5 μg, respectively; lanes 6, 7 and 11, yeast pyruvate dehydrogenase component (E1), 2 μg, 4 μg and 5 μg, respectively; lane S, M_r standards; lane E, yeast total cell extract, 100 μg.

E2, E3, E1 α and E1 β , refer to the component polypeptides of the yeast PDC.


(A)
(B)
10^{-3} x M_r
92
68
43
31
20
14
E2
E3
E1α
E1β
1 2 3 4 5 6 7 S E
8 9 10 11 S

In summary, by immunoblotting analysis with various antisera it has been shown that there is strong immunological cross-reactivity between the yeast and the mammalian PDC, particularly for the subunits E2, E3 and E1 β . These findings suggest that the complexes from lower and higher eukaryotes share many structural features and present similar antigenic determinants which have been highly-conserved during evolution.

The lack of reactivity of the antisera to PDC and E1 α against the E1 α subunit of the yeast enzyme is an interesting observation. The yeast polypeptide is only slightly larger than its mammalian counterpart, however, is not subjected to regulation by phosphorylation/ dephosphorylation. It is possible to speculate that this protein has evolved considerably from the lower to higher eukaryotes to become part of a system responsible for regulation of the complex which involves interactions with additional enzymes.

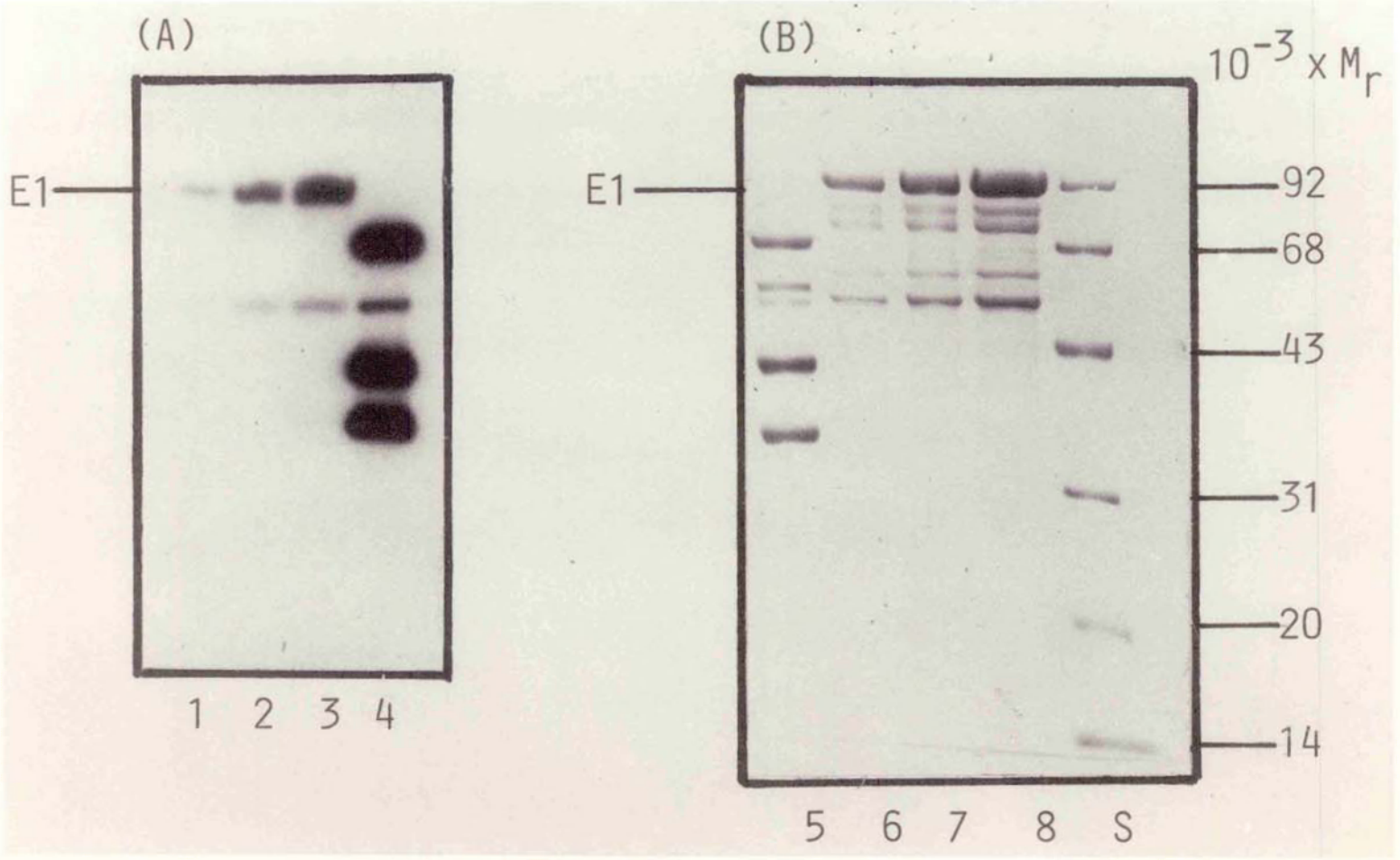
4.7 Cross-reactivity of anti-PDC serum with E. coli complex

Bacterial PDC are also composed of three constituent enzymes, but there are considerable differences in the molecular organisation of the complexes from various sources. In gram-negative bacteria as in E. coli and Azotobacter vinelandii (Reed, 1974; Bosma et al., 1984), the complex consists of three polypeptide chains; the largest, M_r 89,000-110,000 contains the pyruvate dehydrogenase activity, whereas the other two contain the acetyltransferase activity (subunit M_r 65,000-66,000) and the lipoamide dehydrogenase activity (subunit M_r 56,000) respectively. In contrast, complexes from mammals, birds, yeast and gram-positive bacteria possess E1 components composed of two subunits (α and β).

Fig. 4.10 Reactivity of antiserum to ox heart PDC with the E. coli complex

Samples of purified PDC from ox heart and E. coli were electrophoresed on 10% (w/v) SDS/polyacrylamide slab gels. Half of the gel was employed for immunoblotting analysis with anti-PDC serum (A), the other half was stained with Coomassie Blue (B). Lanes 1 and 6, E. coli PDC, 1 μg protein each; lanes 2 and 7, E. coli PDC, 5 μg; lanes 3 and 8, E. coli PDC, 10 μg; lanes 4 and 5, ox heart PDC, 0.2 μg and 12 μg protein respectively. S, M_r markers.

E1 refers to the component enzyme of the E. coli PDC.


(A)
E1
1 2 3 4
(B)
10^{-3} x M_r
E1
92
68
43
31
20
14
5 6 7 8 S

It seemed interesting to explore whether there was immunological cross-reaction between the mammalian complex and the enzymes from a gram-negative bacterium. Samples of *E. coli* PDC were obtained as a gift from Dr. J. Coggins in this Department.

Fig. 4.10 (B) shows the polypeptide pattern of the sample when examined by SDS/polyacrylamide gel electrophoresis. The complex was degraded and a number of fragments were observed in the M_r 56,000-95,000 region, which made it impossible to identify conclusively the components E2 and E3. However, immunoblotting analysis with anti-PDC serum showed strong cross-reaction of the E1 component. As little as 1 µg of the *E. coli* PDC protein could be detected with a 1:100 dilution of the antiserum.

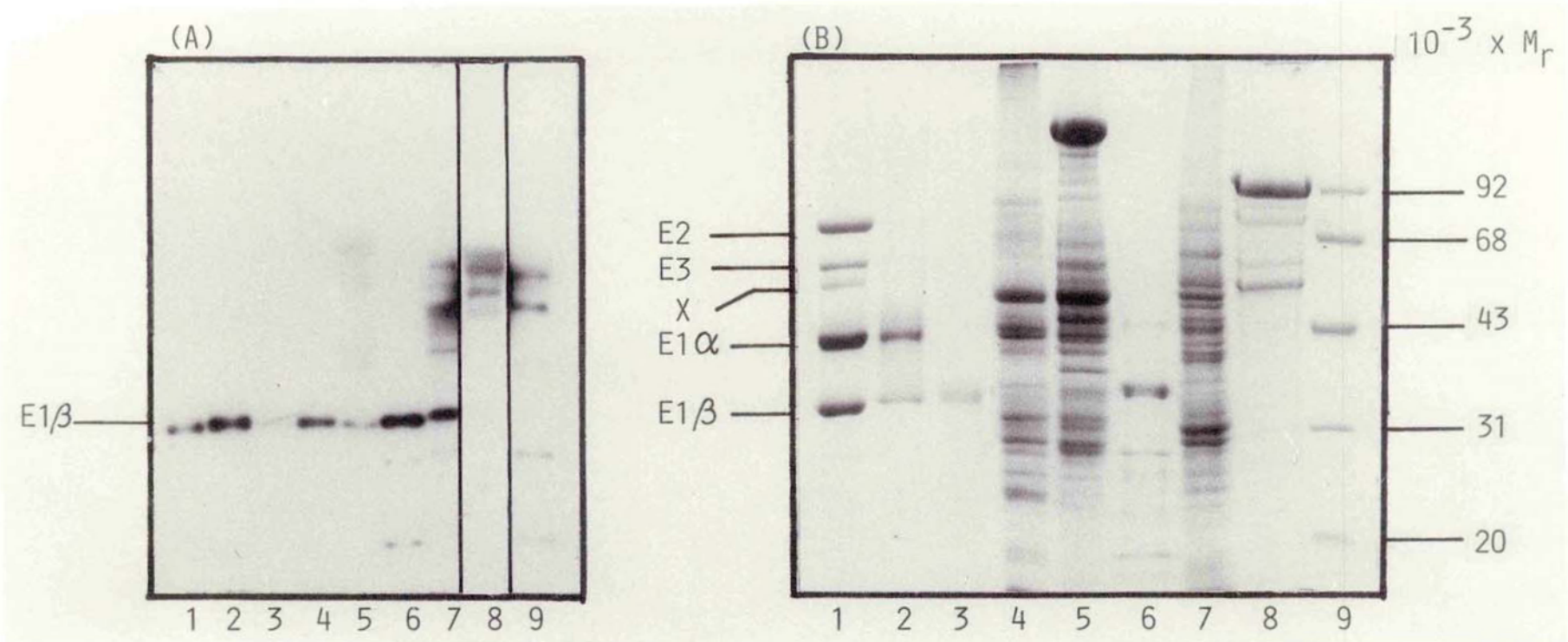
Samples of the yeast components and *E. coli* PDC were immunoblotted against the antiserum to subunit E1 β . Fig. 4.11 shows that subunit E1 β can be detected in rat liver, ox heart and yeast mitochondria as a band of identical M_r. In the *E. coli* enzyme, the antiserum cross-reacts with a bands of M_r 95,000 and another at M_r 50,000, probably corresponding to a fragment of E1. This result seems to indicate that most of the strong cross-reaction observed on the *E. coli* PDC with the anti-PDC serum (Fig. 4.10) is indeed due to the E1 β component. Conclusive data must await the availability of a highly-purified and undegraded *E. coli* PDC.

Fig. 4.11 Cross-reactivity of yeast and E. coli E1 components with antiserum to ox heart subunit E1 β

Purified samples of E. coli PDC, ox heart PDC and subunit E1 β and yeast E1 component were electrophoresed on 10% (w/v) SDS/polyacrylamide slab gels. Half of the gel was employed for transferring resolved proteins onto nitrocellulose paper and subsequent immunoblotting analysis with antiserum to E1 β subunit (A). The parallel gel was stained with Coomassie Blue (B).

(A and B) Lane 1, PDC, 0.25 μg and 10 μg protein, respectively; lane 2, ox heart E1 component, 0.1 μg and 6 μg; lane 3, ox heart E1 β subunit, 0.01 μg and 3 μg; lane 4, ox heart mitochondria, 10 μg and 40 μg; lane 5, rat liver mitochondria, 25 μg and 40 μg; lane 6, yeast E1 component, 2.5 μg and 6 μg; lane 7, yeast mitochondria, 15 μg and 35 μg; lane 8, E. coli PDC, 5 μg and 15 μg; lane 9, ^{125}I-labelled M_r markers.

Track 8 was exposed for a shorter length of time as compared with the other lanes.


(A)
E1β
1 2 3 4 5 6 7 8 9
(B)
10⁻³ x Mr
E2
E3
X
E1α
E1β
92
68
43
31
20
1 2 3 4 5 6 7 8 9

Chapter 5: STUDIES ON THE SUBMITOCHONDRIAL LOCALISATION OF PYRUVATE DEHYDROGENASE COMPLEX BY USING IMMUNOLOGICAL TECHNIQUES

5.1 Introduction

In addition to the well-known multienzyme aggregates that catalyse the oxidation of 2-oxo-acids and fatty acids, many studies indicate that other weak reversible associations exist between metabolically-related enzymes. These transient complexes include the 'soluble' enzymes of the glycolytic pathway and the citric acid cycle enzymes as well as enzymes from other metabolic routes. The existence of these associations were initially based on predictions that closer spatial relationship between the enzymes of a metabolic sequence allowed metabolic "chanelling", resulting in higher efficiencies in the oxidation of substrates.

In recent years, many metabolically significant interactions have been demonstrated between enzymes and between proteins and specific membranes (reviewed by Srere, 1984).

The high protein concentration of the mitochondrial matrix (over 50%) can markedly affect these weak protein interactions, which may have important consequences for the regulation of energy metabolism and in the biogenesis of mitochondria. Srere (1982) has presented a scheme for the hypothetical distribution of proteins in the inner membrane-matrix compartment of rat heart mitochondria, in which most matrix proteins would be adjacent (or perhaps also bound) to the inner membrane. Experimental evidence supporting this model can be summarised as follows:

1) Studies with immobilised enzymes, where several (2-3) metabolically-related enzymes acting consecutively are trapped within the pores of

Sepharose or polyacrylamide matrices (Srere et al., 1973) or cross-linked with glutaraldehyde (Koch-Schmidt et al., 1977), indicated that such systems exhibited catalytic efficiencies several fold higher than that measured with the same amounts of enzymes acting free in solution.

These results have been interpreted in terms of the closer proximity of the enzymes participating in the reaction and/or to the increase of concentration of intermediate around the enzymes resulting from restricted diffusion out of the gel phase (microenvironmental compartmentation).

2) Histochemical and electron microscopic studies of several matrix enzymes e.g. citrate synthase (Srere, 1980) and PDC (Nestorescu et al., 1973) indicated that these enzymes are preferentially associated with the inner face of the inner mitochondrial membrane.

3) The existence of physical interactions between consecutive enzymes of the citric acid cycle and malate-aspartate shuttle have been reported (Srere et al., 1973; Beeckmans & Kanarek, 1981).

4) Recent reports from the laboratory of Srere have shown that enzymes of the β-oxidation pathway, fumarase (mitochondrial), malate dehydrogenase (mitochondrial) and citrate synthase can bind to inverted inner membrane vesicles in a specific manner. The 'receptor' or binding sites seem to be located on the matrix side of the inner mitochondrial membranes. (D' Souza & Srere, 1983; Sümegi & Srere, 1984a).

5.2 Aims of study

The mitochondrial localisation of mammalian PDC has been long established, since the complex can be purified from disrupted mitochondria. (Linn et al., 1972; Cooper et al., 1974), but the difficulty of solubilising the heart complex suggested that it may be bound to the inner membrane (Addink et al., 1972; Pettit & Reed, 1982). Complete solubilisation of

the complex and higher yields were achieved by the inclusion of Triton X-100 in the extraction buffer. This result is consistent with the idea that most PDC activity is bound to the inner mitochondrial membranes (Stanley & Perham, 1980).

Nestorescu et al. (1973) have presented evidence by histochemical methods that PDC activity is associated with the inner membrane of rat heart mitochondria.

By using a variety of procedures for extraction of PDC from pig heart mitochondria, Kenney et al.(1972) concluded that the heart complex appeared to be either in close proximity to the inner membrane or became strongly associated with it on breaking the mitochondria. At present, conclusive evidence on the localisation of PDC within the mitochondrial compartment and its mode of interaction with the inner membrane has not yet been established unequivocally.

It was the aim of these studies to gain additional information on this topic by using immunoblotting as an analytical tool, which permits the detection of small amounts of several mitochondrial proteins present in the various submitochondrial compartments by means of monospecific antisera. The distribution of PDC was compared with that of two 'typical' matrix enzymes: fumarase (mitochondrial) and citrate synthase.

A reasonable hypothesis was that PDC could be associated specifically with the inner mitochondrial membrane to allow "channelling" of NADH directly into the electron transport chain. There was also the question of whether the M_r 50,000 polypeptide (component X) was an integral protein involved in specific binding of PDC to the inner membrane which was released with the complex during its purification.

5.3 Studies on the submitochondrial localization of PDC in rat liver

Preliminary studies showed that PDC components could be detected in both matrix and inner membrane fractions isolated from rat liver mitochondria. These studies were extended in order to verify that this result was not caused by cross-contamination between the fractions.

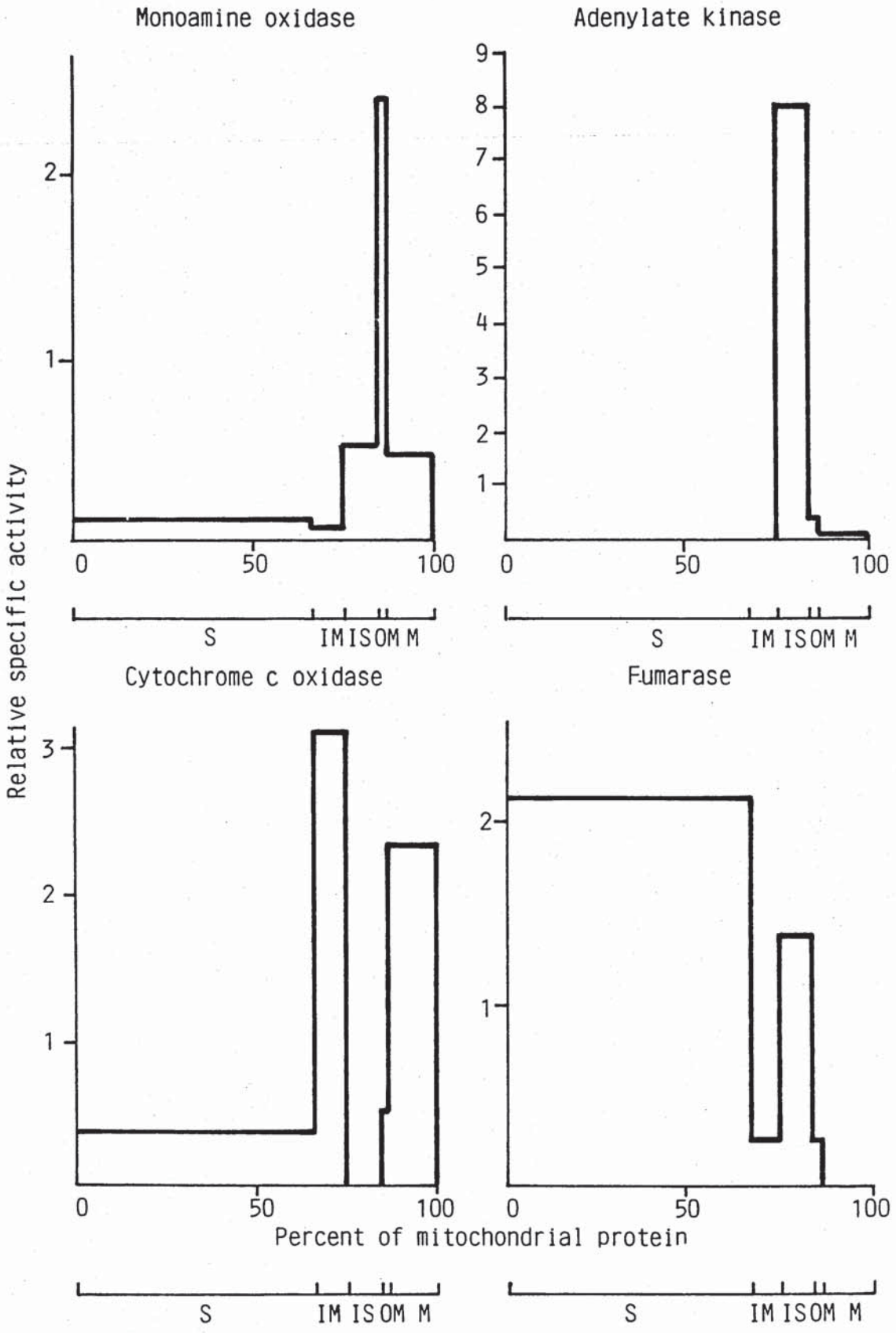
Rat liver mitochondria were purified and fractionated into matrix or soluble, inner membrane, intermembrane space and outer membrane fractions, and a fraction containing mixed membranes by following the procedures described in sections 2.2.7.a and 2.2.7.b.

The distribution of marker enzymes in the subfractions obtained is presented in Fig. 5.1. Recoveries of enzymic activity ranged from 80-101% for each marker. Most of the adenylate kinase activity was recovered in the intermembrane space fraction, which was also contaminated with matrix as shown by the distribution of fumarase activity.

The pattern of distribution of marker enzymes is very similar to that reported previously by Sottocasa et al. (1967) using the same procedure. The small cross-contamination observed between membrane fractions may be explained at least partially, by the existence of "membrane junctions" which have been detected as contact points between the outer and inner mitochondrial membranes in many types of mitochondria. Contamination of inner membranes with matrix may be attributed to entrapment of matrix material into membrane vesicles formed during fractionation or possibly to the existence of matrix polypeptides interacting strongly with the inner face of the inner membrane. Attempts to reduce the contamination of matrix in the intermembrane space fraction by increasing the tonicity of the medium used for swelling the mitochondria helped to reduce the matrix contamination but also led to lower recoveries of adenylate kinase activity.

Fig. 5.1 Distribution of some marker enzymic activities in rat liver submitochondrial fractions

Rat liver mitochondria were fractionated by differential centrifugation into an outer membrane fraction (OM), and intermembrane space fraction (IS) and a pellet containing mitoplasts and unbroken mitochondria. This pellet was sonicated and separated by sucrose-denisty gradient centrifugation into a soluble fraction (S), a membrane fraction (M) containing mixed outer and inner membrane vesicles and an inner membrane fraction (IM) (see section 2.2.7). Enzymic activities were assayed as indicated in section 2.2.8. The ordinates represent relative specific activities of monoamine oxidase, adenylate kinase, fumarase and cytochrome c reductase on a protein basis. The specific activities of the purified mitochondria (sonicated prior to assay) were taken as 1. The abscissas indicate the percentages of total mitochondrial protein recovered in each fraction.


Monoamine oxidase
Adenylate kinase
Cytochrome c oxidase
Fumarase
Relative specific activity
Percent of mitochondrial protein
0
50
100
S
IM
IS
OM
M

Although some contamination between the mitochondrial compartments was unavoidable, the distribution of marker enzymes showed that these fractions were appropriate for studies on the submitochondrial location of PDC in rat liver.

The polypeptide composition of the purified fractions is shown in Fig. 5.2 (B). The distribution of 'typical' matrix markers was checked after electrophoresis and immunoblotting analysis of the fractions. Fig. 5.2 shows the results obtained after immunoblotting with anti-fumarase serum (1A) and anti-citrate synthase serum (2A).

Bands corresponding to the M_r for the subunits of these proteins were detectable in the mitochondrial matrix and more weakly in the intermembrane space fraction (lanes 3 and 5) as well as in the whole mitochondrial fraction (lane 2). Similar bands were not detected in the inner or outer membrane fractions (lanes 4 and 6).

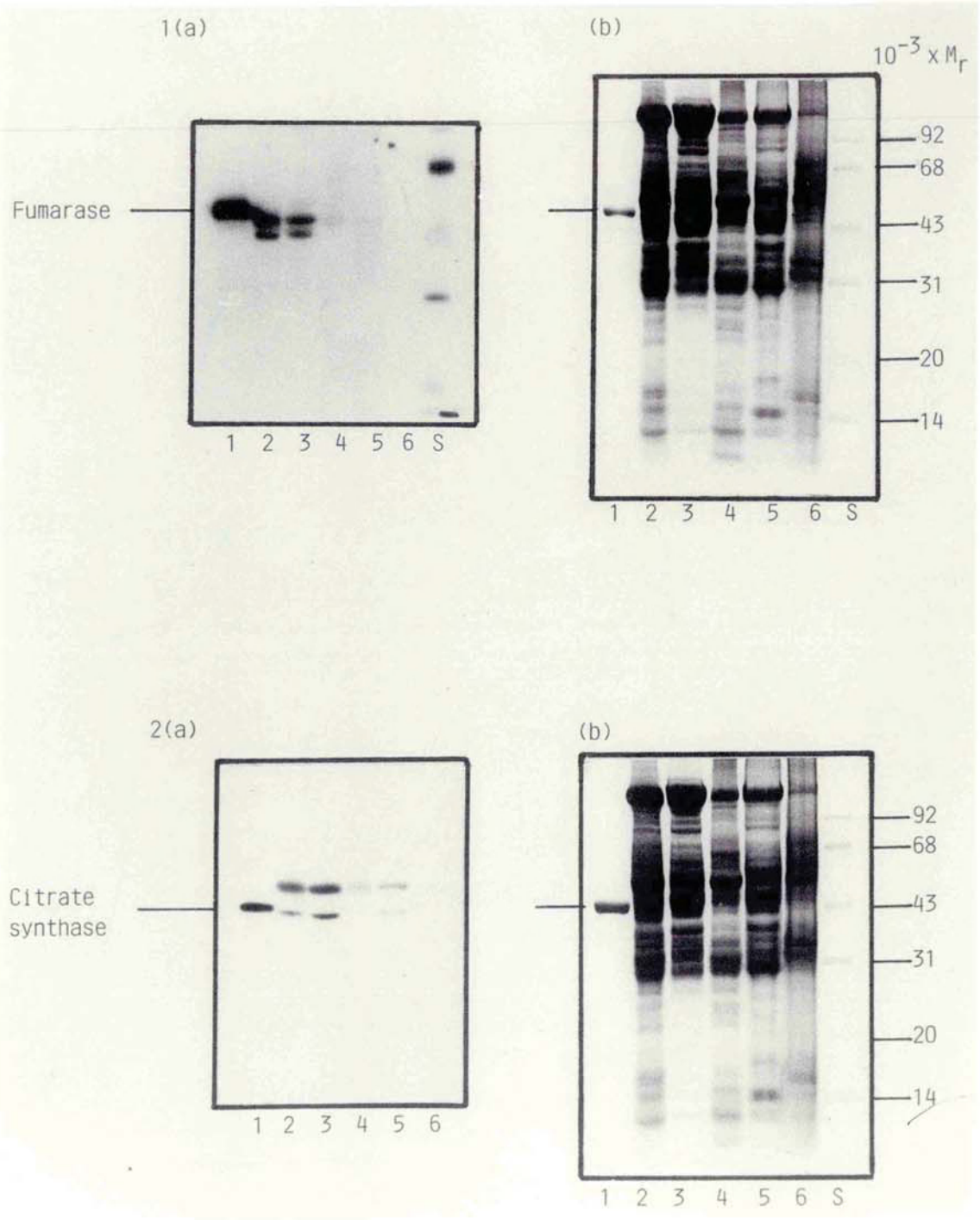
Immunoblots with the anti-fumarase serum also showed a band at lower M_r values. This protein was also present in the commercially-purified pig heart fumarase preparation utilised as antigen. The protein can be detected in Coomassie Blue-stained gels when $\geq 5\,\mu g$ protein are loaded.

Anti-citrate synthase serum also showed the presence of a cross-reacting protein at higher M_r values (approx. 55,000). In this case, no bands of similar electrophoretic mobility could be detected in the purified sample utilised for immunisation when $10\,\mu g$ protein were loaded on a gel. It also seems unlikely that this reaction is due to lack of specificity of the antiserum because the extra band was not detected by immunoblotting analysis in bovine kidney cell extracts (Fig. 5.6, lanes 8, 9 and 10). However, it was found in extracts from cultured rat liver cells (not shown). The enzyme from rat liver is a dimer of two identical subunits M_r 48,000 (Moriyama & Srere, 1971) and no

Fig. 5.2 Immunological detection of fumarase and citrate synthase in rat liver submitochondrial fractions

Fumarase and citrate synthase were electrophoresed with rat liver mitochondria subfractions on 10% (w/v) SDS/polyacrylamide slab gels. Proteins were transferred onto nitrocellulose paper for the immunological detection with anti-fumarase (1A) or anti-citrate synthase (2A) sera. Coomassie Blue pattern of the gels are also shown (1B and 2B, respectively).

(1A and 1B) Lane 1, fumarase, 0.2 µg and 4 µg protein, respectively; (2A and 2B) lane 1, citrate synthase, 0.2 µg and 3 µg; (1A, 1B, 2A and 2B) lanes 2, 3, 4, 5 and 6 contain 50 µg of each, rat liver mitochondria, and fractions S, IM, IS and OM, respectively; S, ^{125}I-labelled M_r markers.


1(a)
(b)
$10^{-3} \times M_r$
Fumarase
92
68
43
31
20
14
1 2 3 4 5 6 S
1 2 3 4 5 6 S
2(a)
(b)
Citrate synthase
92
68
43
31
20
14
1 2 3 4 5 6
1 2 3 4 5 6 S

isoenzymes had been described in this tissue. At present, there is no satisfactory explanation for these results.

When similar amounts of protein from the various fractions obtained were loaded on a gel and analysed by immunoblotting with anti-PDC serum, (Fig. 5.3, A), components E2, X, E1 α and E1 β were detected in both matrix and inner membrane fractions (lanes 3 and 4). Small amounts of the subunits E1 α and E1 β were also seen in the intermembrane space fraction. This can be attributed to matrix contamination in the inter-membrane space fraction as discussed previously.

No reaction was detected with the outer membrane fraction (lane 6). A closer inspection of the immunoblot shown in Fig. 5.3 (A) revealed differences in the relative intensities of the individual subunits of matrix and membrane-bound PDC. A higher proportion of components E2 and X as compared to E1 α and E1 β were observed in the membrane-bound PDC. This result may reflect a higher content of components E2 and X in the membrane bound PDC or to a preferential degradation of these subunits in the soluble form. It was mentioned previously (Chapter 3, section 3.1) that rat liver mitochondria contain a lysosomal protease or "inactivase" that selectively cleaves the transacetylase core of the complex.

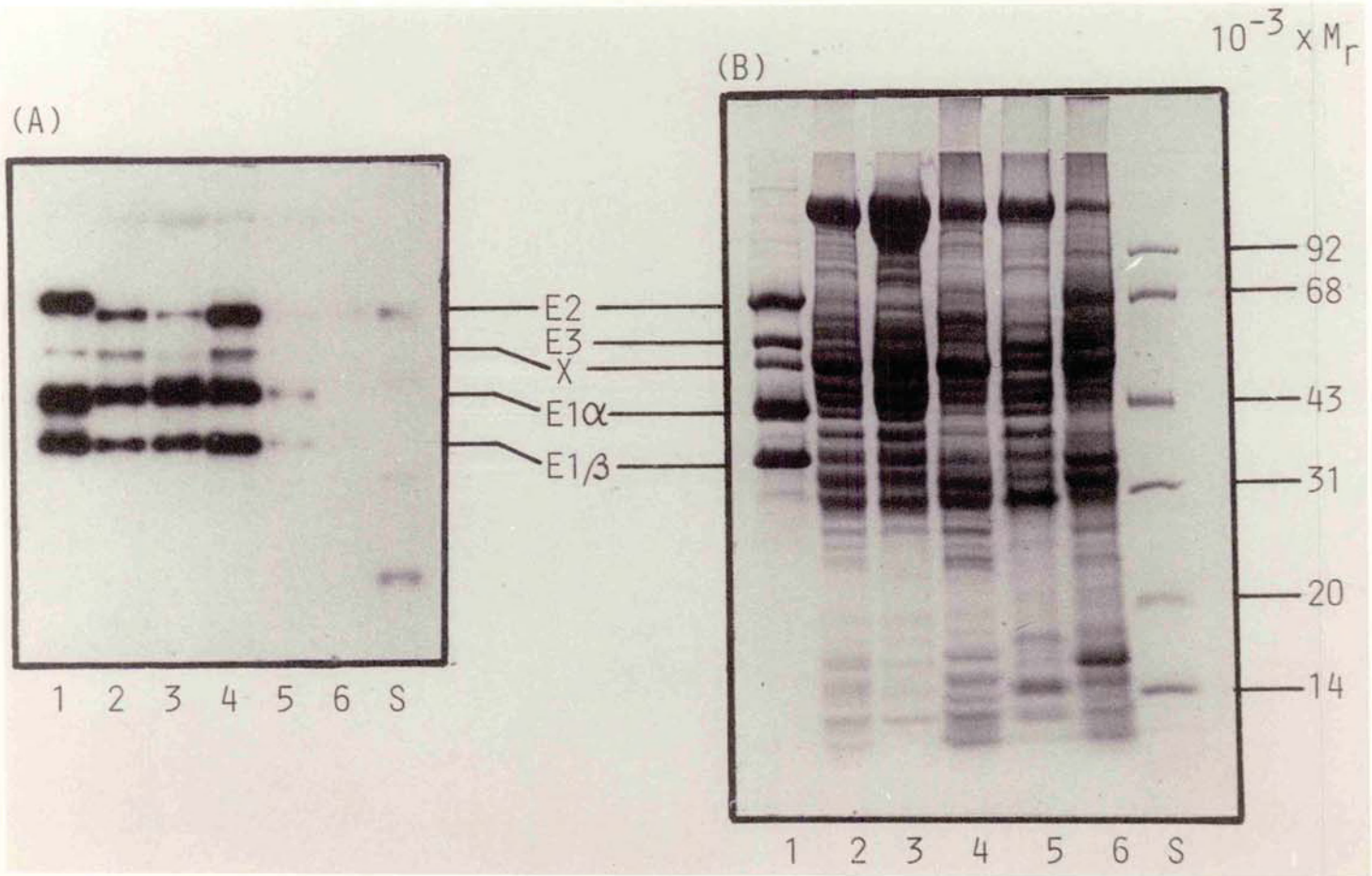
Another possibility was that sonication promoted the preferential release of subunits E1 and E3 from the core enzyme.

The amounts of PDC that can be detected in the soluble and inner membrane fractions seemed to be similar in terms of µg PDC/µg protein in each fraction. However, the amount of protein present in the soluble fraction is about 3 times higher than the amount that corresponds to the inner membrane (Sottocasa et al., 1967). Hence, in rat liver, the distribution of PDC seems to predominantly in the soluble fraction and only 30% of the enzyme may be bound to the inner membrane under these

Fig. 5.3 Immunological detection of PDC polypeptides in rat liver submitochondrial fractions

Samples of purified PDC and submitochondrial fractions from rat liver were electrophoresed in duplicate on 10% (w/v) polyacrylamide gels. Part of the gel was utilized for the detection of antigen proteins with anti-PDC serum (A). The other half was stained with Coomassie Blue (B).

(A and B) Lane 1, PDC 0.5 μg and 14 μg protein, respectively; lane 2, rat liver mitochondria, 50 μg each; lanes 3, 4, 5 and 6 contain 50 μg protein from fractions S, IM, IS and OM, respectively; lane S, ^{125}I-labelled M_r standards.


(A)
(B)
10^{-3} x M_r
E2
E3
X
E1α
E1β
92
68
43
31
20
14
1 2 3 4 5 6 S
1 2 3 4 5 6 S

conditions. A similar study was carried out in ox heart mitochondria.

5.4. Studies on the submitochondrial location of PDC in ox heart

For these experiments ox heart mitochondria were purified as described by Smith (1967). After sonication, mitochondria were fractionated into membrane and soluble fractions. The membrane pellets were extensively washed as described in section 2.2.7.c. and the washings reserved for analysis.

As shown in Fig. 5.4, samples from the membrane, soluble and washing steps fractions were analysed by SDS/polyacrylamide gel electrophoresis. The polypeptide patterns obtained (Fig. 5.4.A) from soluble and membrane fractions were different. The first washing showed essentially the same polypeptide pattern as the soluble fraction, but the second washing contained very few components and showed a main band at M_r 55,000. (Fig. 5.4 B, lane 6).

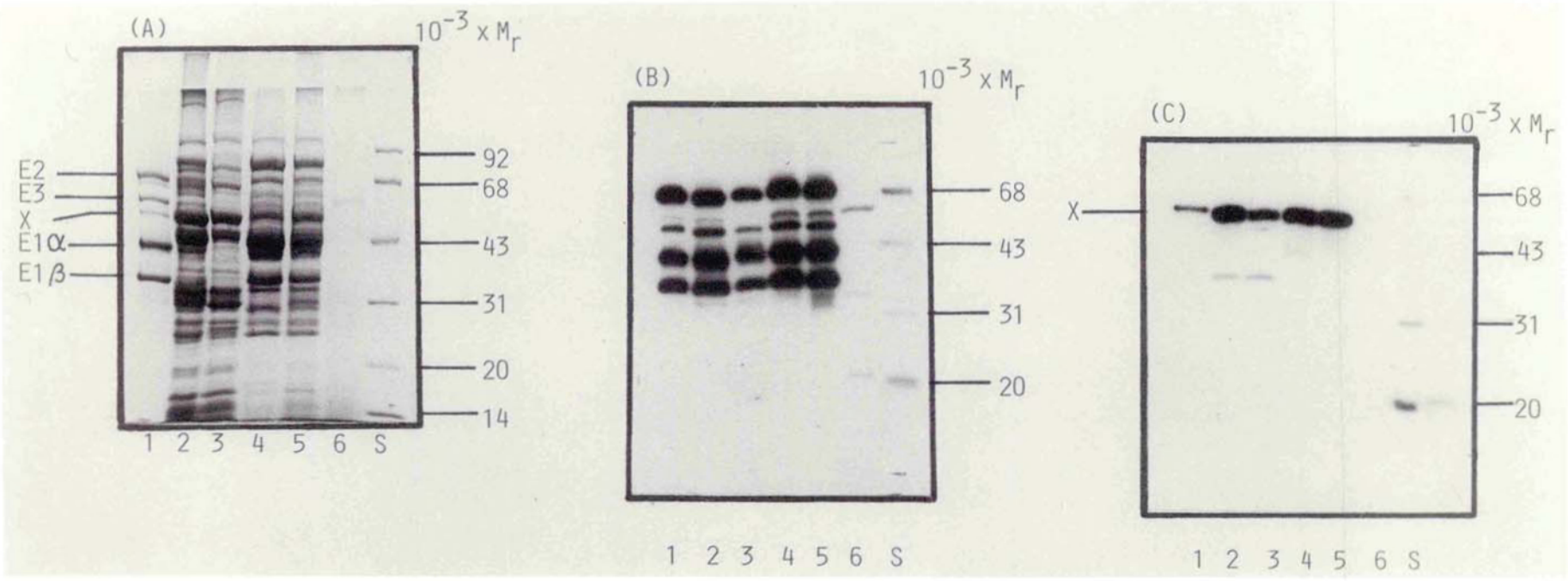
Samples containing equal amounts of protein from each fraction (except from the last washing step) were analysed by immunoblotting with anti-PDC serum (Fig. 5.4 B). All five components of PDC were detected in both soluble and membrane fractions. The band of M_r 55,000 present in the last washing seems to correspond to subunit E3. This could be attributed to partial release of this component during the washing procedure.

Although the immunoblots were not rigorously quantitated, the relative amounts of the individual components of the complex were similar in soluble and membrane-bound PDC (lanes 3 and 4), with the exception of subunit E3 which was less abundant in the membrane fraction. It has been estimated on the basis of protein recovered in each fraction that the amount of PDC that remained associated to the membrane of ox heart mitochondria was about 65-70% (w/w) of the total. These results suggest that a large proportion of PDC is bound to the inner mitochondrial membrane

Fig. 5.4 Immunological detection of PDC polypeptides in ox heart submitochondrial fractions

Ox heart mitochondria were sonicated and fractionated into a membrane and a soluble fraction as indicated in section 2.2.7.c. The membrane fraction was washed 2X with 20 mM-potassium phosphate buffer, pH 6.5, 0.05 M-NaCl and 1% (v/v) rabbit serum. Samples were boiled in Laemmli buffer and loaded on a 10% (w/v) SDS/polyacrylamide slab gel. After transferring onto nitrocellulose paper, the samples were analysed by immunoblotting with anti-PDC (B) or anti-X (C) sera. Protein profiles are shown in (A).

(A, B and C) Lane 1, PDC 8 μg, 0.4 μg and 0.5 μg protein, respectively; lane 2, ox heart mitochondria, 50 μg, 10 μg and 15 μg; lane 3, membrane fraction, 35 μg, 10 μg and 10 μg; lanes 4 and 5, soluble and first washing fractions, 35 μg, 10 μg and 10 μg; lane 6, second washing fraction, 10 μg protein each; S, ^{125}I-labelled mixture of standard proteins.


(A)
10^{-3} x M_r
E2
E3
X
E1α
E1β
92
68
43
31
20
14
1 2 3 4 5 6 S
(B)
10^{-3} x M_r
68
43
31
20
1 2 3 4 5 6 S
(C)
10^{-3} x M_r
X
68
43
31
20
1 2 3 4 5 6 S

and the association is resistant to the extensive washing treatment. Subunit E3 dissociated from the complex and was recovered in the washing solution.

Fig. 5.4. C shows that there is no preferential distribution of component X in the membrane fraction as compared with the soluble fraction (lanes 3 and 4). This result does not exclude the possibility that X may be involved in interactions with the inner membrane.

Immunoblotting analysis of the submitochondrial fractions obtained from ox heart were also performed with anti-fumarase serum (Fig.5.5) and anti-citrate synthase serum (Fig. 5.6).

Fumarase was detected in the matrix or soluble fraction and in the first washing, but was not seen in the membrane fraction (lane 3). Small amounts of a cross-reacting antigen of lower M_r were detected in the soluble fraction.

The distribution of citrate synthase is similar to fumarase, but traces of the enzyme were detected in the membrane fraction (Fig. 5.6 lane 4). Also, in this immune replica, cellular fractions from bovine kidney cells (NBL-1) were analysed to demonstrate the specificity of the antiserum to citrate synthase. Immunoprecipitation studies (not shown) confirmed that this antiserum is capable of specifically immunoprecipitating citrate synthase from NBL-1 cells whereas immunoprecipitates from rat liver cell extracts showed two protein bands of similar M_r to those shown in Fig. 5.2 (2A).

PDC activity was detectable in the initial sonicated mitochondria and in the soluble and first washing fractions. However, activity was not detectable in the final membrane fraction. This result can be explained by loss of component E3 from the membrane-bound complex during the washing steps (Fig. 5.4). Enzymic activity was not restored by adding 5 units of pig heart lipoamide dehydrogenase or by adding antimycin A

Fig. 5.5 Immunoblotting analysis of ox heart submitochondrial fractions with anti-fumarase serum

Ox heart submitochondrial fractions obtained as described in section 2.2.7.c. and purified fumarase were electrophoresed in duplicate on 10% (w/v) SDS/polyacrylamide slab gels. Polypeptides were transferred onto nitrocellulose and subsequently analysed with anti-fumarase serum (A) by immunoblotting technique. A replica of the gel was stained with Coomassie Blue (B).

(A and B), Lane 1, fumarase 1.5 µg each; lane 2, ox heart mitochondria 15 µg and 35 µg; lane 3, membrane fraction 10 µg and 35 µg; lane 4 and 5, fractions soluble and first washing 10 µg and 35 µg each, respectively.

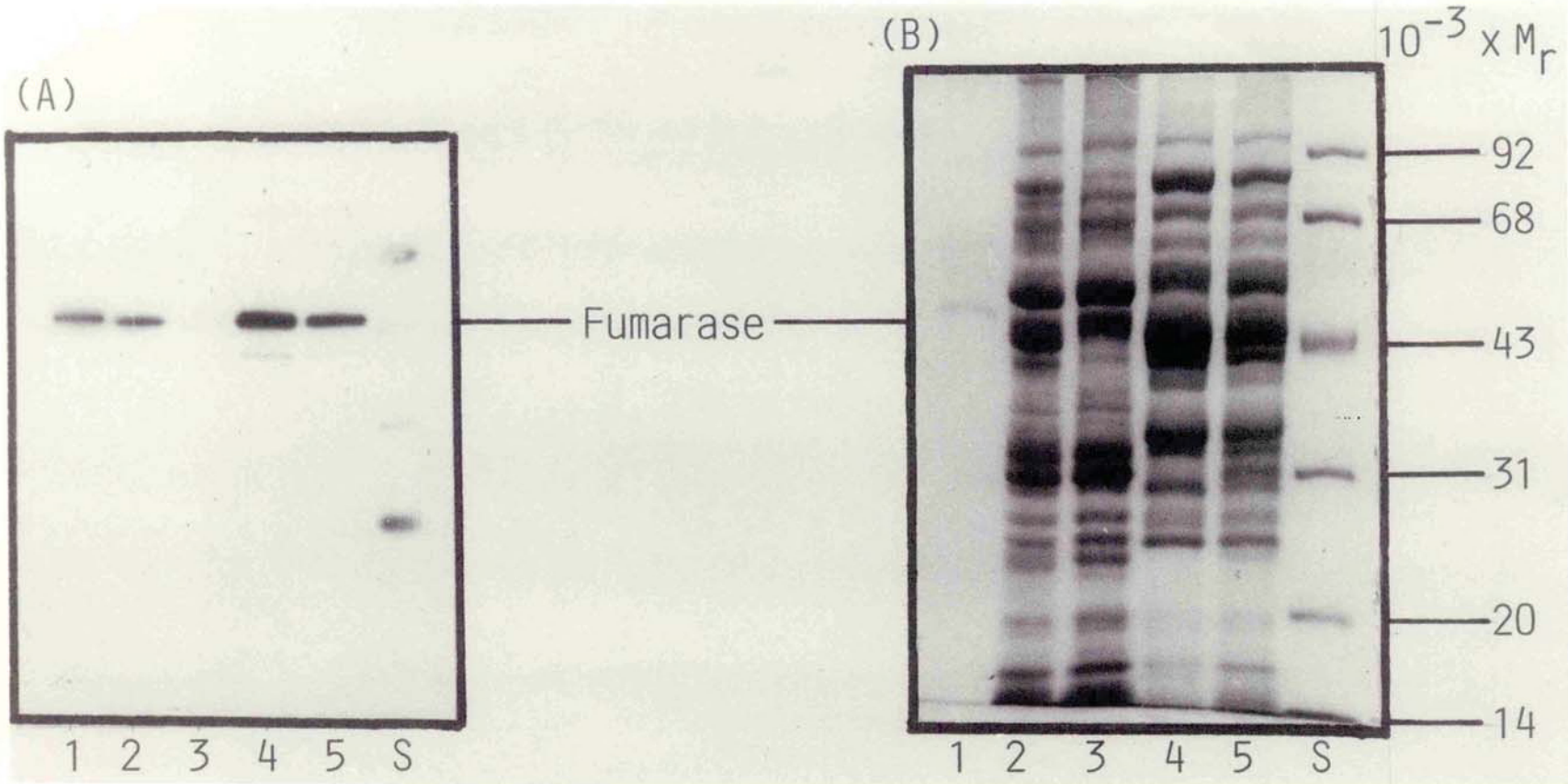
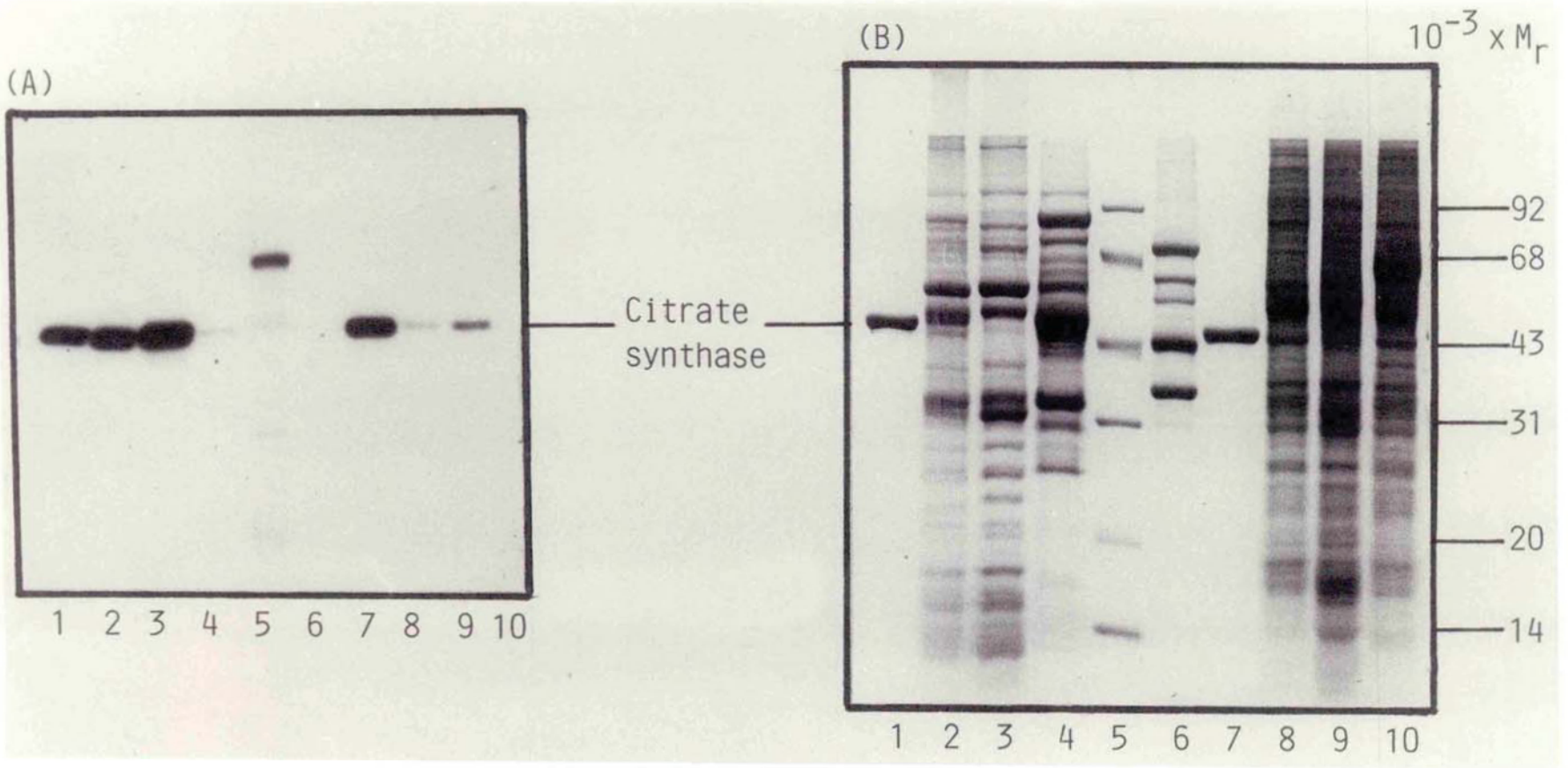

(A)
(B)
10^{-3} x M_r
92
68
43
31
20
14
Fumarase
1 2 3 4 5 S
1 2 3 4 5 S

Fig. 5.6 Immunological detection of citrate synthase in ox heart submitochondrial fractions and subcellular extracts from NBL-1 cells

NBL-1 cell extracts and ox heart submitochondrial fractions were analysed by immunoblotting technique with anti-citrate synthase serum essentially as described in legend to Fig. 5.5. (A) Immunoblot. (B) Coomassie Blue pattern.

(A and B) Lane 1, citrate synthase, 0.2 μg and 3 μg protein, respectively; lane 2, ox heart mitochondria, 15 μg and 35 μg; lane 3, soluble fraction 10 μg and 35 μg; lane 4, membrane fraction, 10 μg and 35 μg; lane 5, ^{125}I-labelled M_r standards; lane 6, PDC, 5 μg and 15 μg; lane 7, citrate synthase, 0.2 μg and 3 μg; lanes 8, 9 and 10, NBL-1-cells, NBL-1 mitochondria and NBL-1 cytosol 40 μg and 60 μg protein each, respectively.


(A)
1 2 3 4 5 6 7 8 9 10
Citrate synthase
(B)
10^{-3} x M_r
92
68
43
31
20
14
1 2 3 4 5 6 7 8 9 10

to inhibit NADH oxidase activity which could interfere with the assay. However, it is possible that correct assembly of the externally-added E3 component did not take place under the conditions tested owing to steric hindrance or because of a slow reassociation process. These preliminary findings deserve further investigation.

5.5. Discussion

Evidence presented in this Chapter suggests that PDC is largely bound to the ox heart inner mitochondrial membranes. It seems also probable that a fraction of the enzyme has a similar distribution in rat liver mitochondria.

This interaction between PDC and the inner mitochondrial membrane is significatively higher than that of two other mitochondrial proteins, fumarase and citrate synthase, which bind weakly or not at all to the inner membranes in both rat liver and ox heart mitochondria.

The ox heart system seems to be more suitable for these types of study than the rat liver, partly owing to the fact that ox heart mitochondria have a heavily invaginated inner membrane comprising about 50% (w/w) of the total mitochondrial protein, whereas in the rat liver is only about 20%. In addition, obvious limitations are imposed by the procedures available for fractionating rat liver mitochondria to obtain preparations with low levels of cross-contamination between adjacent compartments.

Moreover, the rat liver system has the additional disadvantage of containing lower amounts of the complex than ox heart and that selective proteolytic degradation of PDC may occur to an unknown extent during fractionation of the rat liver mitochondria stemming from the presence on this tissue of an "inactivase" of lysosomal origin (Wieland, 1975).

No evidence for degradation of PDC was found in the ox heart fractions.

Results from ox heart mitochondria agree with those obtained after sonication of pig heart mitochondria or treatment with phospholipase followed by subsequent exposure to high ionic strength (Kenney et al., 1972) which resulted in only 20-30% solubilization of PDC activity; 70% of the complex was recovered in the inner membrane fraction. It remains to be established whether the observed distribution of PDC between soluble and membrane-bound fractions is artifactual or reflects functional/structural differences between two dissimilar populations of macromolecules.

The apparent distribution of component X between the soluble and membrane-bound fractions of ox heart mitochondria is similar to that of the other constituents of the complex (Fig. 5.4). This result suggests that component X is an integral component of PDC rather than a mitochondrial protein involved in binding of the complex to the inner membranes.

Lipoamide dehydrogenase seemed to be released from the membrane-bound enzyme during the washing steps (Fig. 5.4). Massey (1966) has suggested that E3 is quite easily dissociated from the complexes. Even when the enzyme is carefully prepared, free uncomplexed flavoprotein is found in the cytosol (Matuda & Saheki, 1982). This could result from the protein being functionally interchangeable with respect to the various 2-oxo-acid dehydrogenase complexes (Sakurai et al., 1970).

Sümegi & Srere (1984b) had recently presented evidence on binding of PDC to pig heart inner mitochondrial membranes. They have also shown that PDC and two other dehydrogenases: 2-oxoglutarate dehydrogenase complex and malate dehydrogenase were able to bind NADH-ubiquinone oxidoreductase (Complex I) under certain in vitro conditions.

These results are very interesting since the binding of these dehydrogenases to Complex I, the integral membrane NADH-ubiquinone oxidoreductase complex could result in a functionally-linked organisation where the NADH-producing enzymes (dehydrogenases) would be associated with the NADH-oxidising system in a highly efficient manner. However, at present, functional evidence for the existence of a coupled system between these complexes has still to be produced.

Many of the quantitative parameters of the interaction of PDC with inner membrane have yet to be determined, as well as the nature of the interaction and the components of the complex involved. Moreover, it will be most important to establish if the inner membrane protein(s) involved in binding are components of Complex I which contains about 26 polypeptides, most of unknown function (Heron et al., 1979).

Chapter 6: STUDIES ON COMPONENT X: AN IMMUNOLOGICALLY DISTINCT POLYPEPTIDE ASSOCIATED WITH MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX

6.1 Introduction

Ox heart PDC is a large macromolecular assembly, M_r 8.5×10^6 containing multiple copies of its three constituent enzymes: pyruvate dehydrogenase (E1), dihydrolipoyl acetyltransferase (E2) and lipoamide dehydrogenase (E3).

When the polypeptide composition of the bovine enzyme is examined after SDS/polyacrylamide gel electrophoresis, four main components are detectable at M_r values of 42,000 (α chain of E1), 36,000 (β chain of E1), 70,000-74,000 (E2) and 55,000 (E3), Linn et al. (1972).

On occasions, an additional polypeptide, M_r 51,000±1,000 has been detected in highly-purified PDC or dihydrolipoyl acetyltransferase preparations isolated from bovine tissues using different procedures (Kresze & Steber, 1979; Machicao & Wieland,1980; Stanley & Perham, 1980; Bleile et al., 1981; Pettit & Reed, 1982) and porcine heart (Roche & Cate, 1977).

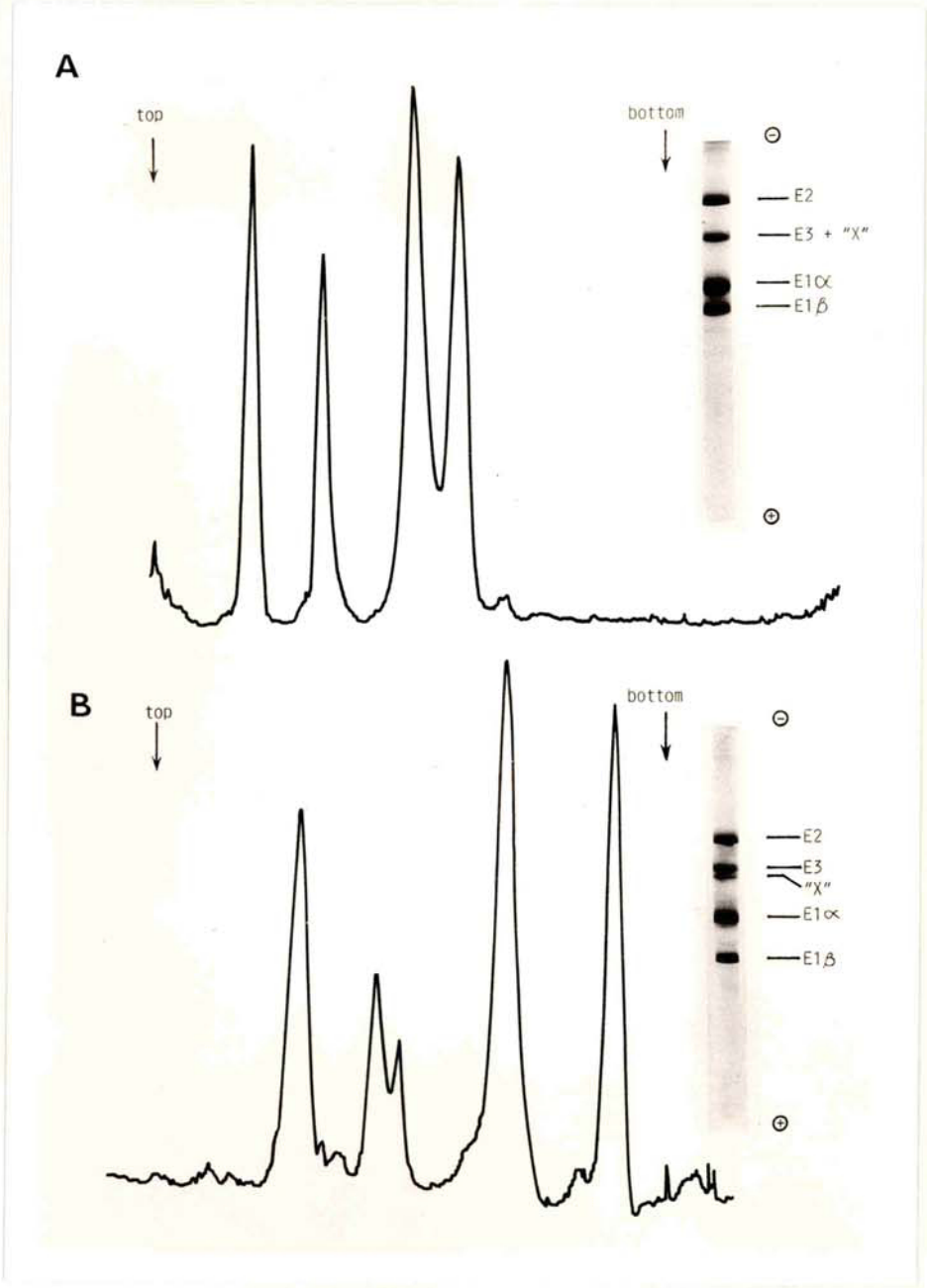
This polypeptide, termed in this study, component X, has been regarded as a proteolytic fragment of E2 (or E3) or possibly the intrinsic pyruvate dehydrogenase kinase of the complex. In the majority of earlier studies (Barrera et al., 1972) this component remained undetected since it does not readily separate from the E3 component on SDS/phosphate gels (Stanley & Perham, 1980).

Fig. 6.1 compares the resolution of the constituent polypeptides of ox heart PDC in two differing SDS/polyacrylamide gel systems. It is clearly shown that separation of component X from the E3 subunit was only achieved in the Tris-glycine buffered system of Laemmli (1970)

Fig. 6.1 The subunit composition of ox heart PDC as revealed on differing SDS/polyacrylamide gel systems

Purified PDC (15 µg) was electrophoresed on 10% (w/v) SDS/polyacrylamide cylindrical gels (12 cmx 0.5 cm) prepared in phosphate buffer (A) or Tris-glycine buffer (B). Electrophoresis was carried out at 7.5 mA/gel until the tracking dye had migrated to 0.5 cm from the bottom. The gels were stained with Coomassie Blue. Densitometric traces of the protein bands resolved on each gel system are also shown.

For experimental details refer to section 2.2.6.b.


A
top
bottom
E2
E3 + "X"
E1α
E1β
B
top
bottom
E2
E3
"X"
E1α
E1β

(Fig. 6.1B) and not in the phosphate-buffered system (Weber & Osborn, 1969) (Fig.6.1A).

Co-migration of components E3 and X in the latter gel system was confirmed after quantitation of densitometric scans of the Coomassie Blue-stained gels. Table 6.1 shows that this composite peak contributed 15.2% of the total absorbance, while on Laemmli gels, these proteins represented about 9% and 6% of the absorbance, respectively. The relative proportions of the other components remained constant in both gel systems.

The proportion of E2 as estimated by this method was lower than expected from the overall composition of the complex since this polypeptide chain has a low colour yield with Coomassie Blue (stain bound per μg protein) (Sugden & Randle, 1978).

In this Chapter, results will be presented which demonstrate the investigations carried out on the possible identity of component X.

6.2 Absence of immunological cross-reaction between component X and the other polypeptides of the complex

Subunit-specific antisera directed against components E2 and X were raised in rabbits as described in section 2.2.12.b. In Fig. 6.2, the precise reactivities of these antisera against their specific antigens were analysed by immunoblotting with varying amounts of PDC resolved by SDS/polyacrylamide gel electrophoresis.

In each case, only the parent antigen was detected by its specific antiserum and no cross-reaction between these polypeptides was observed. This indicated that protein X was not immunologically related to E2 or to the other subunits of the complex. Similar results were obtained by immunoblotting analysis with antiserum to E3 (not shown).

Table 6.1 SUBUNIT COMPOSITION OF THE PYRUVATE DEHYDROGENASE COMPLEX

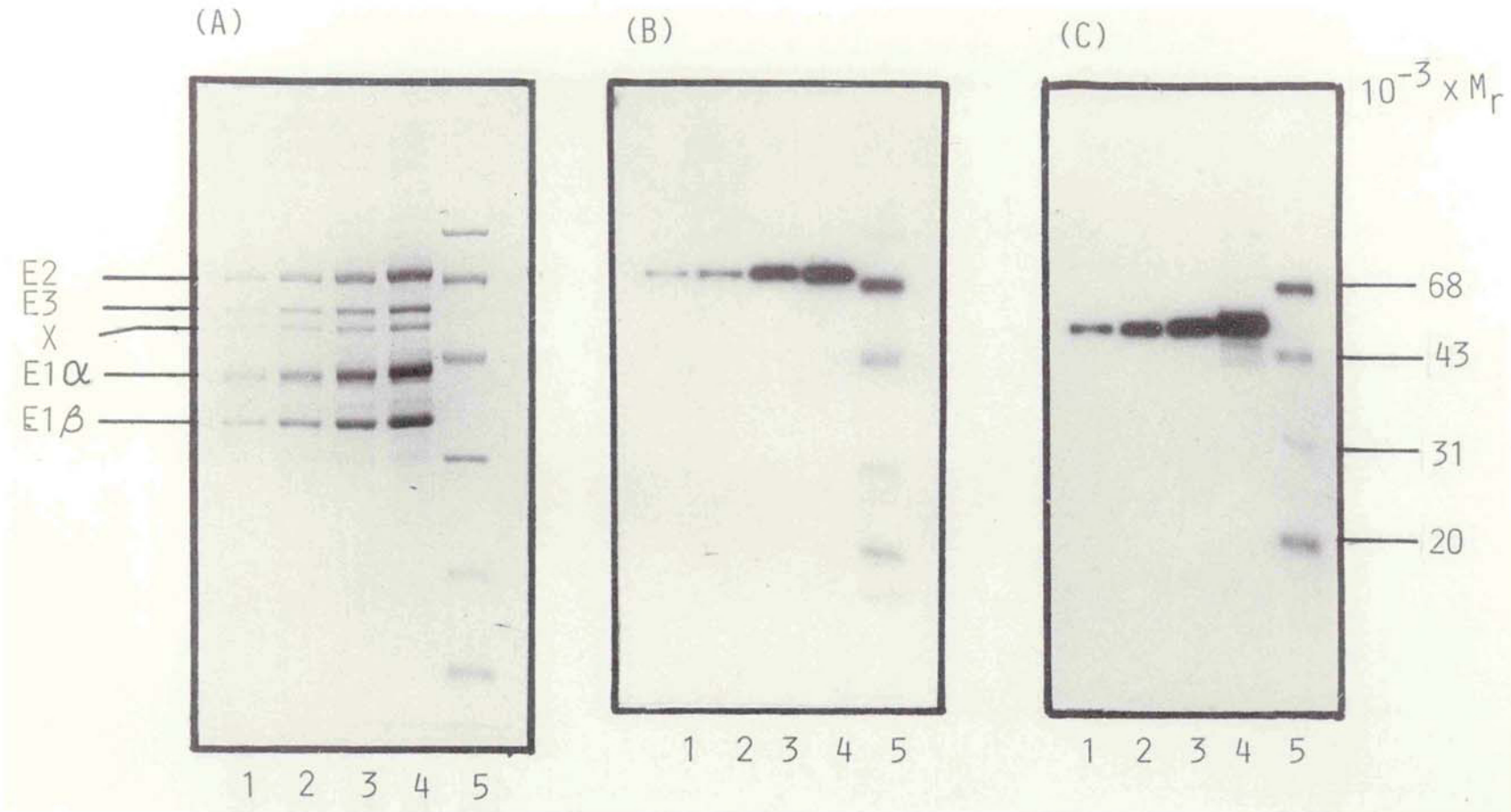
Subunit	Tris-glycine system % Area	Phosphate-buffered system % Area
Dihydrolipoyl acetyltransferase	24.2 ± 1.6	23.2 ± 1.8
Lipoamide dehydrogenase	9.2 ± 1.1	15.2 ± 1.4
Pyruvate dehydrogenase subunit α	34.3 ± 1.1	35.5 ± 1.6
Pyruvate dehydrogenase subunit β	25.9 ± 1.9	26.3 ± 1.7
Component X	6.4 ± 1.4	-----

SDS/polyacrylamide gel electrophoresis was performed in rod gels as described in section 2.2.6.b., using the Tris-glycine buffer system of Laemmli (1970) or the phosphate-buffered system described by Weber & Osborn (1969). Gels were scanned and the areas under the peaks were determined by weighing the traces. The values are means of three determinations using two different preparations of PDC (purity 95-98%).

Fig. 6.2 Immune-replica analysis of PDC with subunit-specific antisera against the dihydrolipoyl acetyltransferase and component X

Varying amounts of PDC were subjected to SDS-polyacrylamide electrophoresis in a 10% (w/v) slab gel. Part of the gel was stained with Coomassie Blue (A). Another set was transferred onto nitrocellulose paper for incubation and subsequent immunological detection with anti-E2 (B) or anti-X (C).

Lanes 1-4 contain 2 µg, 5 µg, 7.5 µg and 15 µg of protein in (A); 0.05 µg, 0.1 µg, 0.25 µg, and 0.5 µg in (B) and 0.25 µg, 0.5 µg, 0.75 µg and 1 µg in (C), respectively. In each case, the right hand track contains a standard mixture of ^{125}I-labelled M_r markers.


(A)
(B)
(C)
E2
E3
X
E1α
E1β
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
10^-3 x Mr
68
43
31
20

6.3 Immunological detection of component X in cell extracts

Detection of component X in SDS-extracts of cultured cells was performed by immunoblotting with antiserum to ox heart PDC. Cell extracts were prepared under conditions which were designed to minimise proteolytic degradation of the complex (see Chapter 4, Fig. 4.4; De Marcucci et al., 1985a). Furthermore, the protein was detected exclusively in the mitochondrial fraction along with components E2, E1α and E1β of the complex. The lack of reactivity of anti-PDC serum with the E3 component was discussed in Chapter 4, section 4.4. These results have been confirmed by similar studies with samples from several rat tissues obtained after freezing in liquid N_2 and extraction of the powdered tissues in hot SDS (not shown).

These results indicate that protein X is a normal cellular component in a variety of tissues since no proteolysis is expected under these conditions, unless occurring naturally in vivo. It also appears that protein X is confined to the mitochondrial compartment.

6.4 Subcellular localisation of component X

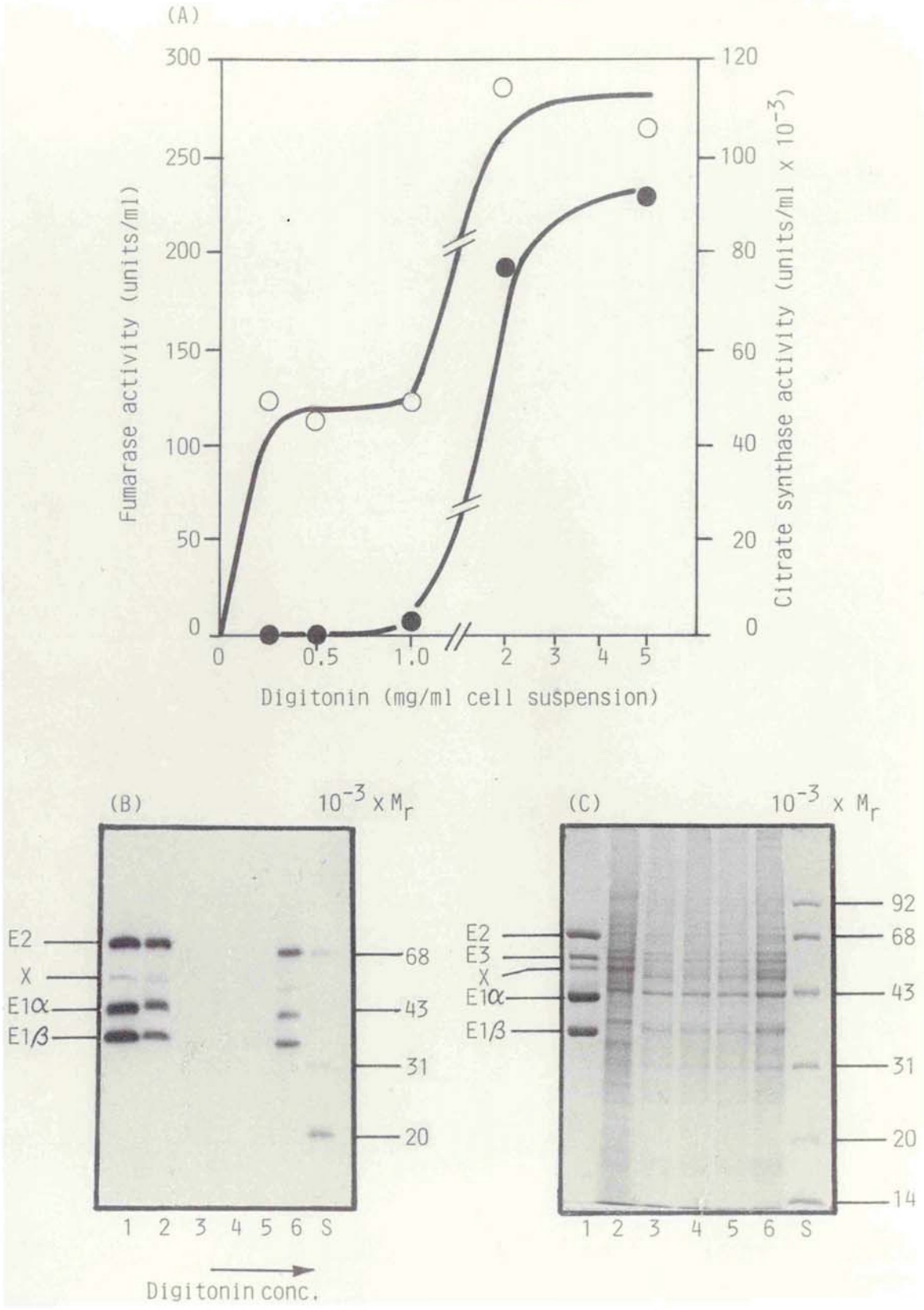
The mitochondrial localisation of protein X was further investigated by selective disruption of cells with digitonin for isolation of cytosolic and particulate fractions (Zuurendonk & Tager, 1974; Mori et al., 1981a). This method exploits the difference in cholesterol content between the plasma and inner mitochondrial membrane (Zuurendonk et al.,1979). As digitonin interacts selectively with cholesterol, conditions can be found in which the plasma membrane is lysed while the mitochondrial inner membrane remains intact. At higher levels of digitonin, mitochondrial disruption is induced with concomitant release of mitochondrial enzymes into the cytosol.

Fig. 6.3 Immunological detection of PDC polypeptides in extracts from pig kidney cells treated with digitonin

Suspensions of PK-15 cells (5×10^6 cells/ml) in 20 mM-potassium Hepes buffer, pH 7.4, 0.25 M-sucrose, 3 mM-EDTA were incubated at 0°C with various concentrations of digitonin as indicated in section 2.2.7.d.

After 2 min, cell suspensions were rapidly centrifuged. Samples from the supernatant fractions (cytosol) were utilised for assaying fumarase, (—O—)and citrate synthase,(—●—) activities as described in sections 2.2.8.c and 2.2.8.f. respectively (A). Aliquots containing approx. 50 µg protein were electrophoresed onto 10% (w/v) SDS/polyacrylamide slab gels. The gel was utilised for the immunochemical identification of PDC polypeptide after incubation with anti-PDC serum (B). A parallel gel was stained with Coomassie Blue (C).

(B and C) Lane 1, PDC, 0.5 µg and 12 µg protein, respectively; lane 2, PK-15 mitochondria, 40 µg and 50 µg; lanes 3, 4, 5 and 6 cytosol from PK-15 cells treated with 0.25 mg, 0.5 mg, 1.0 mg and 2.0 mg of digitonin, respectively (40 µg each); lane S, ^{125}I-labelled M_r standards. Part (A) of this Figure was reproduced with permission from Dr. G.Gibb.


(A)
Fumarase activity (units/ml)
Citrate synthase activity (units/ml x 10^{-3})
Digitonin (mg/ml cell suspension)
(B)
10^{-3} x M_r
E2
X
E1α
E1β
68
43
31
20
1 2 3 4 5 6 S
Digitonin conc.
(C)
10^{-3} x M_r
E2
E3
X
E1α
E1β
92
68
43
31
20
14
1 2 3 4 5 6 S

In these experiments, disruption of mitochondria was monitored by the appearance of citrate synthase and increase of fumarase activities in the cytosolic fractions obtained after treatment of pig kidney cells with increasing concentrations of digitonin.

As shown in Fig. 6.3 (A), at concentrations of digitonin $\geq$ 2mg/ml, citrate synthase activity was detected in the cytosol. Fumarase activity showed a biphasic response. At low levels of digitonin, fumarase activity was detectable owing to the presence of a cytosolic pool of the enzyme. A two-fold increase in fumarase activity was observed when the mitochondrial isoenzyme was released at concentrations of digitonin $\geq$ 2mg/ml.

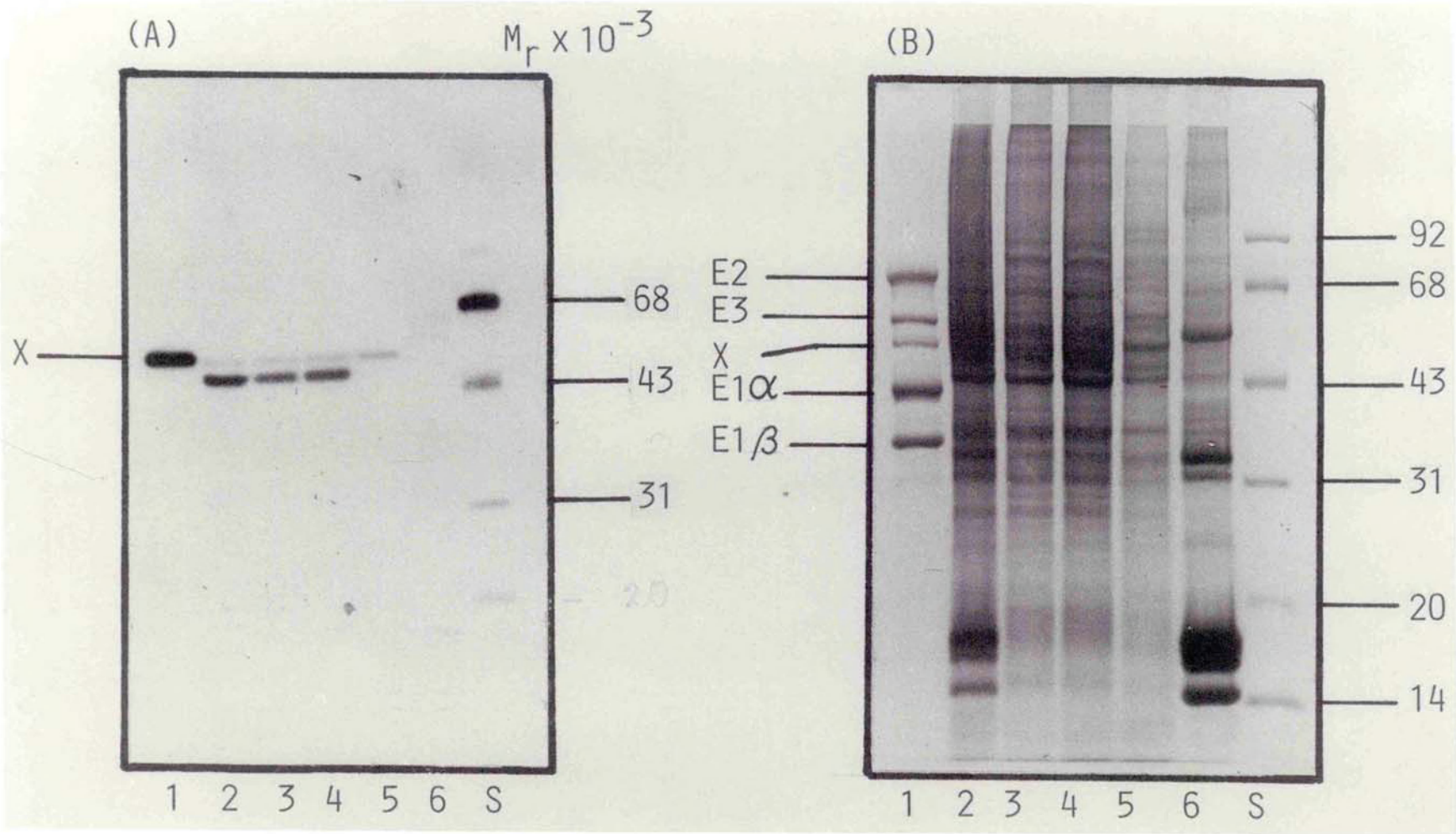
Immunoblotting analysis of the cytosolic fractions with anti-PDC serum, (Fig. 6.3,B) showed that the components of the complex were detectable in the cytosol only when the concentration of digitonin was $\geq$ 2mg/ml. Under these experimental conditions, this level corresponded to the concentration of detergent required for mitochondrial disruption.

A similar study was carried out in Buffalo rat liver cells (BRL). Subcellular fractions were obtained after fractionation by differential centrifugation (Attardi & Ching, 1979) and analysed by immunoblotting with antiserum to component X. Fig. 6.4 shows that the antiserum cross-reacts with an M_r 50,000 polypeptide (lanes 2-5) present in whole cells and in the mitochondrial fraction. An additional cross-reacting antigen of lower M_r value was also observed in whole cell extracts (lane 1) and in postnuclear supernatant fractions (lanes 2 and 3) but was absent from mitochondrial fractions (lane 4). The identity of this strongly cross-reacting antigen has not yet been established. Results with other cell lines confirmed that this protein was a cytosolic component.

Fig.6.4 Immunoblotting analysis of subcellular fractions from BRL cells with antiserum to component X

BRL cells were grown in two large roller bottles (burlers) until they reached confluence. Monolayers were washed with PBS, scraped off and the resulting cell suspension was subjected to subcellular fractionation following the procedure described by Attardi & Ching (1979). Samples of the various fractions obtained were electrophoresed onto 10% (w/v) polyacrylamide gels. (A) Immunoreplica obtained after incubation with antiserum to component X and (B) Coomassie Blue pattern.

(A) and (B) Lane 1, PDC 0.5 µg and 12 µg protein, respectively; lane 2, SDS-extract of whole cells, 80 µg and 60 µg; lanes 3 and 4, postnuclear supernatant 50 µg and 60 µg; lane 5, mitochondrial fraction 40 µg each; lane 6, nuclear fraction 40 µg each; lane 5, ^{125}I-labelled M_r markers.


(A)
$M_r \times 10^{-3}$
X
68
43
31
1 2 3 4 5 6 S
(B)
E2
E3
X
E1α
E1β
92
68
43
31
20
14
1 2 3 4 5 6 S

6.5 Studies on the possible identity of component X with pyruvate dehydrogenase kinase

It has been suggested previously that the unassigned M_r 50,000 polypeptide associated with mammalian PDC could be the pyruvate dehydrogenase kinase (Sugden & Randle,1978; Stanley & Perham, 1980). Stepp et al. (1983) reported that subunits of M_r 48,000 and 47,000 have been observed in highly-purified pyruvate dehydrogenase kinase.

In this study, this possibility was explored initially by measuring the effects of anti-X serum on the ATP-dependent inactivation of the complex catalysed by the intrinsic kinase. Fig. 6.5 shows that anti-X serum did not affect PDC activity but protected the enzyme from inactivation in the presence of 0.2 mM-ATP. This effect was reflected in a significant increase in $t_{\frac{1}{2}}$ for complete (98%) inactivation of the complex from 5 min to 14 min. Antiserum to subunit E1 α produced a similar effect, but the $t_{\frac{1}{2}}$ increased to 8 min only.

The possibility that component X was related to the kinase was further examined by direct measurement of the effects of anti-X serum on the initial rate of phosphorylation of PDC in the presence of [γ-^{32}P] ATP. The results of this experiment are shown in Fig. 6.6. Only antiserum to PDC showed a significant inhibition of the phosphorylation reaction (approx. 30%) as compared with control serum. Anti-X and anti-E1 α slightly decreased phosphorylation, whereas anti-E2 had no measurable effect. Analysis of the products of phosphorylation by autoradiography showed that approx. 95% of the radioactive product had a M_r identical to subunit E1 α .

The reasons behind the lack of agreement between these two types of experiments are unclear. Phosphorylation at site 1 on the E1 α subunit of PDC is sufficient to cause inactivation of the tetramer

Fig. 6.5 Effects of antiserum against component X on the rate of inactivation of PDC in the presence of ATP

PDC (20 µg) was incubated at room temperature in the presence of 100 µl of control serum or antiserum to component X essentially as described in section 2.2.12.d. After 30 min, the sample was transferred into a water bath at 30°C and ATP was added to a final concentration of 0.2 mM. Aliquots (5-100 µl) were withdrawn at the indicated times for spectrophotometric assay of PDC activity. Controls containing no ATP were also included. Results are means of two determinations that differed by less than ± 5%.

(—○—) Control serum (no ATP added); (—△—) anti-X serum (no ATP added); (—●—) control serum (plus ATP); (—▲—) anti-X serum (plus ATP).

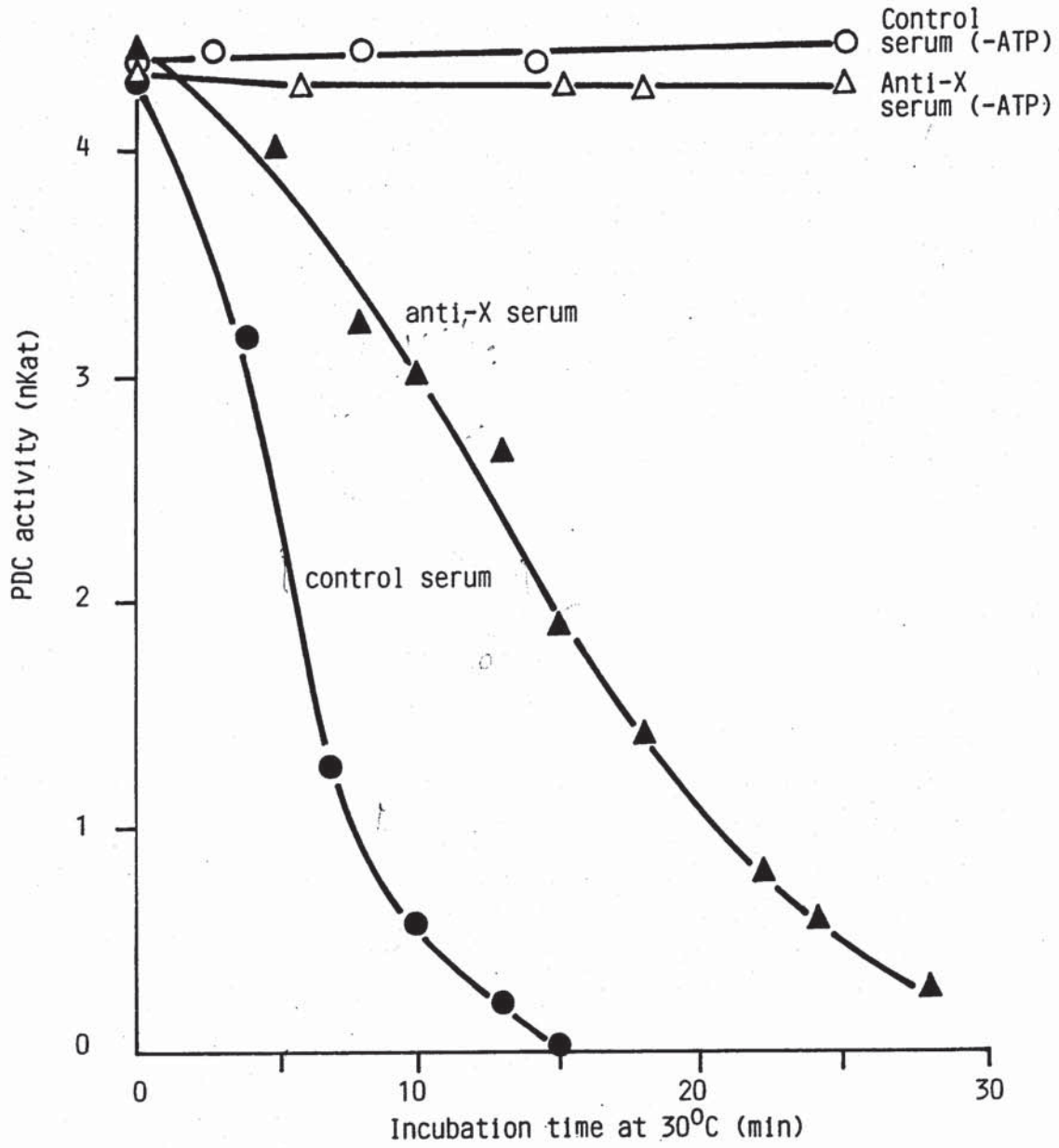

Control serum (-ATP)
Anti-X serum (-ATP)
anti-X serum
control serum
PDC activity (nKat)
0
1
2
3
4
0
10
20
30
Incubation time at 30°C (min)

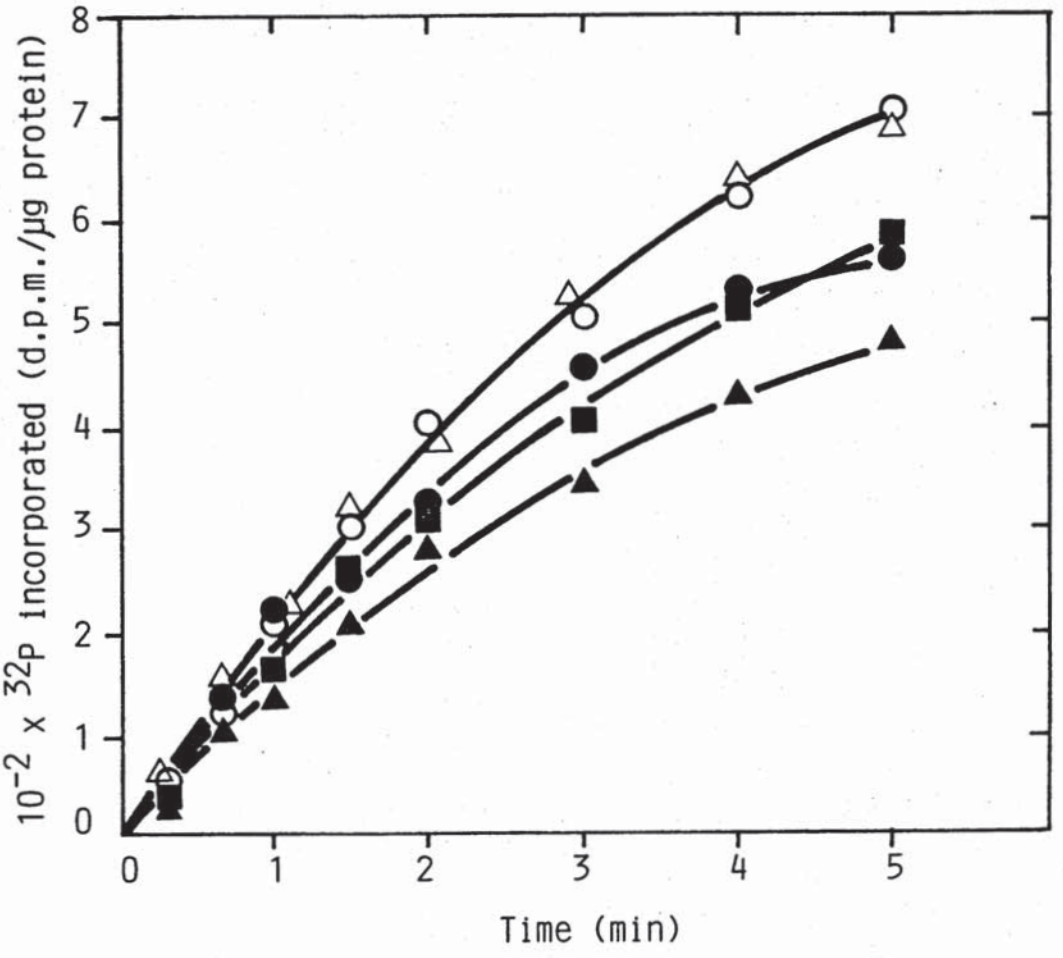
Fig. 6.6 Effects of various antisera on pyruvate dehydrogenase kinase activity

PDC (50 μg) was incubated at room temperature for 30 min with 100 μl of various antisera, as described in section 2.2.12.d. Phosphorylation reactions were initiated by the addition of [γ-^{32}P] ATP (0.2 mM; 132,000 d.p.m./nmol). Pyruvate dehydrogenase kinase was assayed by measuring the initial rate of incorporation of ^{32}P into TCA-precipitable radioactivity by the filter paper discs method. Results are means of three separate determinations differing by less than ± 5%.

(—▲—) anti-PDC serum; (—●—) anti-X serum;

(—■—) anti-E1α serum; (—○—) control serum;

(—△—) anti-E2 serum;


8
7
6
5
4
3
2
1
0
0
1
2
3
4
5
Time (min)
10^{-2} x ^{32}P incorporated (d.p.m./µg protein)

(Yeaman et al., 1978), whereas the physiological significance of phosphorylation at sites 2 and 3 is uncertain. Appreciable phosphorylation at sites 2 and 3 accompanies loss of the final 30% of the PDC activity. It is possible that binding of antiserum to the complex causes a reduction in the rate of phosphorylation at site 1 or prevents conformational changes which are involved in the inactivation of the complex.

A more definitive answer to the question of the identity of component X with the pyruvate dehydrogenase kinase came from studies performed with a sample of purified bovine kidney pyruvate dehydrogenase kinase, generously provided by Prof. Lester Reed, University of Texas, USA.

The presence of component X and the cross-reactivity with the pyruvate dehydrogenase kinase were examined by immunoblotting analysis with antiserum to protein X. Fig. 6.7 shows that anti-X serum failed to react with subunits α and β of the kinase, which migrated with M_r values 47,000 and 46,000, respectively (lane 5). A band corresponding to component X was detected in the subcomplex E2-X (Peak I from gel filtration of the dissociated complex) and in the acetyltransferase fraction obtained after treatment of Peak I with p-chloromercuriphenyl sulphonic acid (lanes 2 and 3), (sections 2.2.10.a and 2.2.10.c).

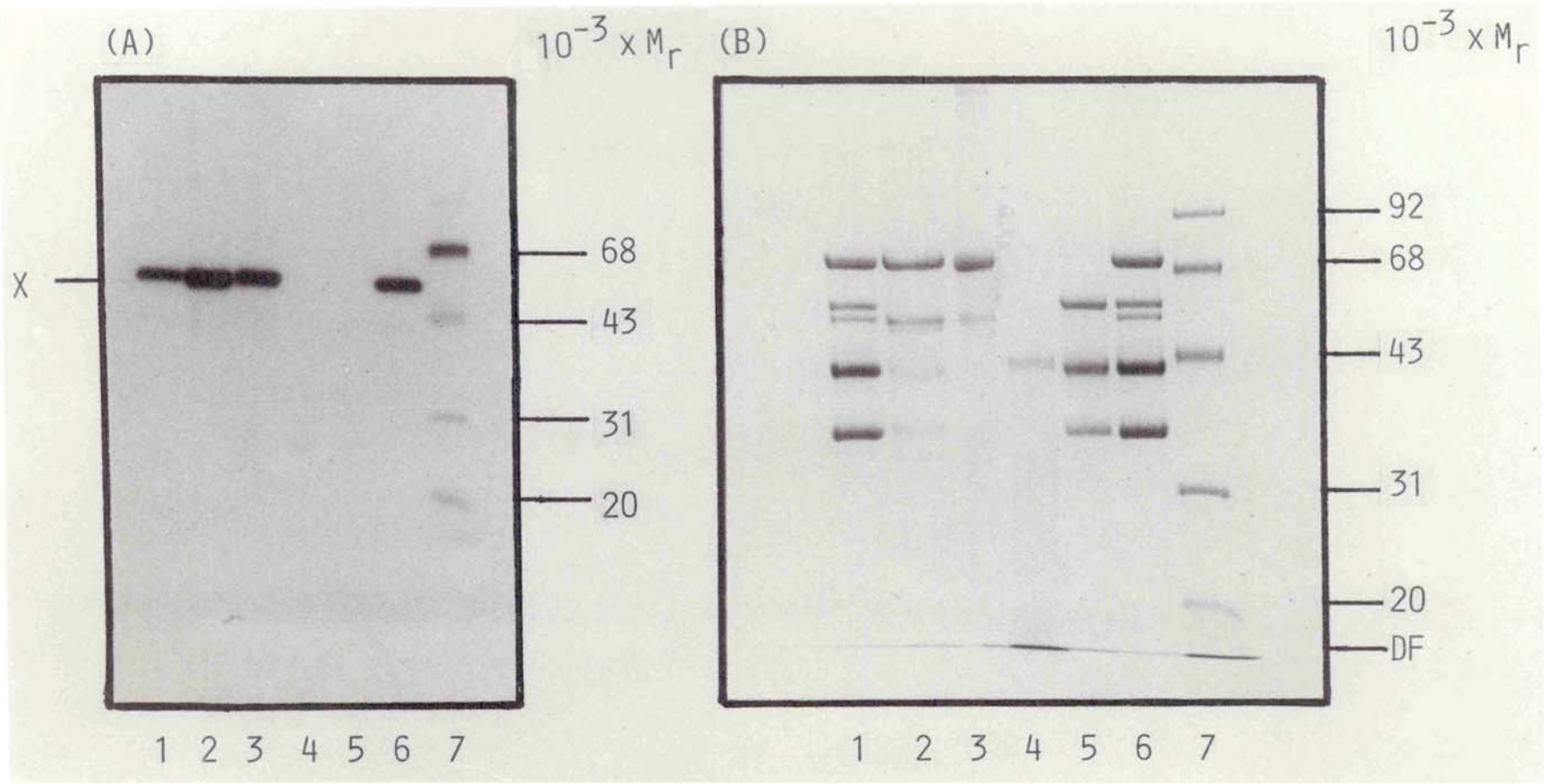
It was clear that component X remained tightly-associated with the core enzyme under conditions in which E1 and E3 were released. In addition protein X was not dissociated after treatment with p-chloromercuriphenyl sulphonic acid, a reagent that promotes dissociation of the pyruvate dehydrogenase kinase (Stepp et al., 1983).

These results provided strong evidence that protein X was not related to either subunit of bovine pyruvate dehydrogenase kinase.

Fig. 6.7 Lack of reactivity of antiserum to component X with purified pyruvate dehydrogenase kinase from bovine kidney

Subcomplexes of PDC, purified by gel filtration of the dissociated multienzyme and bovine kidney pyruvate dehydrogenase kinase were analysed on 10% (w/v) polyacrylamide slab gels. (A) Immunoblotting analysis after incubation with antiserum to component X and (B) Coomassie Blue profile.

(A and B) Lanes 1 and 6, PDC 0.5 μg and 12 μg protein, respectively; lane 2, subcomplex I (E2 plus X) 1 μg and 5 μg; lane 3, subcomplex I treated with p-hydroxymercuriphenyl sulphonate, 4 μg each; lane 4, PDC kinase, 7.5 μg each; lane 5, fraction II (E1 plus E3) 10 μg each; lane 7, ^{125}I-labelled M_r standards. DF, dye front.


(A)
$10^{-3} \times M_r$
X
68
43
31
20
1 2 3 4 5 6 7
(B)
$10^{-3} \times M_r$
92
68
43
31
20
DF
1 2 3 4 5 6 7

Moreover, the strong association of component X with the core enzyme and its mitochondrial localisation argued against the possibility that component X is a protein which became adventitiously bound to the complex during its isolation.

6.6 Localisation of protein X within the complex

It was apparent from the studies described in section 6.5, that component X was firmly bound to the core enzyme. However, its location within the complex has not yet been established.

A reasonable assumption was that if this polypeptide was exposed on the surface of the complex, it would be possible to detect binding of antibodies specifically directed against it in the intact assembly. Since only a small fraction of the total IgG would be expected to bind to the complex (0.05-1.0%), increased sensitivity was attained by radioiodination of purified IgG fractions from control and anti-X sera.

The final specific activities of the preparations were 3.2×10^6 c.p.m./μg anti-X IgG and 3.9×10^6 c.p.m./μg control IgG. Iodination did not alter the immunoreactivity of the IgG as checked by incubation of the ^{125}I-labelled anti-X IgG with nitrocellulose blots containing 2 μg purified protein X followed by autoradiography. Binding of control IgG under these conditions was negligible.

Native PDC was incubated with approx. 600 ng of each ^{125}I-labelled IgG fractions (approx. 2×10^6 c.p.m.) and loaded onto 10-30% (w/v) sucrose-density gradients. After centrifugation, fractions were analysed for their content of ^{125}I and enzymic activity. Fig. 6.8 shows that a small peak of radioactivity co-migrated with the peak of PDC in the sample incubated with ^{125}I-labelled anti-X IgG. The distribution of radioactivity from the sample incubated with the control IgG showed a shallow background with no detectable peak at the same position of the gradient. This experiment was repeated twice with

Fig. 6.8 Sucrose-density gradient centrifugation of PDC incubated with ^{125}I-labelled anti-X IgG

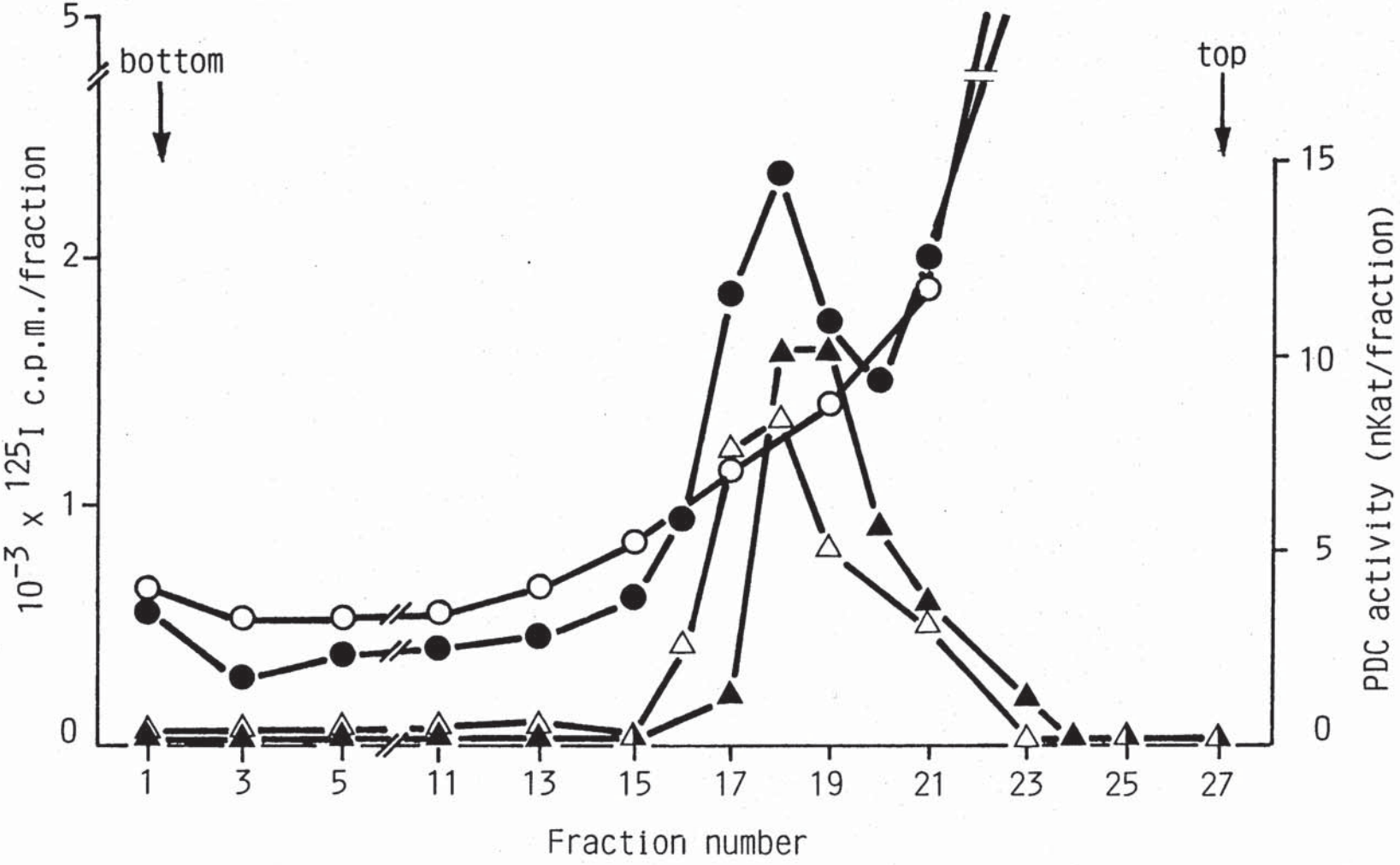
Anti-X and control IgG fractions were purified by affinity chromatography on protein A-Sepharose columns and radioiodinated with ^{125}I as indicated in sections 2.2.12.i and 2.2.14.b.

Native PDC (1.9 mg) was incubated for 1 h at room temperature in the presence of anti-X and control ^{125}I-labelled IgG (approx. 2×10^6 c.p.m. each). Samples were centrifuged for 4 h in a linear 10-30% (w/v) sucrose-density gradient. ^{125}I radioactivity and PDC activity were determined in the fractions collected.

Distribution of ^{125}I radioactivity in the gradients: PDC incubated with control IgG: (—○—); or anti-X IgG:(—●—) .

PDC activity in the gradients; sample incubated with control IgG (—△—) ; or anti-X IgG: (—▲—) .

Additional experimental details in section 2.2.12.j.


bottom
top
10^{-3} x ^{125}I c.p.m./fraction
PDC activity (nKat/fraction)
Fraction number
0
1
2
5
0
5
10
15
1
3
5
11
13
15
17
19
21
23
25
27

longer centrifugation times and with the inclusion of 1% (v/v) Triton X-100 in the sucrose solutions to reduce non-specific binding. Results were essentially the same as shown in Fig. 6.8.

Approx. 0.05% of the total radioactivity in the ^{125}I-labelled anti-X IgG was recovered in the peak fractions (after background correction from the control value). Hence, these results suggest that antibodies to protein X are able to bind to antigenic determinants on the intact assembly, indicating that this polypeptide is at least partially exposed on the surface of the complex. The precise localisation of this protein within the complex would require other types of study, such as reconstitution or cross-linking experiments.

6.7 Studies on the possible identity of component X with a fragment of the E2 subunit

Although earlier data (sections 6.2 and 6.3) suggested that component X is not immunologically related to subunit E2, further studies were performed to clarify this point. Fig. 6.9 shows the characteristic pattern of proteolytic fragments generated by elastase digestion after immunodetection with anti-E2 (B) or anti-X (C) sera.

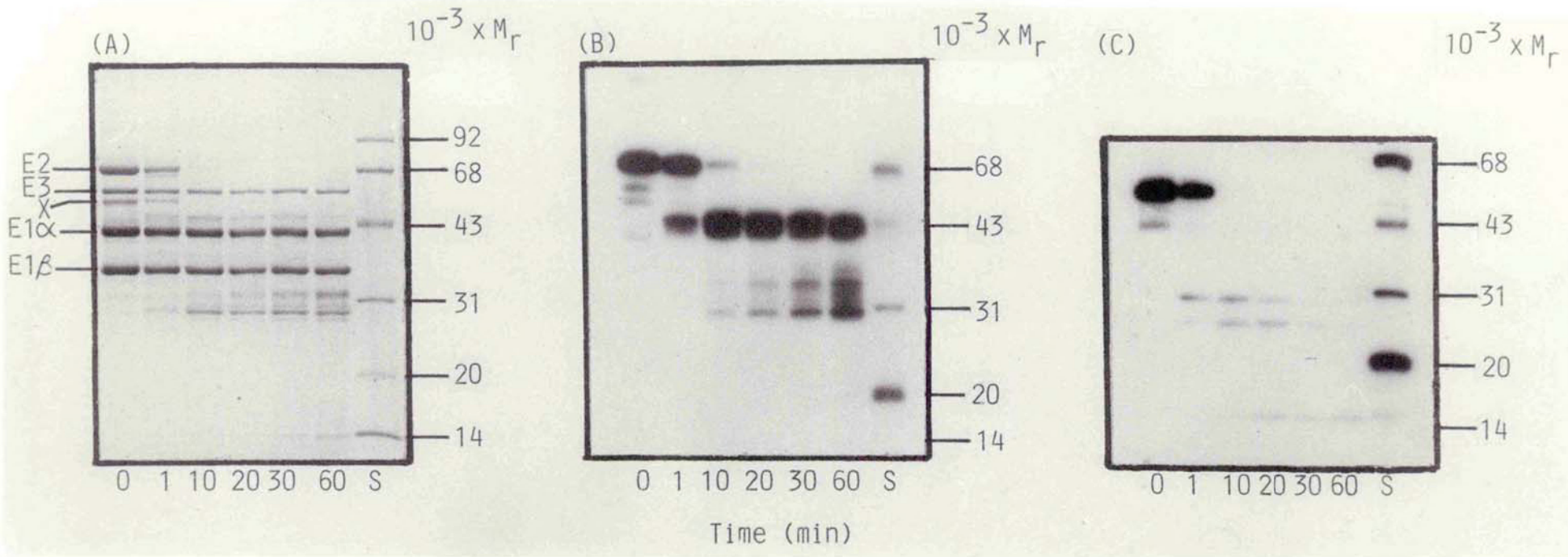
It is apparent that components E2 and X were more susceptible to elastase attack than subunits E3, E1 α and E1 β (Fig. 6.9, A). The main cleavage products of E2 were two strongly immunoreactive peptides M_r 45,000 and 42,000 which were largely resistant to further degradation under the experimental conditions chosen. A less immunologically-reactive peptide appeared at M_r 29,000 at longer time intervals.

Fragments of similar size have been observed previously by Coomassie Blue staining after digestion of PDC or purified acetyltransferase. The M_r 45,000 fragment contains the lipoyl domain whereas the 29,000 M_r peptide contains the subunit binding domain (Kresze & Ronft, 1980). The pattern of protein X-derived fragments showed two peptides

Fig. 6.9 Immunological detection of peptides derived from E2 and component X after proteolysis of native PDC with elastase

A sample of PDC (1 mg/ml) was incubated with elastase (4.5 μg/ml) for the indicated times in 0.1 M-potassium phosphate buffer, pH 7.0 at 25°C.

Samples containing 15 μg protein were resolved by SDS/polyacrylamide gel electrophoresis on 10% (w/v) slabs and stained with Coomassie Blue (A). Similar gels were loaded with 0.1 μg protein (B) or 1 μg protein (C) and subjected to immunoblotting analysis with anti-E2 or anti-X sera, respectively. Lane S corresponds to ^{125}I-labelled M_r standards.


(A)
$10^{-3} \times M_r$
E2
E3
X
E1α
E1β
92
68
43
31
20
14
0 1 10 20 30 60 S
(B)
$10^{-3} \times M_r$
68
43
31
20
14
0 1 10 20 30 60 S
(C)
$10^{-3} \times M_r$
68
43
31
20
14
0 1 10 20 30 60 S
Time (min)

of M_r values 29,000 and 27,000 which were cleaved to smaller peptides M_r 14,000 after 20 min (De Marcucci et al., 1985b).

As revealed by this 'immune mapping' technique, there were clear differences in the pattern of peptides generated from E2 and X after elastase treatment. To obtain more definitive 'maps' to compare these proteins, peptide mapping of the ^{125}I-labelled proteins, purified by extraction from polyacrylamide gels, were obtained by the procedure devised by Cleveland et al. (1977).

Fig. 6.10 (A) illustrates the 'maps' obtained after proteolysis of subunits E2 and X with elastase. It is apparent that E2 was initially cleaved into two fragments of M_r 45,000 and 29,000. At longer time intervals, the M_r 45,000 peptide was degraded whereas the band at 29,000 increased. Other peptides at M_r values 25,000 and 18,000 appeared at longer incubation times. Component X was relatively more resistant to hydrolysis and was cleaved into a M_r 30,000 peptide, which appeared to degrade slowly into a smaller fragment of M_r 27,000 after longer periods of digestion.

Following incubation with V8 protease, subunit E2 was fragmented into a large peptide M_r 45,000 (indicated with an arrow), whereas component X was degraded into several peptides of M_r 40,000-28,000, which were not detectable in the maps of the E2 subunit.

Limited proteolysis by α-chymotrypsin (Fig. 6.11) showed that component X was cleaved into two principal fragments of M_r values 35,000 and 18,000, which were rather resistant to further proteolysis, whereas subunit E2 was fragmented into a number of peptides of M_r values in the range 45,000-25,000.

In summary, one-dimensional peptide maps from subunits E2 and X, obtained with similar amounts of radioactively-labelled purified proteins, indicated no apparent similarities in the patterns generated by various

Fig. 6.10 One-dimensional peptide maps of subunits E2 and X during limited proteolysis with elastase and V8 protease (S.aureus)

^{125}I-labelled subunits E2 and X were obtained after resolution of the dissociated PDC, extraction from SDS-gels and radioiodination with ^{125}I by the Iodogen method, as described in sections 2.2.10.f and 2.2.14.c. Samples containing approx. 2 x 10^5 c.p.m. of each protein were incubated at 35° C with elastase (0.03 µg) or V8 protease (0.15 µg) as outlined in section 2.2.16.a.

At the indicated times, 10 µl aliquots were removed and proteolysis stopped after boiling for 5 min with Laemmli sample buffer. Samples were subjected to electrophoresis on 15% (w/v) polyacrylamide slab gels. Characteristic peptide patterns were observed after autoradiography of the dried gels.

(A) Autoradiogram of samples obtained during proteolysis with elastase.

(B) Autoradiogram of samples obtained during incubation with V8 protease.

Lanes E2 and X correspond to the radioactive material before incubation. S, ^{125}I-labelled M_r standards.

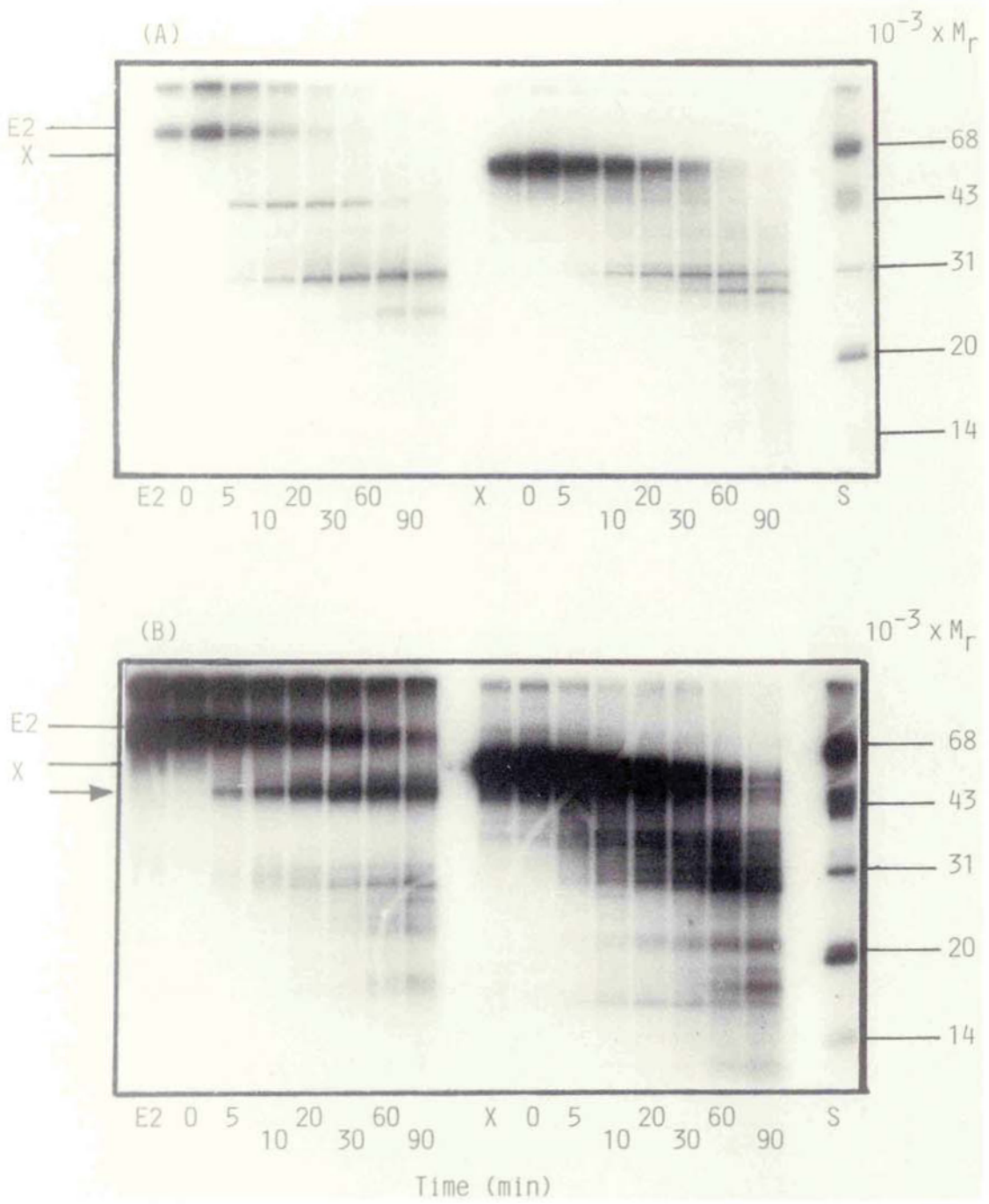
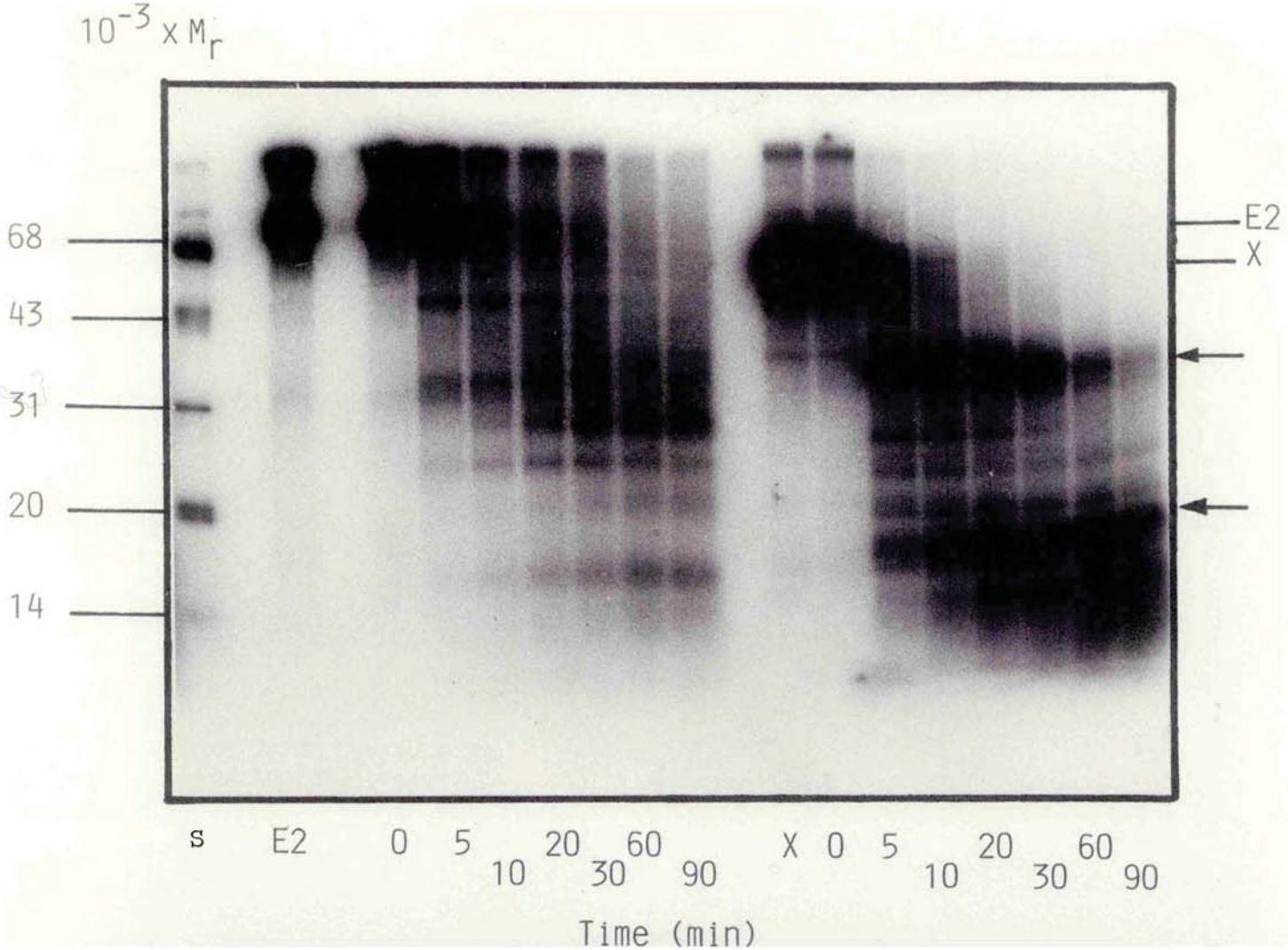

(A)
10^{-3} x M_r
E2
X
68
43
31
20
14
E2 0 5 10 20 30 60 90 X 0 5 10 20 30 60 90 S
(B)
10^{-3} x M_r
E2
X
68
43
31
20
14
E2 0 5 10 20 30 60 90 X 0 5 10 20 30 60 90 S
Time (min)

Fig. 6.11 <u>One-dimensional peptide map of subunits E2 and X during limited proteolysis with α-chymotrypsin</u>

A time course of proteolysis of ^{125}I-labelled subunits E2 and X with α-chymotrypsin (0.06 μg) was performed under similar conditions as described for elastase and V8 protease in Fig. 6.10. Samples were electrophoresed on a 15% (w/v) polyacrylamide slab gel. Peptide bands were detected after autoradiography of the dried gel.

Lane S, ^{125}I-labelled M_r standards.


10⁻³ x Mr
68
43
31
20
14
E2
X
S
E2
0
5
10
20
30
60
90
X
0
5
10
20
30
60
90
Time (min)

proteases of differing specificities. These results clearly argue against the possibility that component X is a large fragment derived from the E2 subunit.

In Chapter 7, section 7.5, peptide maps of ^{14}C-labelled subunits E2 and X are presented. Results in these sections provide additional support for this conclusion

6.8 Studies on the possible function of component X

On the basis of the main features of protein X, e.g. its M_r value of 51,000± 1,000, subcellular localisation and strong association with the core enzyme, studies were carried out to establish its possible relationship to known mitochondrial proteins which share these characteristics. It has been reported that citrate synthase, a functionally-linked enzyme of the citric acid cycle is capable of association with PDC under certain conditions. (Sümegi et al., 1980; Sümegi & Alkonyi, 1983). This association was demonstrated by co-precipitation with poly(ethylene glycol) and by gel chromatography of the purified proteins. In this interaction, citrate synthase does not bind preferentially to the core enzyme, but seems to bind to all components of PDC with similar affinities. Citrate synthase is also a dimer of two identical subunits M_r 48,000. (Singh et al., 1970).

In spite of these reports citrate synthase activity was not detectable in purified ox heart PDC fractions; this result was also confirmed after immunoblotting analysis with anti-citrate synthase serum such as shown in Chapter 5 Fig. 5.6, lane 6.

The acetyltransferase component of the branched-chain 2-oxo-acid dehydrogenase complex has identical subunits with an estimated M_r 52,000 (Pettit et al., 1978) which also possess structural similarities to the acetyltransferase of PDC (Chuang et al., 1984).

After immunoblotting analysis using the specific antiserum against the acetyltransferase subunit of the branched-chain 2-oxo-acid dehydrogenase complex (Fig. 6.12, B), there was no cross-reaction with PDC or purified component X. Moreover, anti-X serum failed to cross-react with any subunit of the purified branched-chain 2-oxo-acid dehydrogenase complex (Fig. 6.12, C).

This result (see also Chapter 4, Fig. 4.5) was in line with evidence from reconstitution studies with the components from the bovine pyruvate dehydrogenase and branched-chain 2-oxo-acid dehydrogenase complexes which showed that the isolated enzymes do not interact with each other to any significant extent. (Cook et al., 1985).

Regarding the possibility that protein X corresponded to the catalytic subunit of the PDC phosphatase M_r value 50,000, (Teague et al., 1982; Pratt et al., 1982) evidence for the absence of detectable phosphatase activity in the purified PDC fractions has been discussed in Chapter 3, section 3.2.2.

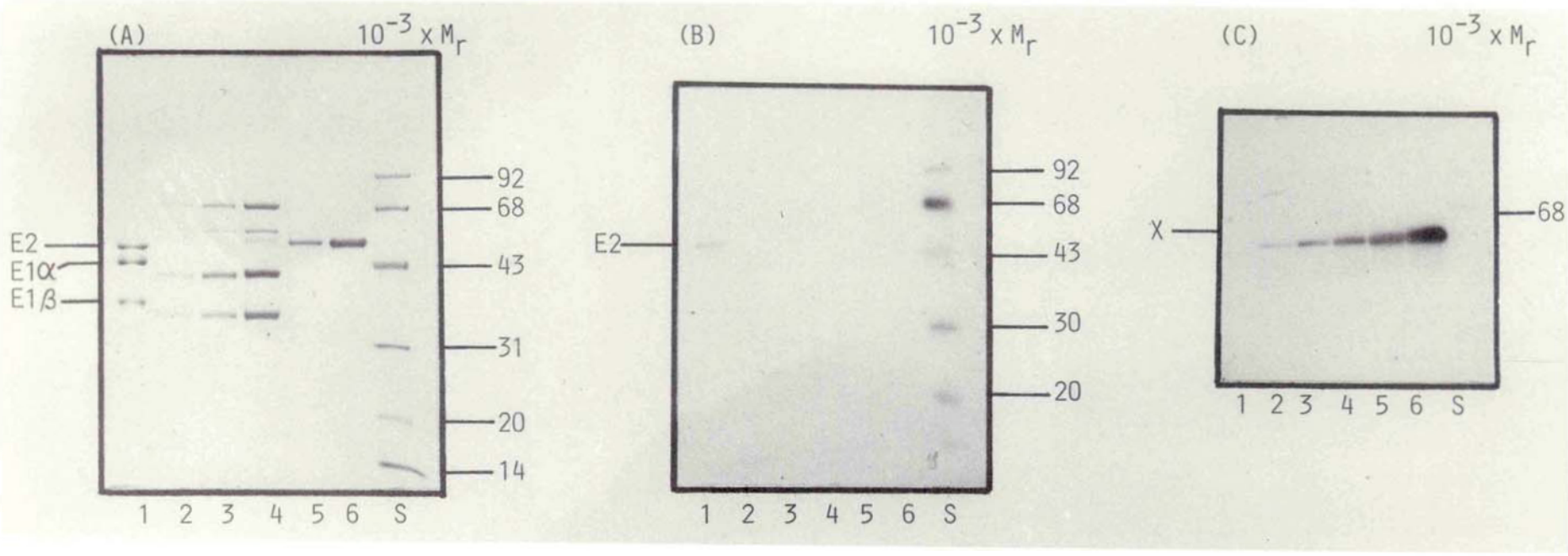
An additional feature of the 50,000 M_r polypeptide that may provide a clue for its specific function was provided from studies in which incubation of PDC with [2-^{14}C] pyruvate, resulted in the covalent association of the label, presumably in the form of acetyl groups, with both components E2 and X. Studies on the characteristics of this reaction and the involvement of component X will be the subject of Chapter 7.

Fig. 6.12 Immunological analysis of PDC and branched-chain 2-oxo acid dehydrogenase complex with antisera against component X and E2 component of branched-chain 2-oxo-acid dehydrogenase complex

Samples of purified branched-chain 2-oxo-acid dehydrogenase complex, PDC and component X were electrophoresed on a 10% (w/v) polyacrylamide slab gel and analysed by immunoblotting with the antiserum against the E2 component of the branched-chain 2-oxo-acid dehydrogenase complex (B). The same blot was then processed with antiserum directed to component X (C). A similar gel was stained with Coomassie Blue (A).

(A) and (B or C), Lane 1, branched-chain 2-oxo-acid dehydrogenase complex 10 μg and 0.5 μg protein, respectively; lanes 2, 3 and 4, PDC 2.5 μg, 5 μg and 10 μg, respectively; lanes 5 and 6, purified component X, 1.5 μg and 3 μg; lane S, ^{125}I-labelled M_r standards.

E2, E1 α and E1 β refer to the acetyltransferase and branched-chain amino acid dehydrogenase subunits of the branched-chain 2-oxo-acid dehydrogenase complex, respectively.


(A)
$10^{-3} \times M_r$
E2
E1α
E1β
92
68
43
31
20
14
1 2 3 4 5 6 S
(B)
$10^{-3} \times M_r$
E2
92
68
43
30
20
1 2 3 4 5 6 S
(C)
$10^{-3} \times M_r$
X
68
1 2 3 4 5 6 S

Chapter 7: STUDIES ON THE INVOLVEMENT OF COMPONENT X IN THE ACETYLATION REACTIONS OF PYRUVATE DEHYDROGENASE COMPLEX

7.1 Introduction

Investigations on the dihydrolipoyl acetyltransferase from both prokaryotic and eukaryotic cells have shown that, besides its structural role, the core enzyme contains lipoic acid moieties covalently attached by amide bonds to the ε -amino groups of lysine residues. This cofactor participates in the transfer of electrons and acyl groups between the catalytic sites of E1 and E3.

Current evidence suggests also that the acetyl transfer reactions in PDC occur after the initial decarboxylation step, which is catalysed by E1 with the production of CO_2 and an enzyme-linked hydroxyethyl TPP derivative. Acetyl group generation may be considered as a reductive acetylation of E2-bound lipoic acid. It is believed that the reactive hydroxyethyl derivative attacks the dithiolane ring of lipoamide in a nucleophilic displacement reaction followed by a reverse condensation to form 6,S-acetyl dihydrolipoamide-E2 (Koike & Koike, 1976).

Enzyme E1 catalyses both the decarboxylation step and the subsequent acetylation of the lipoyl groups bound to E2, which mediates the transfer of acetyl groups to CoA in a displacement reaction. The final step catalysed by a flavoprotein, consists of an oxidation of the E2-bound dihydrolipoamide followed by reduction of NAD^+.

Evidence for the scheme illustrated in Fig. 7.1. is based largely on model reactions carried out with resolved components (Barrera et al., 1972; Bleile et al., 1981); identification of the thioester intermediate (Gunsalus et al., 1956; Reed & Cox, 1966) and kinetic analysis and electron microscopy of the isolated complex and acetyltransferase component (Reed & Oliver, 1968; Reed, 1974).

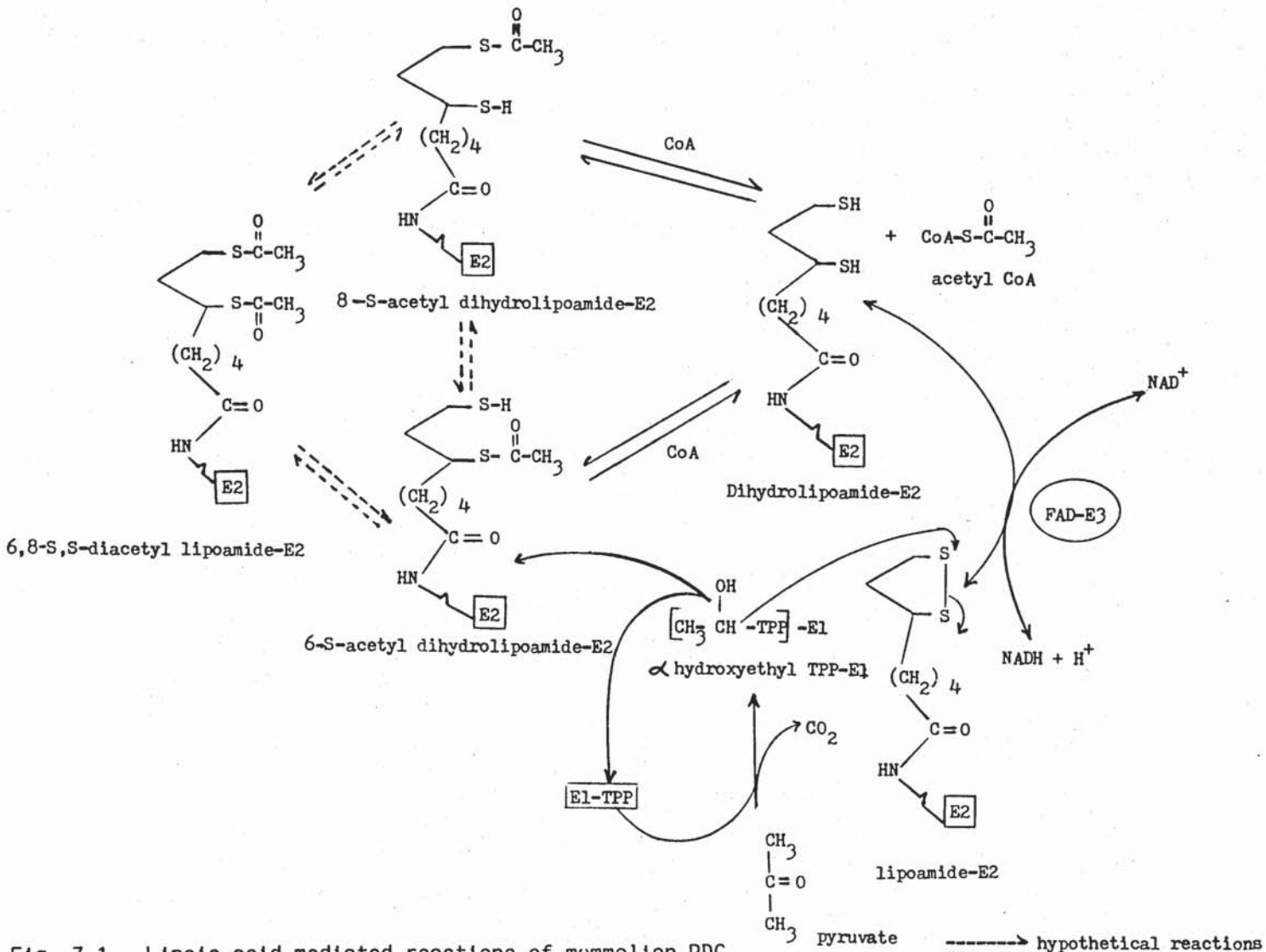


Fig. 7.1. Lipoic acid-mediated reactions of mammalian PDC

O'Connor et al. (1982) have presented ^{13}C n.m.r. data which indicate that both 6- and 8- thiols of dihydrolipoamide are acetylated, following reaction of the dihydrolipoamide with [1-^{13}C] AcSCoA in the presence of the E2 component of bovine kidney. These findings are in conflict with earlier data which indicated that only the 6-S-acetyl dihydrolipoamide was formed.

It was reported (Brown & Perham, 1976; Danson & Perham, 1976) that the acetyltransferase component of E. coli PDC can be inactivated selectively by maleimides in the presence of pyruvate or NADH. The E2-bound lipoyl moieties became reductively acetylated and the S-acetyl dihydrolipoamide in turn reacted with the maleimide, inactivating the enzyme.

Substrate or product-induced sensitisation of the complex to sulphydryl reagents has afforded a simple method for determining the functionally-active lipoyl content of multienzyme complexes (Danson & Perham, 1976; Collins & Reed, 1977), the number of E2 chains per mole of complex (Brown & Perham,1976) and exploring the mobility of lipoyl residues in the intact assembly (Ambrose-Griffin & Perham,1976).

The assembly of the E2 subunits into highly-mobile lipoyl domains and an inner core constitute unique architectural features of this protein that facilitate the interaction of lipoyl moieties with successive active sites on the complex in a multiple random coupling mechanism (Hackert et al., 1983).

7.2 Studies on the effects of NEM on the acetylation of PDC

Initial experiments showed that incubation of native PDC in the presence of the substrate [2-^{14}C] pyruvate, TPP and Mg^{++} in the absence of CoA was accompanied by a rapid incorporation of the label into a TCA-precipitable form. Essentially 95% of the radioactivity incorporated

was released when the labelled complex was exposed to vapours of performic acid as described by Barrera et al. (1972). Based on previous studies these TCA acid-stable groups were assumed to be acetylated lipoyl moieties of the complex (Barrera et al., 1972; Danson & Perham, 1976). As shown in Fig. 7.2, acetylation of native PDC occurred in a biphasic manner. A rapid incorporation of label (completed within 1 min) was followed by a slower phase of acetylation, which was sensitive to the presence of 0.5 mM-NEM.

After examination of the acetylated proteins by fluorography, most of the radioactivity was found associated with the E2 component, while a second acetylated species of M_r 50,000 co-migrating with the band of component X, was also detected (Fig. 7.3).

The instability of the S-acetyl dihydrolipoamide group, particularly in the absence of NEM, was demonstrated by the decrease in radioactivity associated with these proteins following addition of DTT to the Laemmli sample buffer before electrophoresis on SDS/polyacrylamide gels (A) or boiling in this buffer (B) as compared with samples in which no sulphydryl reagents were added before electrophoresis (C).

The incorporation of radioactivity into E2 and X was very rapid as the labelling pattern observed after a 1 min incubation remained essentially unchanged after 30 min.

A similar pattern to that shown in Fig. 7.3 was obtained with samples incubated in the absence of NEM (not shown).

Approx. 50-60 mol acetyl groups were incorporated per mol of complex in the presence of NEM. In spite of some variability, higher levels of acetylation could be obtained routinely after incubation of the complex in the absence of NEM for relatively long periods of time (approx. 50-100% increase above the values observed in the presence of NEM).

Fig. 7.2 Time course of incorporation of acetyl groups from [2-^{14}C] pyruvate into PDC: effects of NEM

Native, NEM-treated or pyruvate-treated PDC were incubated in 50 mM-potassium phosphate buffer, pH 7.2 containing 0.5 mM-$MgCl_2$, 2.5 mM-EDTA, 0.2 mM-TPP in a final volume of 0.2 ml. [2-^{14}C] pyruvate was added at a final concentration of 0.2 mM after a 1 min preincubation at 30°C. Pretreatment of the enzyme with NEM or pyruvate were performed as described in section 2.2.15. Reactions were terminated at the indicated times and 10 µl aliquots were assayed for protein-bound radioactivity by the filter paper discs method.

(—○—) Native PDC (0.2 mg); (—●—) NEM-treated PDC (0.2 mg); (—□—) pyruvate-treated PDC (0.2 mg); (—△—) native PDC (0.2 mg) incubated with NEM added to 0.5 mM before incubation.

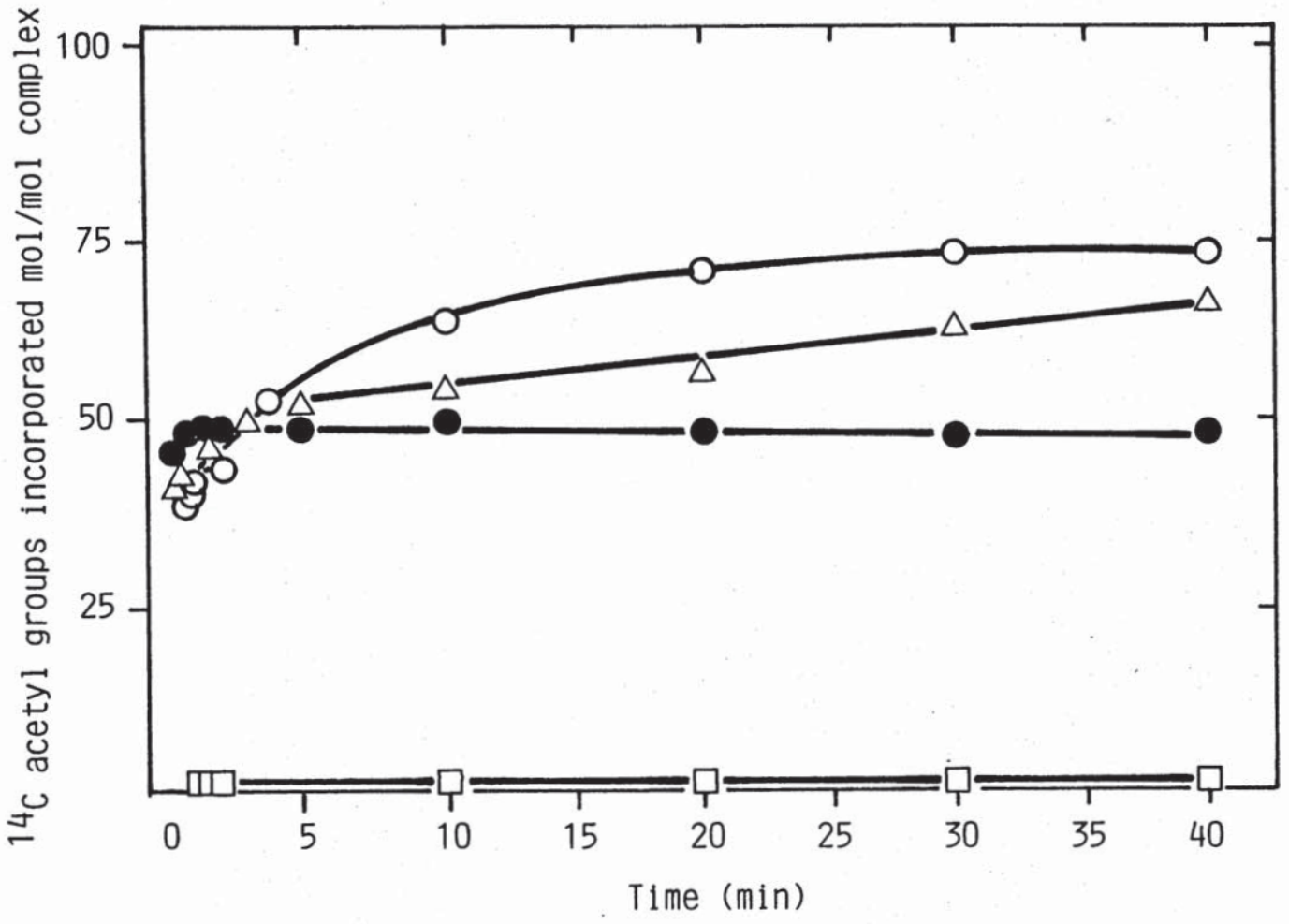
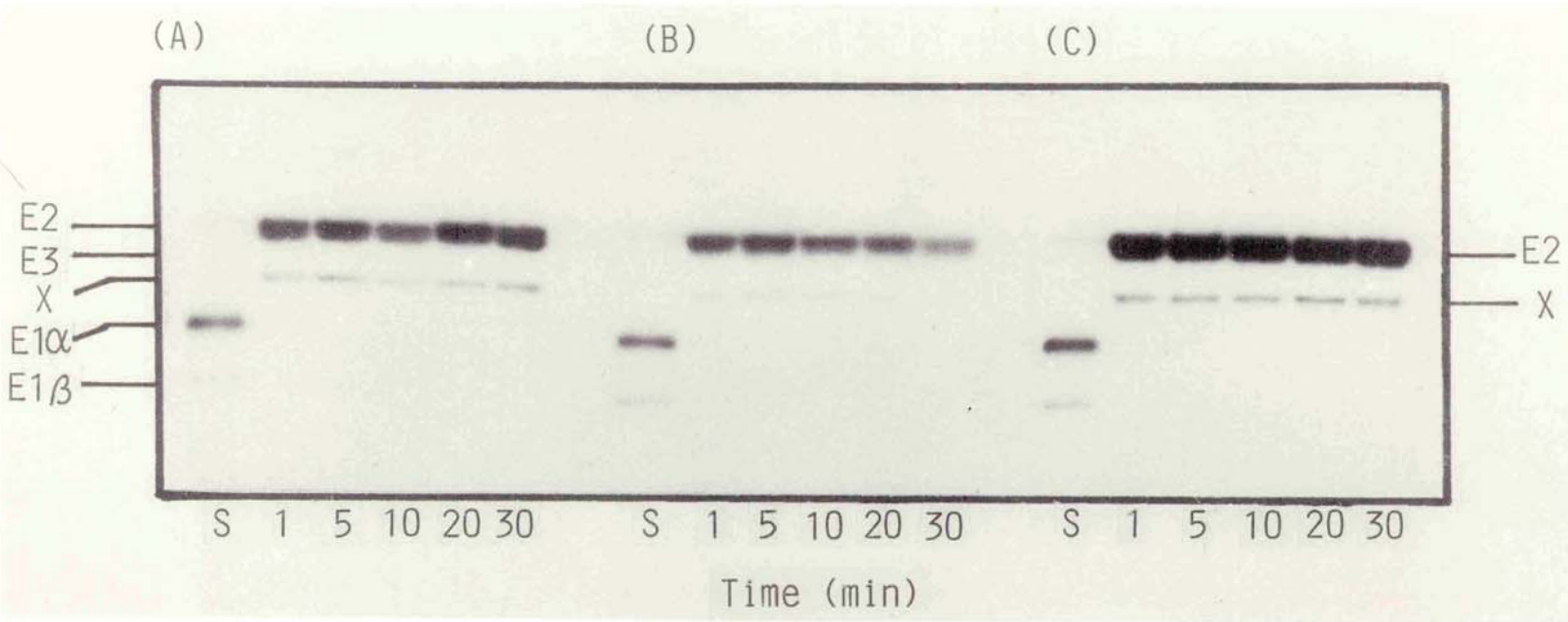

^{14}C acetyl groups incorporated mol/mol complex
100
75
50
25
0
5
10
15
20
25
30
35
40
Time (min)

Fig. 7.3 Analysis of acetylated protein after incubation of native PDC with [2-^{14}C] pyruvate

PDC (0.2 mg) was incubated as described in legend to Fig. 7.2 in presence of 0.5 mM-NEM. Samples containing 25 µg protein were withdrawn at the times indicated, mixed with an equal volume of Laemmli sample buffer containing 10 mM-DTT and boiled for 5 min (B), not boiled (A) or mixed with the same buffer containing no sulphydryl reagents (C) before resolution by electrophoresis on 10% (w/v) SDS/polyacrylamide gels. After staining with Coomassie Blue, the gel was processed for fluorography and dried. Radiolabelled bands were detected after 5 days. S, ^{3}H-labelled PDC.


(A)
(B)
(C)
E2
E3
X
E1α
E1β
E2
X
S 1 5 10 20 30
S 1 5 10 20 30
S 1 5 10 20 30
Time (min)

Cate & Roche (1979) have reported maximal values of 100 mol acetyl groups/mol of complex. These authors interpreted these results as due to the presence of two lipoyl groups/E2 chain, This value is difficult to reconcile with values approaching 60 acetyl groups/mol of complex reported by other authors (Barrera et al., 1972; Stanley et al., 1981) and with the value of 1.11 lipoyl residues/E2 chain reported by White et al. (1980) by isotopic dilution analysis of the bovine kidney and heart PDC.

Although component X participates in the acetylation reactions of the complex it is clear that it is not responsible for the slow phase of acetylation, since labelling of this protein is not increased with respect to E2 as a function of time.

The increased levels of acetylation attained in the absence of NEM, as compared with those in the presence of NEM cannot be readily explained on the basis of two lipoyl groups/chain. As shown for the E. coli complex (Collins & Reed,1977; Danson et al., 1981), the incubation of native PDC with [2-^{14}C] pyruvate resulted in the incorporation of 2.0 acetyl groups per E2 chain. Addition of 0.5 mM-NEM to the incubation mixtures did not modify the extent of acetylation. Results shown in Fig. 7.2 can be ascribed to either the formation of a diacetyl lipoamide derivative or to the acetylation of a secondary site on proteins E2 and/or X (see Fig. 7.1).

O'Connor et al. (1982) have also presented data suggesting that incubation of [1-^{13}C] AcSCoA with dihydrolipoamide produced the 6- and 8- monoacetyl derivatives plus low levels of the 6,8-S,S- [1-^{13}C] diacetyl dihydrolipoamide. The accumulation of this latter species was enhanced after long incubation times. They have proposed that enzymatic acetylation may occur initially in either thiol. The diacetyl derivative may be then a result from a slow intermolecular migration of the acetyl

groups (Fig. 7.1).

NEM-sensitive acetylation sites were generated only in the presence of the substrate, as pretreatment of the complex with NEM did not affect the ability of the complex to attain higher levels of acetylation. A lower rate of incorporation was observed in the NEM pretreated sample in comparison to untreated PDC (Fig.7.2). A similar effect was obtained with a control sample of PDC treated in the same way. These results could be due to the partial loss of enzyme activity observed after ultracentrifugation since the extent of acetylation attained after prolonged incubation was the same for NEM pretreated and control PDC.

The incorporation of ^{14}C-labelled acetyl groups in proteins E2 and X was quantitated in the Coomassie Blue-stained bands of protein after resolution of the acetylated complex by SDS/polyacrylamide gel electrophoresis.

Table 7.1 shows that the incorporation of radioactivity into the isolated E2 and X components when the complex was incubated in the presence of 0.5 mM-NEM was about half of the value obtained when acetylation was performed in the absence of the reagent. Approx. 15% of the total counts were incorporated into protein X both in the presence or absence of NEM.

7.3 Acetylation of components E2 and X at low levels of active PDC

Fig. 7.4 shows that the rate of acetylation of PDC after incubation with [2-^{14}C] pyruvate decreased when assays were performed in the absence of added TPP. Decreasing the proportion of active E1 component by prior incubation of the complex in the presence of 0.2 mM-ATP, markedly decreased the rate of acetylation as the rate-limiting step is the generation of the intermediate hydroxyethyl TPP by E1. Walsh *et al.* (1976)

Table 7.1 Incorporation of ^{14}C-labelled acetyl groups into subunits E2 and X

Subunit	Additions	Incorporation of [2-^{14}C] pyruvate c.p.m./gel slice	% total
Dihydrolipoyl acetyltransferase	None	1,446 ± 247	85.2 ± 2.8
	NEM	613 ± 11	83.6 ± 1.0
Component X	None	250 ± 20	14.7 ± 1.3
	NEM	123 ± 9	16.4 ± 1.1

Native PDC was incubated with [2-^{14}C] pyruvate in the absence or presence of 0.5 mM-NEM as described in section 2.2.15.a. After 30 min incubation, samples containing approx. 22 µg protein were mixed with Laemmli sample buffer before resolution on a 10% (w/v) SDS/polyacrylamide slab gel. Radioactivity in ^{14}C-labelled subunits E2 and X present as Coomassie Blue-stained bands was determined by scintillation counting as described in section 2.2.5.b.

Pyruvate-treated PDC was used as a control for determination of background radioactivity.

Values represent means of three separate determinations.

Fig. 7.4 Time course of acetylation of native or ATP-inactivated PDC: effects of TPP

Active PDC or ATP-inactivated complex were incubated with [2-^{14}C] pyruvate as described in section 2.2.15.a. Other details as described in legend to Fig. 7.2 except those indicated below. Incubation mixtures contained active PDC (0.2 mg) in the presence of 0.5 mM-NEM (—■—) or in the presence (—○—) or absence (—●—) of TPP. ATP-inactivated PDC (0.2 mg) was prepared as described in section 2.2.15.d and incubated in the presence (—△—) or absence (—▲—) of TPP.

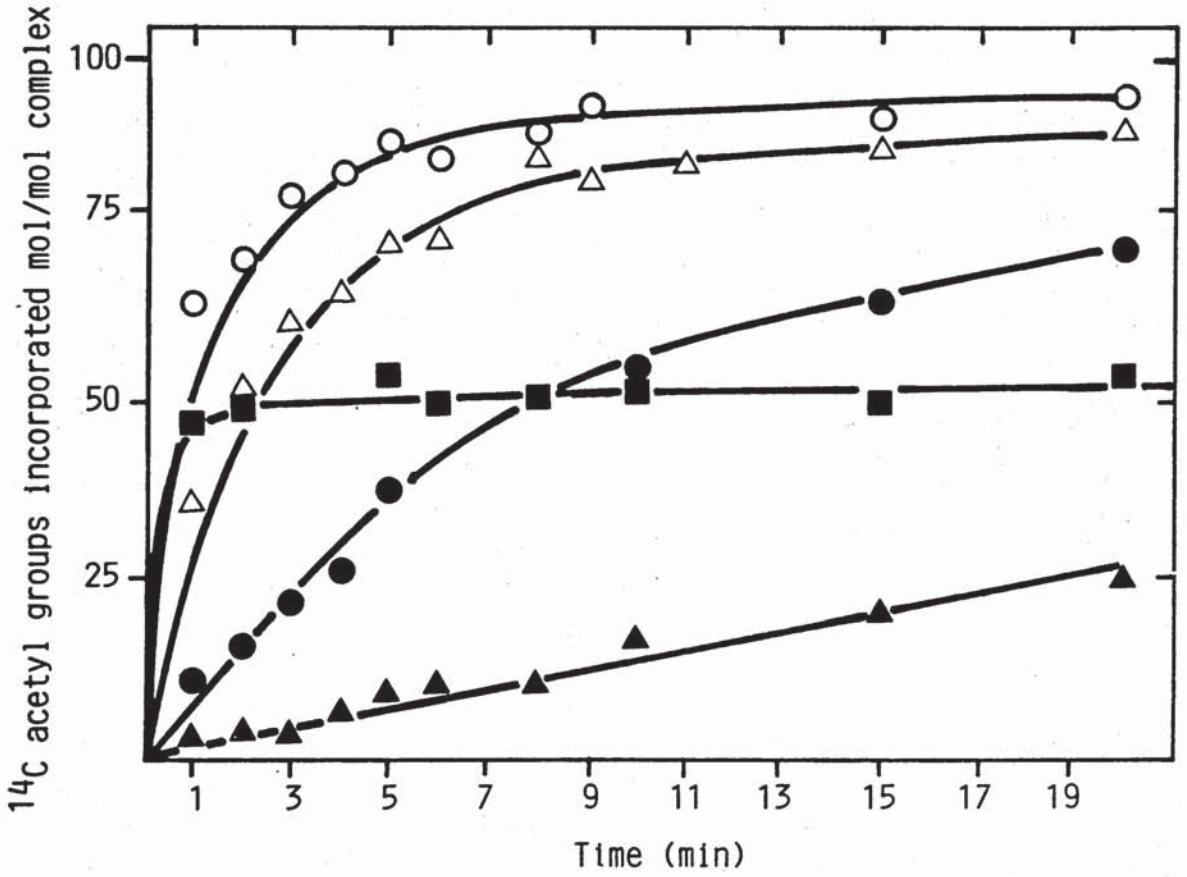
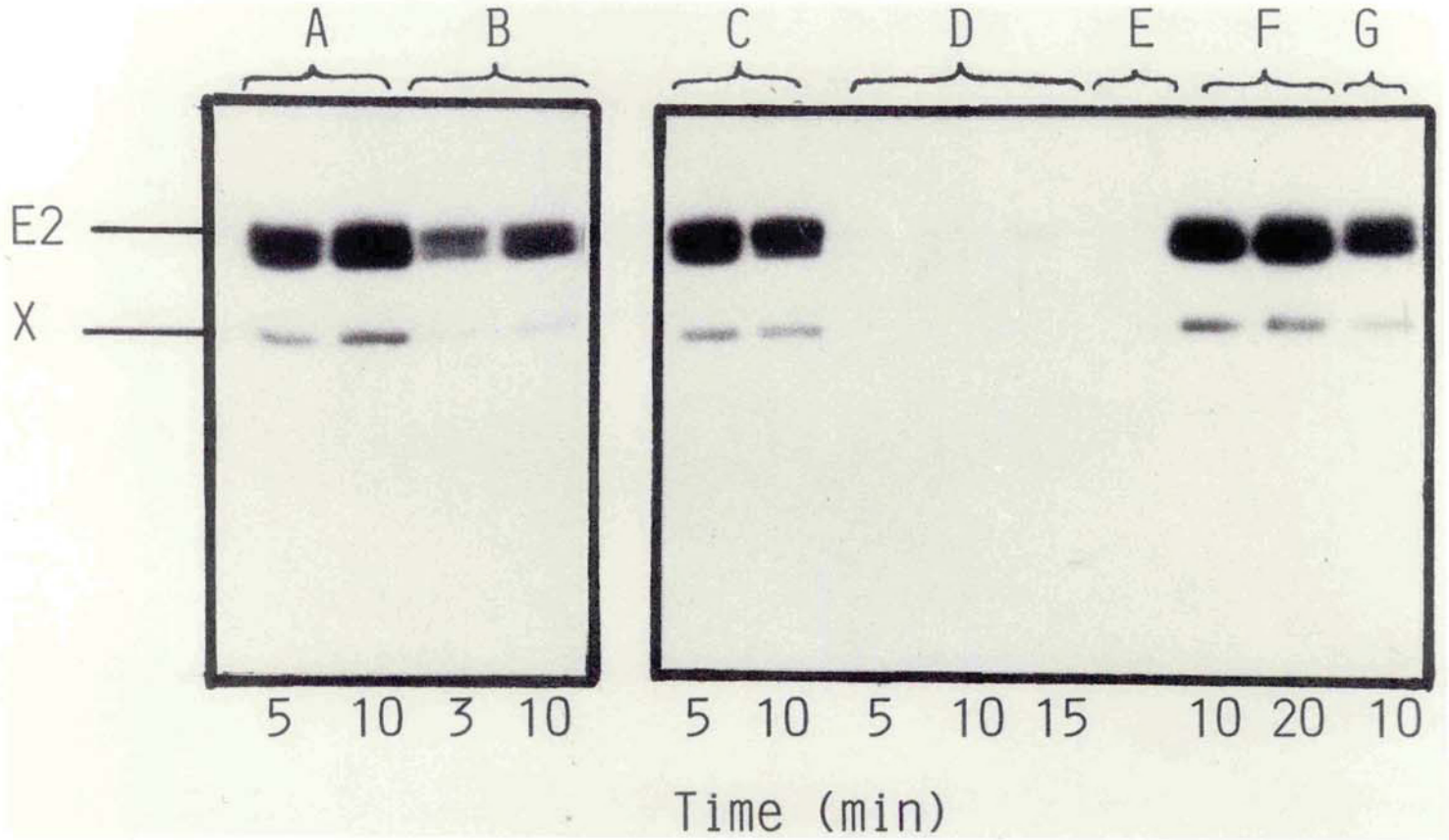

^{14}C acetyl groups incorporated mol/mol complex
100
75
50
25
1
3
5
7
9
11
13
15
17
19
Time (min)

Fig. 7.5 Analysis of acetylated proteins after incubation of native or ATP-inactivated PDC with [2-^{14}C] pyruvate

Samples of native or ATP-inactivated PDC were incubated as described in legend to Fig. 7.4. At the indicated times, samples containing 25 µg protein were mixed with an equal volume of Laemmli sample buffer (without reducing agents) and the samples loaded on a 10% (w/v) polyacrylamide slab gel. Radiolabelled proteins were detected after fluorographic analysis of the dried gel. Native PDC incubated in the presence (A) or absence (B) of TPP. ATP-inactivated PDC incubated in the presence (C) or absence (D) of TPP.

Heat-inactivated PDC (E). Native PDC incubated in the presence of TPP (F) or NEM (G).


A
B
C
D
E
F
G
E2
X
5
10
3
10
5
10
5
10
15
10
20
10
Time (min)

reported that phosphorylation of PDC mainly affects the rate of formation of this compound whereas other reactions such as oxido-reduction of lipoyl groups and binding of pyruvate are unlikely to be modified. However, Roche & Reed, (1972) observed that phosphorylation of PDC decreases its ability to bind TPP suggesting that TPP binding sites and phosphorylation sites on pyruvate dehydrogenase influence each other.

Collins & Reed (1977) and Cate & Roche (1979) have suggested that the high levels of acetylation that can be obtained when only a few E1 molecules are active are achieved by intramolecular transfer of acetyl groups and electron pairs between the lipoyl residues of E2. Cate et al. (1980) have reported also that a slower transfer of E1 components between acetyltransferase cores could also have taken place.

Analysis of the products of acetylation by fluorography (Fig. 7.5) revealed that incorporation of radioactivity into E2 and X components was affected in a similar way.

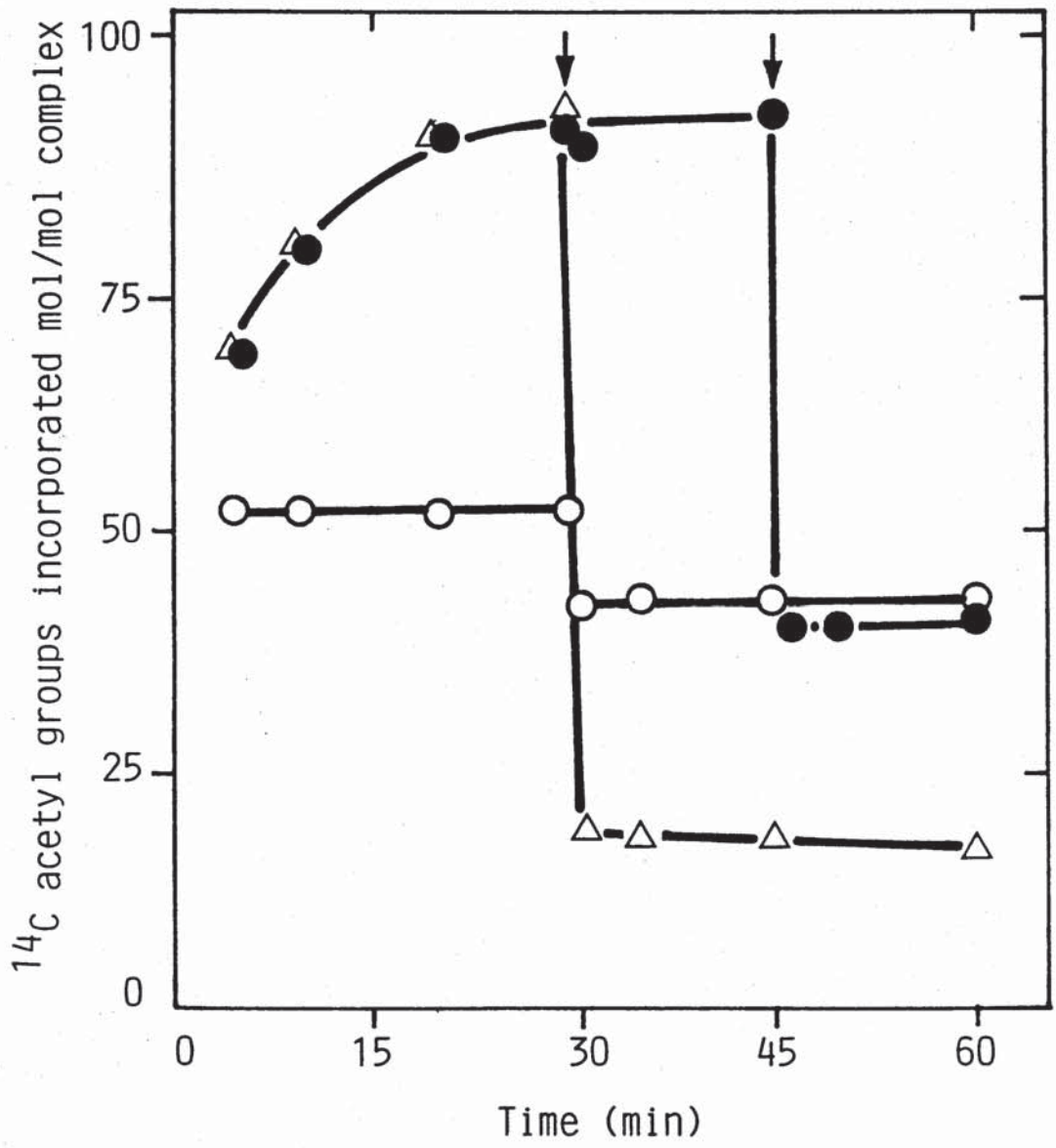
7.4 Deacetylation of PDC

Deacetylation of ^{14}C-labelled PDC was followed by similar assay procedures to those used for acetylation (section 2.2.15.e). In these experiments, PDC was acetylated after incubation with [2-^{14}C] pyruvate for about 30 min in the presence or absence of NEM. At the times indicated by vertical arrows, CoA was added to a final concentration of 1 mM (Fig. 7.6). As shown previously, the ^{14}C-labelled acetyl groups incorporated cannot be removed by CoA from S-acetyl dihydrolipoic acid residues that have been modified with NEM (Collins & Reed, 1977), whereas the protein-bound acetyl groups are almost totally released by CoA in the absence of NEM.

When NEM was added to the incubation mixtures 15 min before CoA,

Fig. 7.6 Time course of deacetylation of ^{14}C-labelled PDC on incubation with CoA

Native PDC (0.2 mg) was incubated with [2-^{14}C] pyruvate at 30°C in the presence or absence of 0.5 mM-NEM. Aliquots of 10 µl were assayed for protein-bound radioactivity. Other details as described in section 2.2.15.e. At the time indicated by the first vertical arrow, CoA was added to a sample of native PDC incubated in absence of NEM (—△—) and to the sample incubated in presence of NEM (—○—) To a duplicate sample of native PDC, NEM was added at a final concentration of 0.5 mM after 30 min incubation (—●—) CoA was added to this sample to a final concentration of 1 mM, at the time indicated by the second vertical arrow.


^{14}C acetyl groups incorporated mol/mol complex
100
75
50
25
0
0
15
30
45
60
Time (min)

it was observed that deacetylation was not totally inhibited. This was an unexpected result suggesting that at least some of the NEM sensitive thiol groups were not accessible to the reagent following reductive acetylation by pyruvate. However, it should be emphasized that these experiments must be interpreted with caution as CoA also reacted with free NEM. (CoA was present in 2-fold excess with respect to NEM).

To circumvent this difficulty, a similar experiment was performed (Fig. 7.7). After acetylation of the complex for 30 min and incubation with 0.5 mM-NEM for 15 min at 30°C, excess reagent was removed by centrifugation of the complex prior to incubation in the presence of CoA. It was demonstrated that the NEM-pretreated PDC was capable of releasing the acetyl groups bound to the protein in the presence of CoA to the same extent as control PDC.

Analysis of the products obtained after deacetylation of the complex in the presence of CoA by SDS/polyacrylamide gel electrophoresis and fluorography (Fig. 7.8) indicated that radioactivity associated with the subunits E2 and X was released in a similar way from NEM-pretreated and control PDC (lanes 2 and 7).

One plausible explanation for these results in that thiol groups sensitive to NEM modification are generated only transiently during the reactions of acetylation and deacetylation. It is possible that a second acetylation site becomes available in the complex under these conditions, like a cysteine thiol group. Alternatively, one must consider the possible formation of diacetyl dihydrolipoamide during prolonged incubation of the complex. The existence of these species would explain the high levels of acetylation and the insensitivity of the complex to NEM modification after full acetylation. In this respect

Fig. 7.7 Time course of deactylation of ^{14}C-labelled PDC: effects of NEM pretreatment

Native PDC (2.6 mg) was incubated for 30 min at 30° with [2-^{14}C] pyruvate under standard conditions. The sample was separated into two portions which were incubated for 15 min at 30°C in the absence (control sample) or presence of 0.5 mM-NEM. PDC was recovered after ultra-centrifugation as described in section 2.2.15.b and incubated in the presence of 1 mM-CoA for 30 min as described in section 2.2.15.e. Aliquots of 10 µl were assayed for protein-bound radioactivity at the indicated times.

Curves correspond to (—▲—) NEM-pretreated PDC; (—○—) control PDC; and (—△—) control PDC incubated in the absence of CoA.

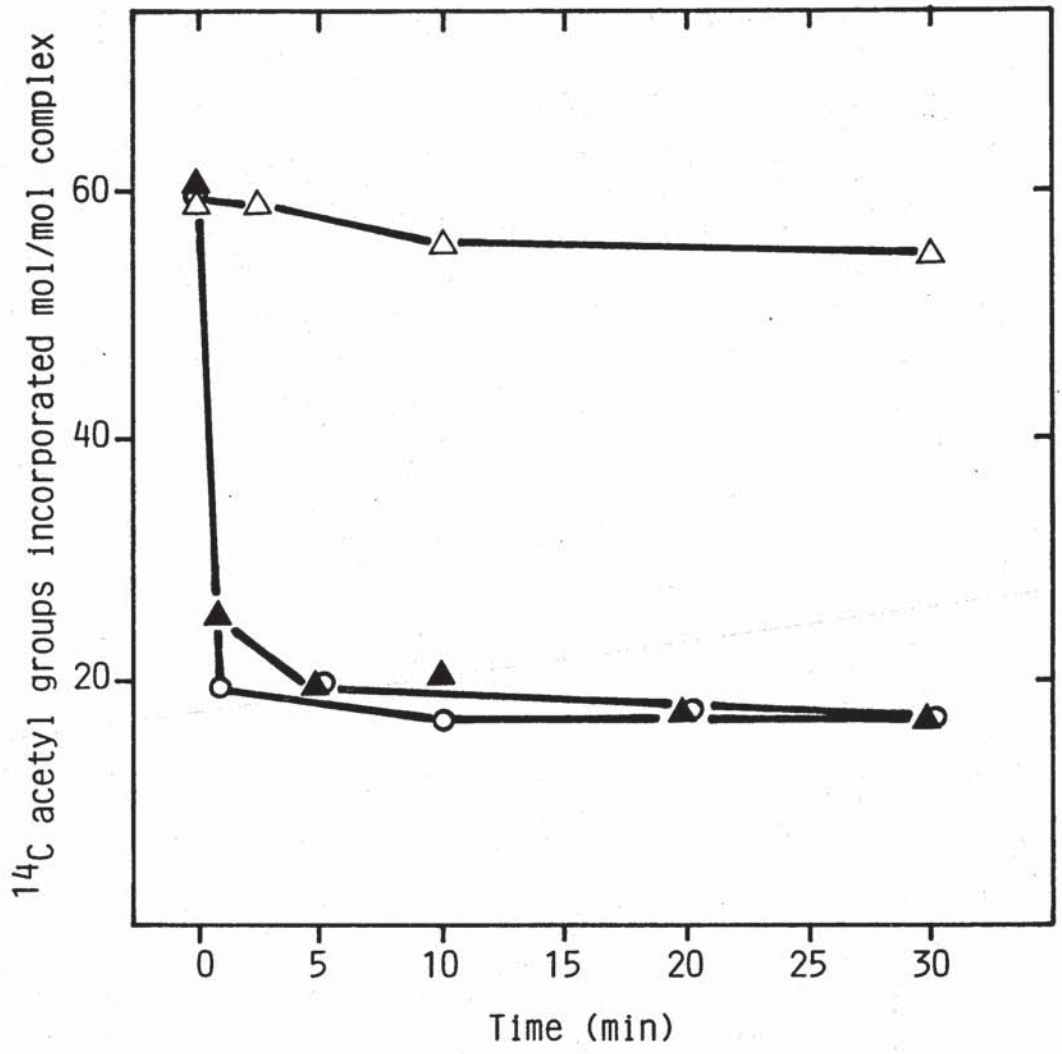
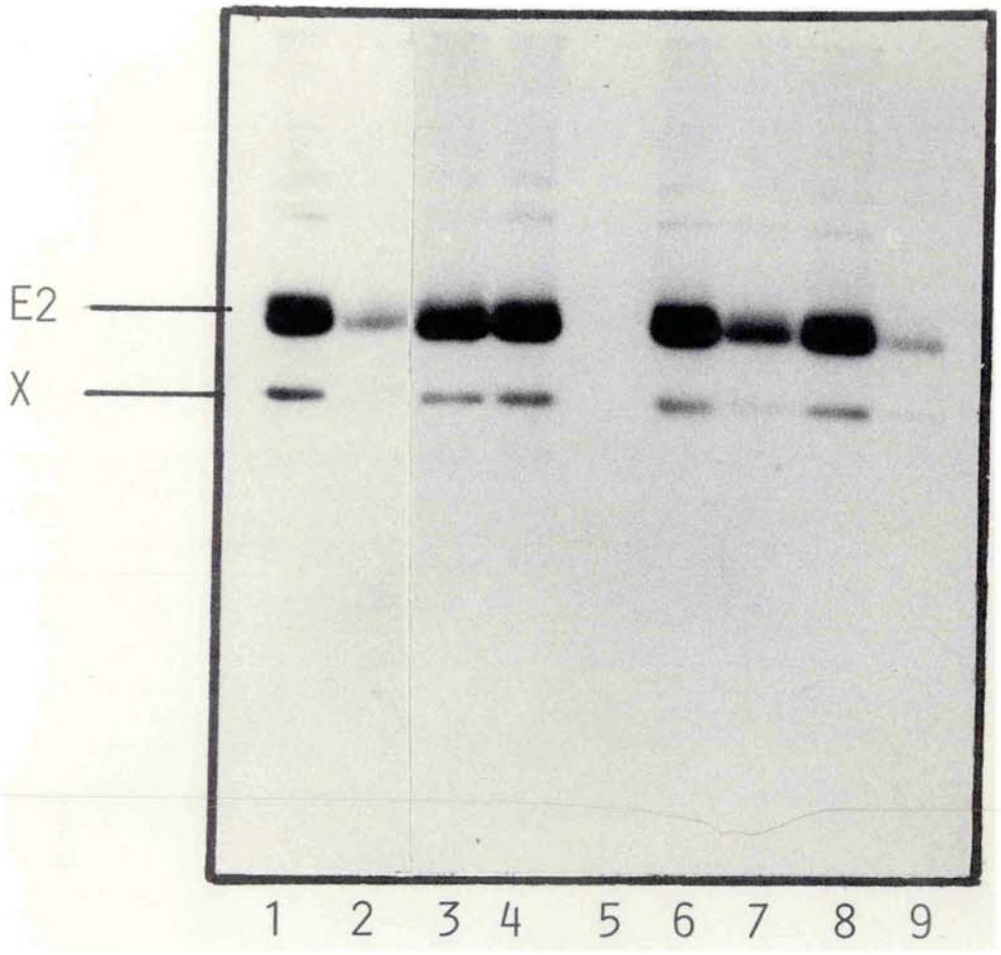

^{14}C acetyl groups incorporated mol/mol complex
60
40
20
0
5
10
15
20
25
30
Time (min)

Fig. 7.8 Fluorographic analysis of the products of deacetylation of PDC in the presence of CoA

Samples from experiments described in Fig. 7.6 and 7.7 containing 25 µg protein were analysed by electrophoresis on 10% (w/v) polyacrylamide gels followed by fluorography.

For each sample, it is shown the material labelled, before or 15 min after the addition of CoA to 1 mM. Lanes 1 and 2, native PDC acetylated in the absence of NEM; lanes 3 and 4, PDC sample acetylated in the presence of NEM; lane 5, heat-inactivated PDC; lanes 6 and 7, NEM-pretreated PDC deacetylated in the presence of NEM; lanes 8 and 9, same as 6 and 7 but deacetylation took place in the absence of NEM in the assays.


E2
X
1 2 3 4 5 6 7 8 9

it may be of interest to determine whether the extent of deacetylation depends on the time of incubation of the complex in the presence of [2-^{14}C] pyruvate.

The identification of the groups involved in the second acetylation in E2 (and/or X) is necessary to determine the physiological importance of this reaction.

7.5 Peptide mapping of acetylated E2 and X subunits

Immunological studies carried out with specific antisera as well as other studies presented in Chapter 6 strongly support the idea that component X is structurally different from the E2 component. However, in functional terms, there is a strong resemblance between these proteins: i) the incorporation of ^{14}C-labelled acetyl groups is dependent on the activity of the E1 component; ii) the incorporation of these groups is reduced in a similar degree when thiol or lipoyl groups associated with the complex are modified by NEM (approx. 50%); iii) ^{14}C-labelled acetyl groups are released from both proteins after incubation with CoA. These characteristic properties of the acetylation reaction suggest that lipoyl groups are present in both E2 and X components.

Experiments were designed to establish peptide maps of the ^{14}C-labelled subunits E2 and X to obtain information about the M_r values and time course of degradation of peptides containing the radiolabelled acetyl groups from each protein.

For this purpose, approx. 3 mg PDC were incubated in the presence of [2-^{14}C] pyruvate, concentrated by precipitation with 5% (w/v) poly(ethylene glycol), dissociated and resolved on a SDS/ polyacrylamide preparative gel. Peptide-mapping was performed as described by Cleveland et al. (1977) after elastase digestion of the proteins in the stacking gel of a second polyacrylamide slab. The ^{14}C-labelled peptides derived

from subunits E2 (A) and X (B) were detected by fluorography (Fig. 7.9).

In spite of the high amount of elastase used for these experiments, subunit E2 remained largely undegraded. The main product appeared as a 45,000-M_r peptide that was relatively resistant to further hydrolysis. ^{14}C-labelled peptide present in component X appeared initially to have an M_r value of 30,000 and was further degraded to a 25,000-M_r fragment and then to peptides of M_r values $\leq$ 10,000 at higher elastase/protein ratios. These results clearly indicate that the peptides containing the ^{14}C-labelled acetyl groups are different in component X as compared with E2. This constitutes further evidence for structural differences between these proteins.

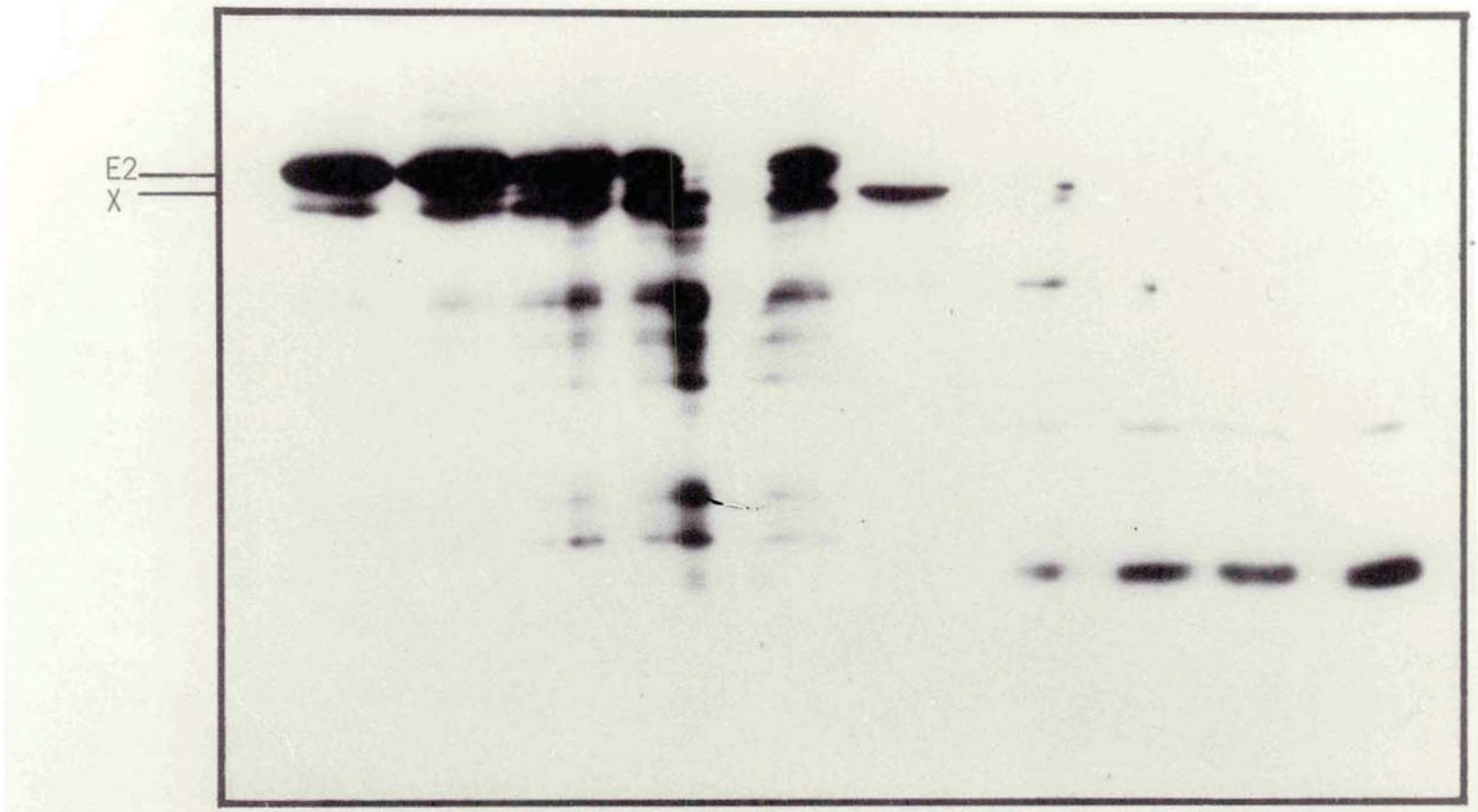
In a refinement of this approach, peptide maps of subunits E2 and X were obtained after isolating the ^{14}C-labelled proteins from SDS/ polyacrylamide gels.

PDC was labelled after incubation in the presence of [2-^{14}C] pyruvate and N-ethyl [2,3-^{14}C] maleimide. Under the experimental conditions used approx. 95% of the incorporated radioactivity was detected in components E2 and X (J.A. Hodgson, unpublished results).

This procedure led to stablisation of the radioactivity incorporated allowing the electrophoretic resolution of the complex and elution of proteins from the gel slices without the large losses observed when the complex was labelled with [2-^{14}C] pyruvate and NEM. These losses were probably due to the heating and long incubation of the gel slices at alkaline pH involved in the elution procedure (see also Fig. 7.3). Preliminary experiments showed that elastase activity was inhibited shortly after addition of the enzyme to the extracted proteins, presumably caused by the high levels of SDS present in the samples. Therefore, the protease was added in successive portions (0.5 µg protein each).

Fig. 7.9 Peptide mapping by limited protelysis of subunits E2 and X in gel slices

Subunits E2 and X were labelled after incubation of native PDC (2 mg) with 0.1 mM- [2-^{14}C] pyruvate under standard conditions for acetylation (section 2.2.15.a.). The complex was resolved into its components in a 10% (w/v) SDS/polyacrylamide preparative gel. Bands corresponding to subunits E2 and X were visualised after brief staining with Coomassie Blue. Gel slices of these bands were placed on the sample wells of a second 15% (w/v) polyacrylamide slab gel and each slice was overlayed with the indicated amount of elastase. Digestions were carried out as described in section 2.2.16.a ^{14}C-labelled peptides from subunit E2 (lanes 1-5) and X (lanes 6-10) were detected after fluorography. Ratios protease/protein (w/w) are also indicated.



	1	2	3	4	5	6	7	8	9	10
Elastase (µg)	6.6	13.2	26.4	37.6	66.6	3.3	6.6	13.2	26.4	52.8
Ratio $\frac{\text{protease}}{\text{protein}}$ (w/w)	0.12	0.24	0.48	0.68	1.21	0.16	0.33	0.66	1.32	2.11

Fig. 7.10 shows the course of degradation of the ^{14}C-labelled peptides from subunits E2 (lanes 1-6) and X (lanes 7-12) with increasing amounts of elastase.

Subunit E2 was rapidly cleaved into a fragment of M_r 45,000 containing the ^{14}C-label and an unlabelled peptide M_r 29,000 detectable in the Coomassie Blue-stained gel (not shown). The former fragment was relatively resistant to further hydrolysis and was cleaved to a fragment of M_r 30,000. At higher elastase levels, this fragment was cleaved into smaller peptides $M_r \leq 25,000$.

Component X was more resistant to digestion than subunit E2, under these experimental conditions. This protein was initially cleaved into a fragment of M_r 28,000 which was then converted into a smaller peptide M_r 25,000.

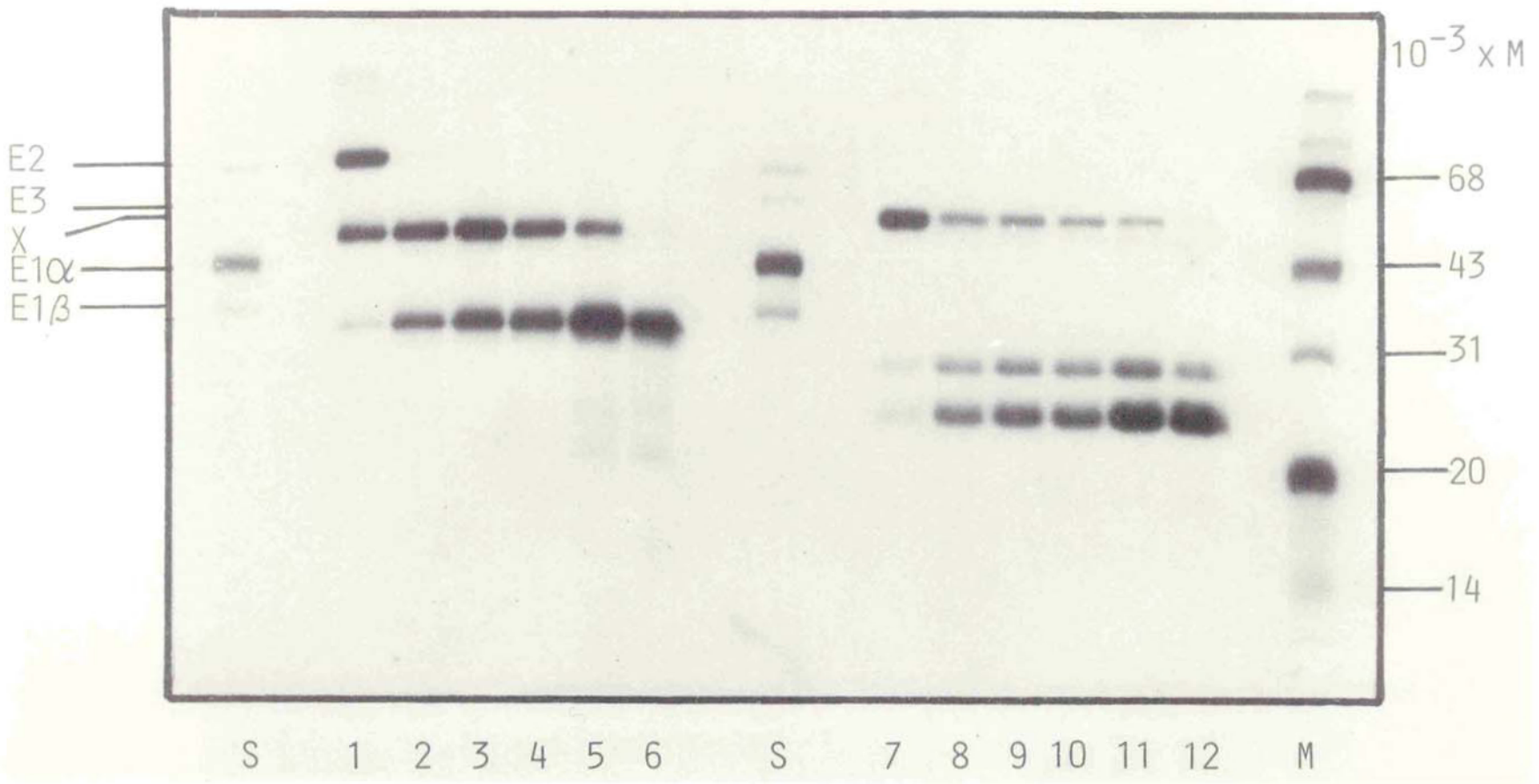
These results are remarkably similar to the peptide maps obtained by partial digestion with elastase by the Cleveland et al. (1977) technique (Fig. 7.9), with the maps from ^{125}I-labelled subunits E2 and X (Chapter 6, Fig. 6.10,A) and with the immunomaps shown in Fig. 6.9. Taken together, these results reveal that antisera raised to subunits E2 and X strongly cross-reacted with fragments of M_r similar to the peptides carrying the acetyl groups. Therefore it is possible to speculate that regions of these proteins capable of bearing acetyl groups (possible lipoyl domains) are highly immunogenic. These results also indicate that the M_r values for these polypeptide regions are quite different between the components E2 and X.

Fig. 7.10 Peptide mapping by limited elastase proteolysis of ^{14}C-labelled subunits E2 and X

^{14}C-labelled subunits E2 and X were purified by extraction from SDS/polyacrylamide gels as described in section 2.2.10.f, after incubation of PDC with 5 µCi each [2-^{14}C] pyruvate and N-[2,3-^{14}C] ethylmaleimide. Samples from the extracts containing 6000 c.p.m. of each protein were incubated in a final volume of 160 µl in 0.125 M-Tris/HCl buffer, pH 7.4, 0.5% (v/v) Triton X-100 at 35°C. Digestions were carried out by successive additions of small amounts of elastase (0.5 µg) up to 3.0 µg. Aliquots (25-50 µl) containing equal amounts (approx. 1000 c.p.m) of subunit E2 (lanes 1-6) or component X (lanes 7-12) were removed 5 min after each protease addition, mixed with Laemmli sample buffer and boiled for 3 min. Samples were loaded onto a 10% (w/v) SDS/polyacrylamide slab gel which was stained with Coomassie Blue before fluorography.

Fluorograph was obtained after 15 days.

S, ^{3}H-labelled PDC; M, ^{125}I-labelled M_r markers.


E2
E3
X
E1α
E1β
10⁻³ x M
68
43
31
20
14
S 1 2 3 4 5 6 S 7 8 9 10 11 12 M

Chapter 8: STUDIES ON THE BIOSYNTHESIS OF MAMMALIAN PYRUVATE DEHYDROGENASE COMPLEX

8.1 Introduction

Mitochondria depend upon the nucleo-cytoplasmic system of gene expression for the biosynthesis of the vast majority of their polypeptides. Therefore, eukaryotic cells must possess mechanisms for sorting newly-formed polypeptides to ensure that the appropriate macromolecules reach their mitochondrial location. Once a specific polypeptide is recognised, it becomes associated with this organelle and routed to its proper intramitochondrial compartment.

Most, but not all, cytoplasmically-synthesised mitochondrial matrix proteins are translated initially as larger precursors of M_r 500-10,000 higher than their corresponding mature forms. A specific matrix protease is responsible for processing translocated precursors to mature mitochondrial proteins during or shortly after entry. (Neupert & Schatz, 1981).

On examining the import of a particular mitochondrial protein it is necessary to determine initially if the polypeptide exists as a transient entity of higher M_r value. There are two general approaches to this problem, both of which rely on the availability of high-titre monospecific antiserum directed against the mature form of the protein of interest (see also section 1.6.4).

A) In vitro protein synthesis. Total or poly(A) containing RNA is isolated and translated under suitable conditions with [^{35}S] methionine in a rabbit reticulocyte lysate or wheat-germ system. After addition of detergents and protease inhibitors, the samples are subjected to immunoprecipitation, SDS/polyacrylamide gel electrophoresis and fluorography.

Radioactive products are compared directly by co-electrophoresis with a sample of radiolabelled mature protein, since the M_r value is the most prominent feature of the precursor polypeptides which permits them to be distinguished from their mature forms.

B) In vivo accumulation of precursors. The biosynthetic pathway of proteins can be examined in vivo by incorporating radioactive amino acids into newly-synthesised polypeptides followed by immunoprecipitation, SDS/polyacrylamide gel electrophoresis and fluorography. After short pulses with [^{35}S]methionine, labelled precursor forms are found in the cytosol (Morita et al., 1981; Reid & Schatz,1982b). However, most precursors have short half-lives (approx. 1-5 min) and their pool sizes are very small.

Import into the mitochondrial matrix, inner membrane and intermembrane space requires an electrochemical potential across the inner membrane. Precursors destined for these compartments, can be accumulated in the cytoplasm if cells are labelled in the presence of uncouplers such as CCCP, DNP and FCCP.

If translocation is blocked while translation is allowed to continue, the pool size of precursors becomes large enough to be detected in short-term labelling experiments. Although the stability of different mitochondrial precursors accumulating in the cytosol varies considerably, many of them appear to be sufficiently stable to allow detection.

Another in vivo strategy has been employed with petite (rho^-) mutant yeast cells in which import of mitochondrial proteins has been blocked with the uncoupler CCCP. Rho^- mutants of S. cerevisiae contain defective mitochondria but normal import/processing mechanisms. In the presence of CCCP, these cells can accumulate precursors in substantial

amounts which allow their detection directly by immunoblotting techniques. (Reid & Schatz, 1982a).

The physiological importance of a biosynthetic pathway is better demonstrated in the in vivo systems using pulse-labelled yeast spheroplasts, primary hepatocytes and various cultured cells. However, only a limited number of studies has been carried out in animal cells using this approach to date.

The availability of high-quality antisera directed specifically against PDC and its individual subunits as well as established cell lines that expressed the enzyme were vital prerequisites for undertaking these studies using the in vivo approach. Optimisation of biosynthetic labelling of cells with radioactive methionine, extraction of cell proteins and antigen immunoprecipitation were gradually developed during this study until they produced consistent and satisfactory results.

8.2 Immunoprecipitation studies with ^{3}H-labelled PDC

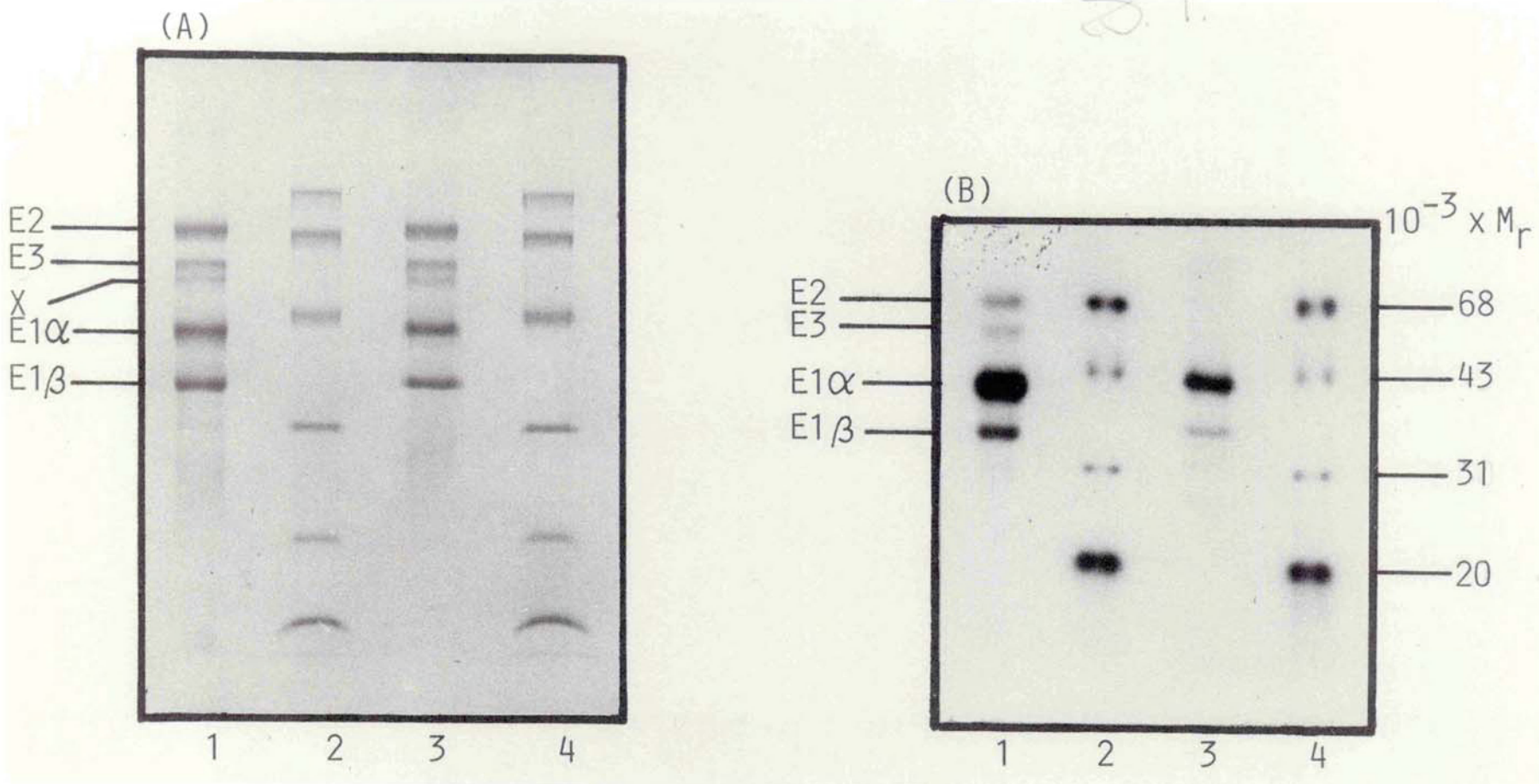
In order to standardise the immunoprecipitation studies, preliminary experiments were conducted with purified PDC using various immunoprecipitation protocols. Detection of the immunoprecipitated products was facilitated by labelling free thiol groups on the enzyme with N-[^{3}H] ethylmaleimide.

Fig. 8.1 demonstrates the pattern of radioactive polypeptides obtained from the native and denatured complex. Fluorographic analysis indicated that in the native complex only the E1 component was labelled to a significant extent (in the absence of pyruvate), whereas in the dissociated state subunits E2 and E3 were labelled also. This difference probably arises from exposure of thiol groups in the E2 and E3 polypeptide chains following unfolding of the proteins in the presence of SDS. No significant incorporation of radioactivity into component X could be detected under these conditions.

Fig. 8.1 Subunit labelling of denatured and native PDC with N-[^{3}H]ethylmaleimide

SDS-dissociated and native PDC (100 ug protein) were labelled by treatment with 50 μCi N-[^{3}H] ethylmaleimide as described in section 2.2.13. Samples of PDC were mixed with ^{3}H-labelled enzyme (approx. 5,000 d.p.m.) before resolution on a 10% (w/v) polyacrylamide slab gel. After staining with Coomassie Blue (A), the gel was processed for fluorography (B).

(A and B) Lane 1, SDS-denatured PDC sample; lane 3, native PDC sample; lanes 2 and 4, ^{125}I-labelled M_r standards.


(A)
E2
E3
X
E1α
E1β
1
2
3
4
(B)
E2
E3
E1α
E1β
10⁻³ x Mr
68
43
31
20
1
2
3
4

Immunoprecipitations were performed initially with ^{3}H-labelled PDC in Triton buffer (see section 2.2.11.e) by double immunoprecipitation and S. aureus techniques. The advantages of the latter technique in terms of rapidity and lower background due to non-specific binding were soon obvious. Furthermore, the large amounts of precipitating immunoglobulins present in the immunocomplexes using the double-antibody technique caused some distortion of resolution in the region of M_r 50,000 owing to migration of heavy chains of IgG.

Some of the parameters investigated in this sytem were as follows: a) amount of antibody required for quantitative recovery of antigen, b) effects of the composition of the washing buffer and c) type of subunit precipitated by the antisera.

Fig. 8.2 shows the polypeptide composition of the immunoprecipitated products obtained with antiserum to native PDC (A), anti-E1 α serum (B) and anti-E2 serum (C).

Antiserum to PDC was capable of immunoprecipitating all the components of the complex except subunit E3. Analysis of the supernatant fraction after immunoprecipitation showed that intact E3 subunit remained in this fraction and was not selectively degraded during the procedure.

Immunoprecipitated products from ^{3}H-labelled PDC with antisera to E1 α and E2 subunits showed that in each case only the corresponding specific protein was selectively immunoprecipitated.

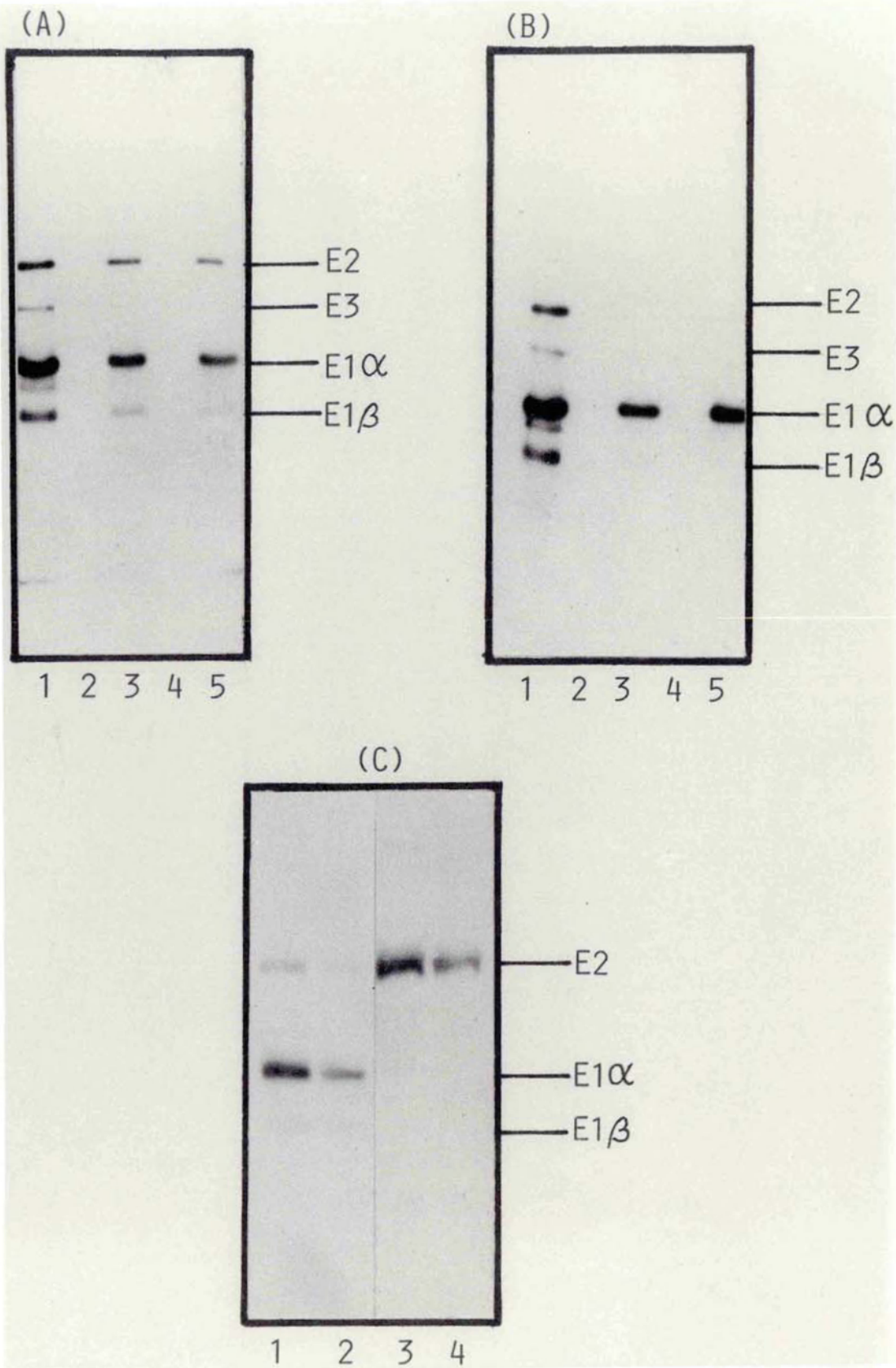
8.3 Biosynthetic labelling and extraction of cell proteins

For studies on the biosynthesis of PDC, it was necessary to incorporate radioactive amino acids into the newly-synthesised components of the complex, before monitoring the fate of radioactivity by selective immunoprecipitation, SDS/polyacrylamide gel electrophoresis and fluorography.

Fig. 8.2 Immunoprecipitation of subunits from dissociated ^{3}H-labelled PDC

SDS-denatured PDC labelled with N-[^{3}H] ethylmaleimide (approx. 30,000-50,000 c.p.m.) was incubated with anti-PDC (A), anti-E1 α (B) or anti-E2 sera in Triton-buffer (0.01 M-Tris/HCl buffer, pH 7.4, 1% (v/v) Triton X-100, 0.3 M-NaCl, 5 mM-EDTA) as described in section 2.2.12.f. Immune complexes were adsorbed onto formalin-treated S.aureus cells before fluorographic analysis of the proteins resolved on 10% (w/v) polyacrylamide slab gels.

(A and B) Lane 1, ^{3}H-labelled PDC before immunoprecipitation; lanes 2 and 4, control immunoprecipitates with 10 μl and 5 μl pre-immune serum, respectively. (A only) Lanes 3 and 5, immunoprecipitates with 10 μl and 5 μl anti-PDC serum. (B) Lanes 3 and 5, immunoprecipitates with anti-E1 α serum, 20 μl and 10 μl, respectively. (C) Lanes 1 and 2, immunoprecipitates with 10 μl and 5 μl of anti-PDC serum, respectively; lanes 3 and 4, immunoprecipitates with 20 μl and 10 μl anti-E2 serum, respectively.


(A)
E2
E3
E1α
E1β
1 2 3 4 5
(B)
E2
E3
E1α
E1β
1 2 3 4 5
(C)
E2
E1α
E1β
1 2 3 4

Cultured cells were labelled with [^{35}S] methionine (100-250 µCi/dish). This isotope was chosen since it is available at high specific activities (> 1000 Ci/mmol) and its high energy of emission enhanced the sensitivity of detection by fluorography. Amino acid composition data has revealed a relative abundance of methionine in all PDC components (Barrera et al., 1972; Hamada et al., 1975; 1976).

Cells were grown at 37°C in standard medium and labelled in semi-confluent monolayers at which stage, the cells were still growing rapidly.

Linear incorporation of [^{35}S] methionine into TCA-precipitable material was obtained for periods of up to 8 h. After 16-20 h labelling between 80-95% of the tracer was incorporated into protein. For short labelling periods (up to 4 h) optimal incorporation was found after preincubation for 1 h in a methionine-deficient medium.

Optimisation studies on the kinetics of incorporation of radioactivity into proteins in various labelling media indicated that radiolabelling was almost as rapid and high in low methionine medium (see section 2.1.2) than in a medium containing no added methionine and no serum.

Solubilisation of cells was performed by quickly mixing the washed monolayers with sterile lysis buffer containing protease inhibitors and inhibitors of processing (PMSF, leupeptin and 1,10-phenanthroline) as indicated in section 2.2.11.e. The non-ionic detergent Triton X-100 completely solubilised the cells while allowing the removal of nuclei and many elements of the cytoskeleton by a short centrifugation. Preliminary studies also indicated that direct solubilisation of the cells in hot SDS, a procedure normally used for yeast spheroplasts and Neurospora hyphae, led to high backgrounds in

immunoprecipitates caused by non-specific binding. The nuclear pellets lacked any detectable PDC as checked by immunoblotting analysis of this fraction.

Incorporation of radioactivity into cell protein was usually determined by direct counting of 5 µl of the labelled extract. Approx. 95-98% of the counts were TCA-precipitable.

8.4 Immunoprecipitation of mature PDC polypeptides

Preliminary studies showed that the composition of the extraction-washing buffer was critical in optimising the detection of PDC polypeptides by immunoprecipitation techniques from ^{35}S-labelled cell extracts. Even when good yields (70-80%) of immunoprecipitated ^{3}H-labelled PDC were obtained in Triton buffer, immunoprecipitates from ^{35}S-labelled cell lysates using this buffer system were not satisfactory. High levels of background radioactivity were found which could not be ascribed to poor specificity of the antiserum.

The background radioactivity in the form of contaminating polypeptides that associated non-specifically with immunocomplexes was very characteristic of the type of cell. Persistent contamination with the 44,000-M_r actin band which appears to have strong affinity for immunocomplexes was found (Barber & Delovitch,1978).

Preincubation of the extracts with fixed S. aureus cells for 2 h at room temperature followed by removal of the cells before adding the antibody (pre-adsorption) was not effective in reducing contamination and led to a decrease in recovery of antigen. Therefore, a number of modifications were attempted by changing the pH, salt concentration and type and concentration of detergents.

A 3-D TKM immunoprecipitation system implemented in this study (section 2.2.12.f) gave satisfactory results. The increased sensitivity of the detection following reduction of the background by

stringent washing conditions did not seem to alter the binding of the antibody and also promoted dissociation of the complex. Good yields (approx. 80%) of immunoprecipitated ^{3}H-labelled PDC were also obtained in the 3-D TKM buffer system.

Cultured cells were labelled for 16-20 h in the presence of 100 μCi [^{35}S] methionine. Aliquots of the cell extracts (100-200 μl) containing 10 x 10^{6} c.p.m. ^{35}S-labelled proteins were immunoprecipitated with 10 μl anti-PDC serum and 50 μl of a 10% (w/v) suspension of S. aureus cells.

Fig. 8.3 shows the results obtained with bovine kidney cells. Bands corresponding to the M_r values for subunits E2, E3, E1 α and E1 β were clearly seen in the immunoprecipitation products. Identity of these bands with PDC polypeptides was confirmed as excess unlabelled PDC or isolated PDC components competed with the in vivo immunoprecipitated products for interaction with the antibody.

Effective immunocompetition was dependent on the amount of unlabelled protein. Binding of subunit E2 was poorly competed out by either PDC or subcomplex E2-X at the levels of protein used in the experiment shown in Fig. 8.3.

No bands corresponding to the M_r values of component X could be identified in the immunoprecipitates.

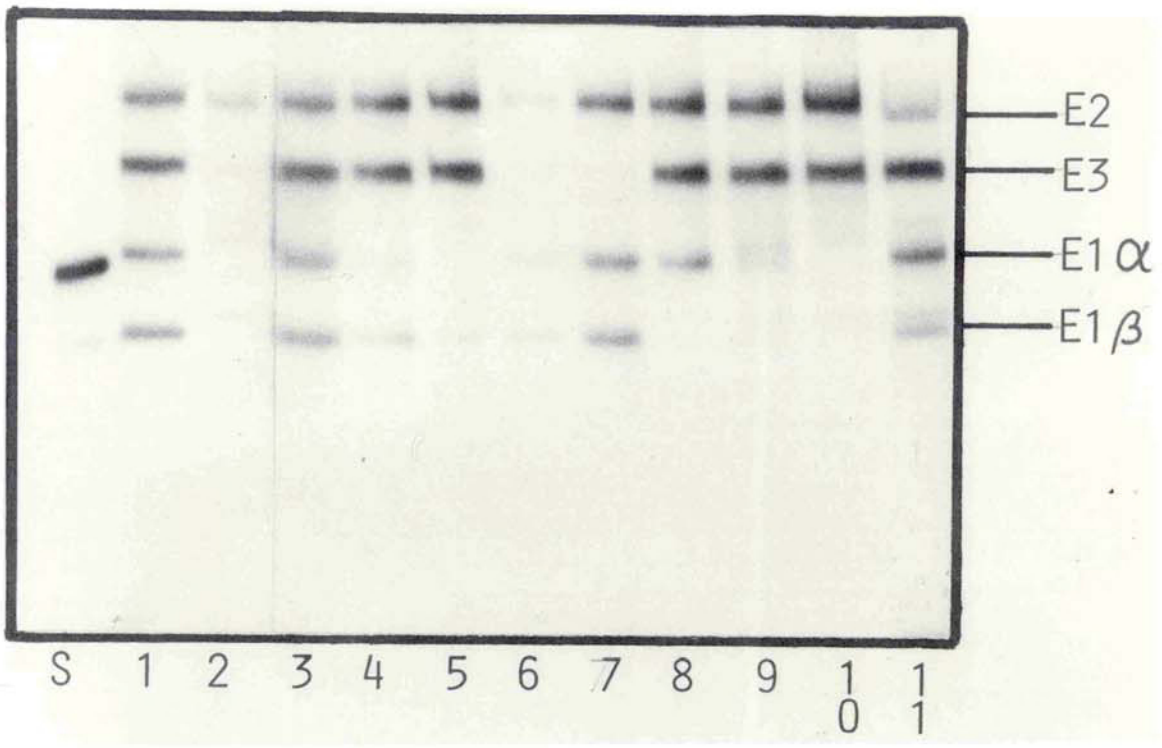
8.5 In vivo synthesis of precursors to PDC

It has been reported that the import of some mitochondrial matrix proteins can be inhibited by CCCP or DNP (Reid & Schatz, 1982b; Jaussi et al., 1982; Fenton et al., 1984) or p-aminobenzamidine (Raymond & Shore, 1981). Since preliminary pulse-labelling experiments using high levels of [^{35}S] methionine were unsuccessful, precursors to PDC were accumulated after incubation of cultured cells in the presence of varying concentrations of FCCP and DNP during the labelling period.

Fig. 8.3 Identification of mature PDC components in bovine kidney cells by immunocompetition

Semi-confluent monolayers of bovine kidney cells (NBL-1) were labelled in low-methionine medium for 18 h with 100 µCi [^{35}S] methionine per dish. Cells were disrupted and aliquots (10 x 10^6 c.p.m.) of the extracts were subjected to immunoprecipitation with 10 µl of anti-PDC serum as described in sections 2.2.11.e and 2.2.12.f. Before the addition of antiserum, various subunits of PDC were added at the levels indicated below. Labelled proteins present in the immunocomplexes were adsorbed to S.aureus cells and analysed by fluorography after resolution on SDS/polyacrylamide gels.

Lane S, ^{3}H-labelled PDC; lane 1, immunoprecipitate with anti-PDC serum with no additions; lane 2, plus 5 µg PDC; lane 3, plus 5 µg component X; lane 4 and 5 plus 2.5 µg and 5 µg Peak II from gel filtration (E1 plus E3) respectively; lanes 6 and 7, plus pig heart E3 5 µg and 10 µg, respectively; lane 8, plus 5 µg of subunit E1 β ; lanes 9 and 10, plus pyruvate dehydrogenase component (E1) 6 µg and 12 µg, respectively; lane 11, plus Peak I from gel filtration (E2 plus X) 15 µg.


E2
E3
E1α
E1β
S
1
2
3
4
5
6
7
8
9
10
11

Approx. 150-250 x 10^6 c.p.m. [^{35}S] methionine were incorporated/ dish in the absence of uncouplers. The inclusion of FCCP or DNP caused marked morphological changes in the cells and reduced the incorporation of label significantly (up to 50%).

Fig. 8.4 shows that in the presence of 10 μM-FCCP, four slowly migrating bands are observed in the immunoprecipitation when compared with the mature subunits (lanes P and P_L) detected in the absence of uncoupler. Similar results were obtained with extracts from cells labelled in the presence of 1-2 mM-DNP. This narrow range of effective uncoupler concentration was chosen after preliminary experiments showing that concentrations of FCCP below 5 μM had no effect on processing of precursors. Similarly, concentrations of 4 mM-DNP, used by previous authors, proved to be toxic and drastically reduced the viability of the cells.

Labelling in the presence of 2 mM-DNP for 4 h, followed by a 40 min chase in the absence of uncoupler showed that conversion of these bands into the mature proteins was complete (Fig. 8.3, B, lane ch).

Similar results were obtained with pig kidney cells with one major difference. While Fig. 8.5 shows that the subunit E3 was absent from the final immune complexes isolated from untreated cells (lanes P and P_L), this band was present in the immunoprecipitates from NBL-1 and BRL cells.

In Fig. 8.5, it was also possible to detect that only partial inhibition of processing had occurred in the presence of 1 mM-DNP. Higher M_r bands were clearly detectable as well as bands corresponding to the mature proteins. If cells were incubated in 2 mM-DNP, the mature bands disappeared and only bands corresponding to the unprocessed polypeptides were visible. Partial processing was also observed after incubation in the presence of 10 μM-FCCP. After a subsequent chase

Fig. 8.4 Dectection of precursor and mature forms of PDC components in cultured bovine kidney cells

Semi-confluent monolayers of bovine kidney cells (NBL-1) were labelled for 4 h in a medium containing 200 µCi [^{35}S] methionine in the absence or presence of FCCP (A) or DNP (B) at the final concentrations indicated.

Immunoprecipitation with anti-PDC serum, electrophoresis of the dissociated immunocomplexes and fluorography were performed as indicated in sections 2.2.12.f and 2.2.6.e (see also legend Fig. 8.3).

Lane S, ^{3}H-labelled PDC; lane C, immunoprecipitates with pre-immune serum; lanes P and P_L, immunoprecipitates from cells labelled in the absence of uncouplers for 4 h or 18 h, respectively; lane ch, immunoprecipitates from extracts of DNP-treated cells after a 40 min chase in the absence of uncoupler.

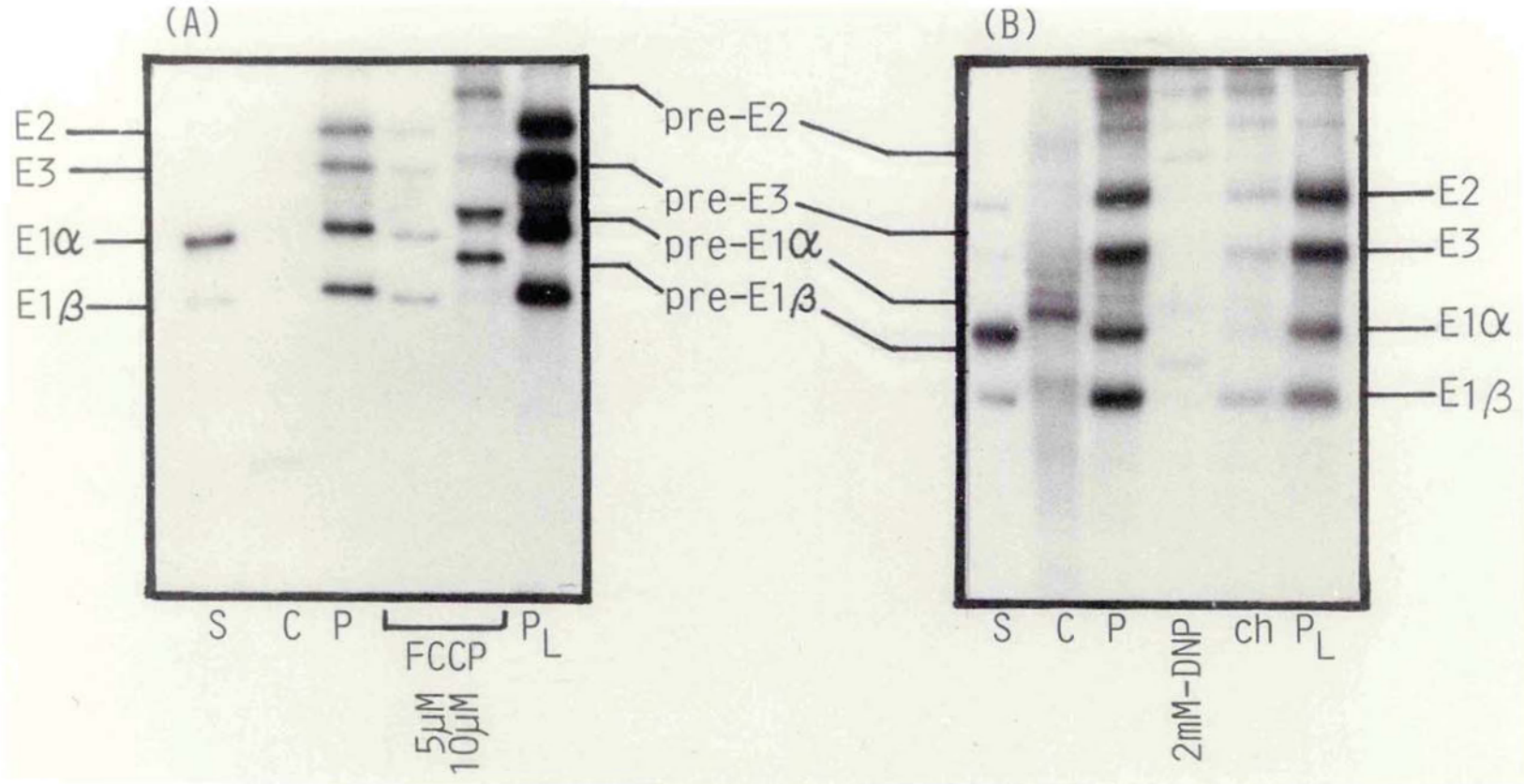
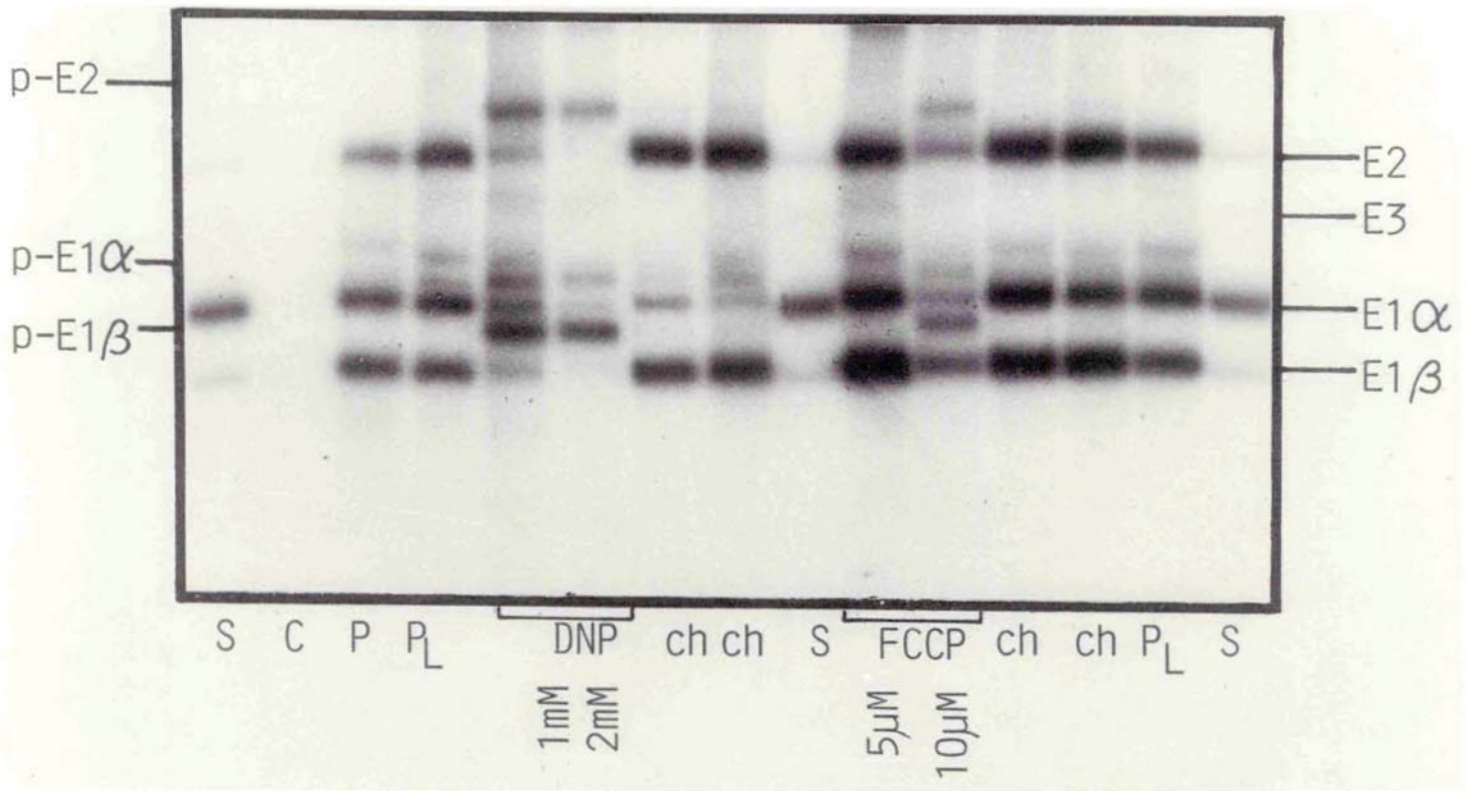

(A)
E2
E3
E1α
E1β
pre-E2
pre-E3
pre-E1α
pre-E1β
S
C
P
FCCP
5μM
10μM
PL
(B)
E2
E3
E1α
E1β
S
C
P
2mM-DNP
ch
PL

Fig. 8.5 Biogenesis of PDC components in cultured pig kidney cells

Monolayers of pig kidney cells (PK-15) were incubated for 4 h in a medium containing 200 µCi [^{35}S] methionine in the presence of DNP or FCCP at the indicated concentrations. Some dishes were "chased" for a 40 min period in the absence of uncouplers. Immunoprecipitation with anti-PDC serum or pre-immune serum, electrophoresis and fluorography were performed as described in sections 2.2.12.f and 2.2.6.e.

Lane S, ^{3}H-labelled PDC; lane C, control immunoprecipitates; lanes P and P_L; immunoprecipitates from cells labelled in the absence of uncouplers for 4 and 18 h, respectively; lanes ch, immunoprecipitates after chase from DNP-treated or FCCP-treated cells, respectively.


p-E2
p-E1α
p-E1β
E2
E3
E1α
E1β
S C P PL DNP ch ch S FCCP ch ch PL S
1mM
2mM
5μM
10μM

period of 30 min the precursor forms were converted completely to the mature forms (lanes ch).

It was obvious that small changes in the concentration of uncouplers seemed to cause dramatic effects in the processing of precursors to PDC polypeptides. Moreover, in different cell types, differential effects on processing could be achieved for a given concentration of inhibitor. This can be attributed to particular characteristics of the cells regarding their sensitivity to the uncoupler and/or variability in the experimental parameters e.g. cell density, viability and rate of growth.

8.6 Time course of precursor processing in cultured cells

The kinetics of disappearance of precursor forms was determined after incubating the cells with [^{35}S] methionine in the presence of 2 mM-DNP, followed by different times of chase after release of inhibition.

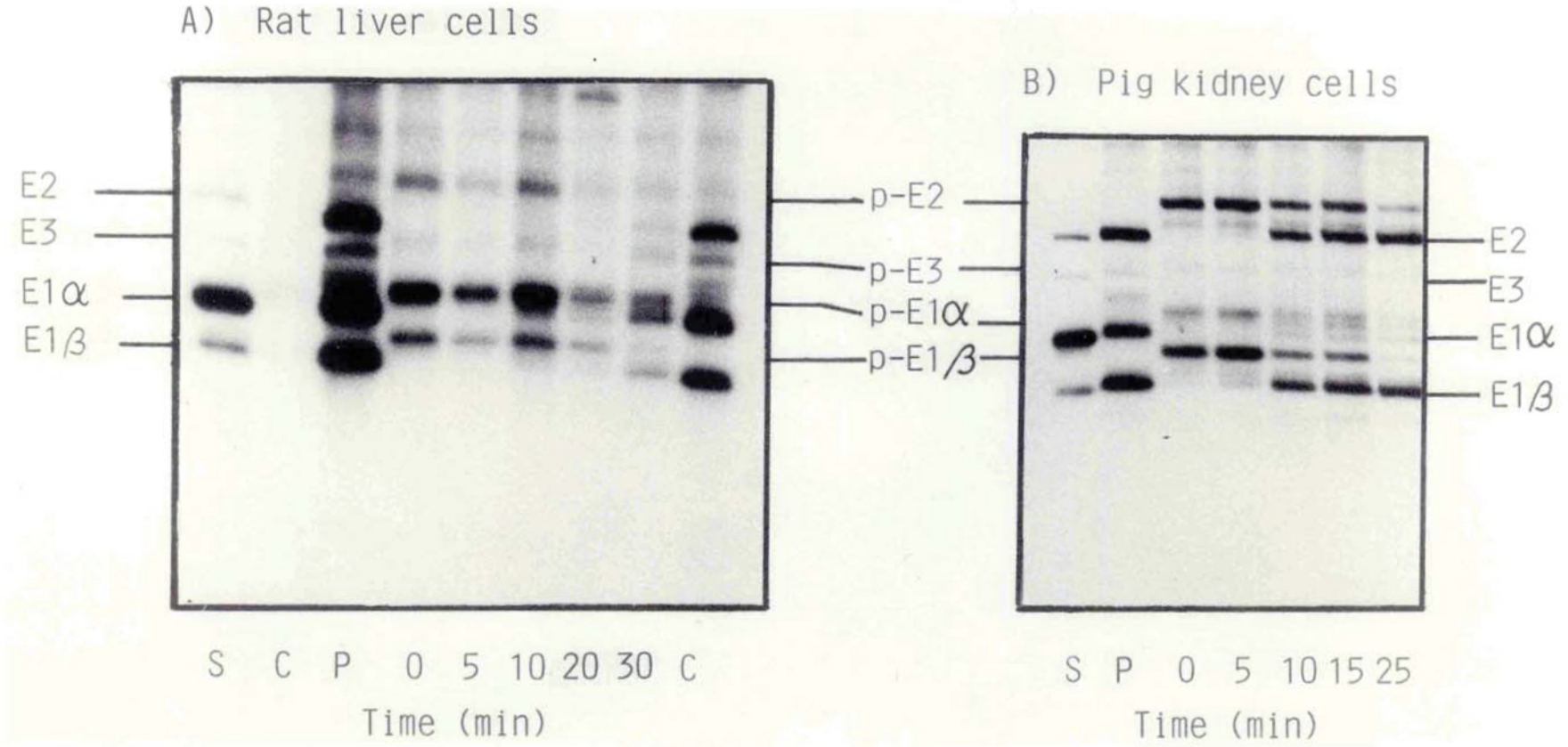
Fig. 8.6 shows the results of such experiments performed in rat liver (A) and pig kidney (B) cells. Lane 0 indicates the accumulated precursors present at the start of the chase while the following lanes correspond to the products which were recovered at various times thereafter. In the absence of the uncoupler, newly-processed enzyme was observed with a concomitant decrease in the amount of precursor. This strongly suggested that the import was an energy-dependent process, as has been shown previously for many other matrix proteins.

Precursor forms were rapidly converted into the mature forms with a half-lives ($t_{\frac{1}{2}}$) of 15-20 min in rat liver and 10-12 min in pig kidney cells. At present, it is not known if these differences arise from experimental variability or reflect differences in the rate of processing of PDC precursors in the two types of cell.

Fig. 8.6 Kinetics of processing of precursors to mature PDC subunits in pulse-chase experiments

Rat liver (A) or pig kidney cells (B) were labelled for 4 h with 200 µCi [^{35}S] methionine in the presence of 2 mM-DNP. The medium was removed and the cells chased in normal growth medium containing no uncoupler. Cell extracts were obtained at the indicated times (0-40 min) and subjected to immunoprecipitation with anti-PDC serum followed by electrophoresis and fluorography as described in section 2.2.12.f.

Other lanes; lane S, ^{3}H-labelled PDC; lane C, control immunoprecipitates; lane P, immunoprecipitates from cells labelled in the absence of DNP.


A) Rat liver cells
E2
E3
E1α
E1β
p-E2
p-E3
p-E1α
p-E1β
S C P 0 5 10 20 30 C
Time (min)
B) Pig kidney cells
E2
E3
E1α
E1β
S P 0 5 10 15 25
Time (min)

Half times for *in vivo* processing of precursor to mature mitochondrial matrix enzymes have been estimated as 2-10 min. These include pre-ornitine transcarbamylase (approx. 2 min; Mori *et al.*, 1981a); pre-carbamyl phosphate synthetase (2 min; Raymond & Shore, 1981; Mori *et al.*, 1981a); pre-aspartate aminotransferase (1 min; Jaussi *et al.*, 1982); pre-methyl-malonyl-CoA mutase (6-9 min; Fenton *et al.*, 1984) and 10 min for serine: pyruvate aminotransferase (Oda *et al.*, 1984). It is possible that the half times for processing and transport of mitochondrial precursors differ between mitochondrial proteins. Another possibility is that the inhibitor may have additional toxic effects on the cells that may prevent rapid reversal. In this respect, it may be of interest to determine the kinetics of processing after shorter labelling periods in the presence of uncouplers.

The kinetics observed for the individual precursors seem to be similar, which suggests that processing *in vivo* occurs in a coordinated manner for the various components of the complex.

8.7 Stability of precursor forms in cultured cells

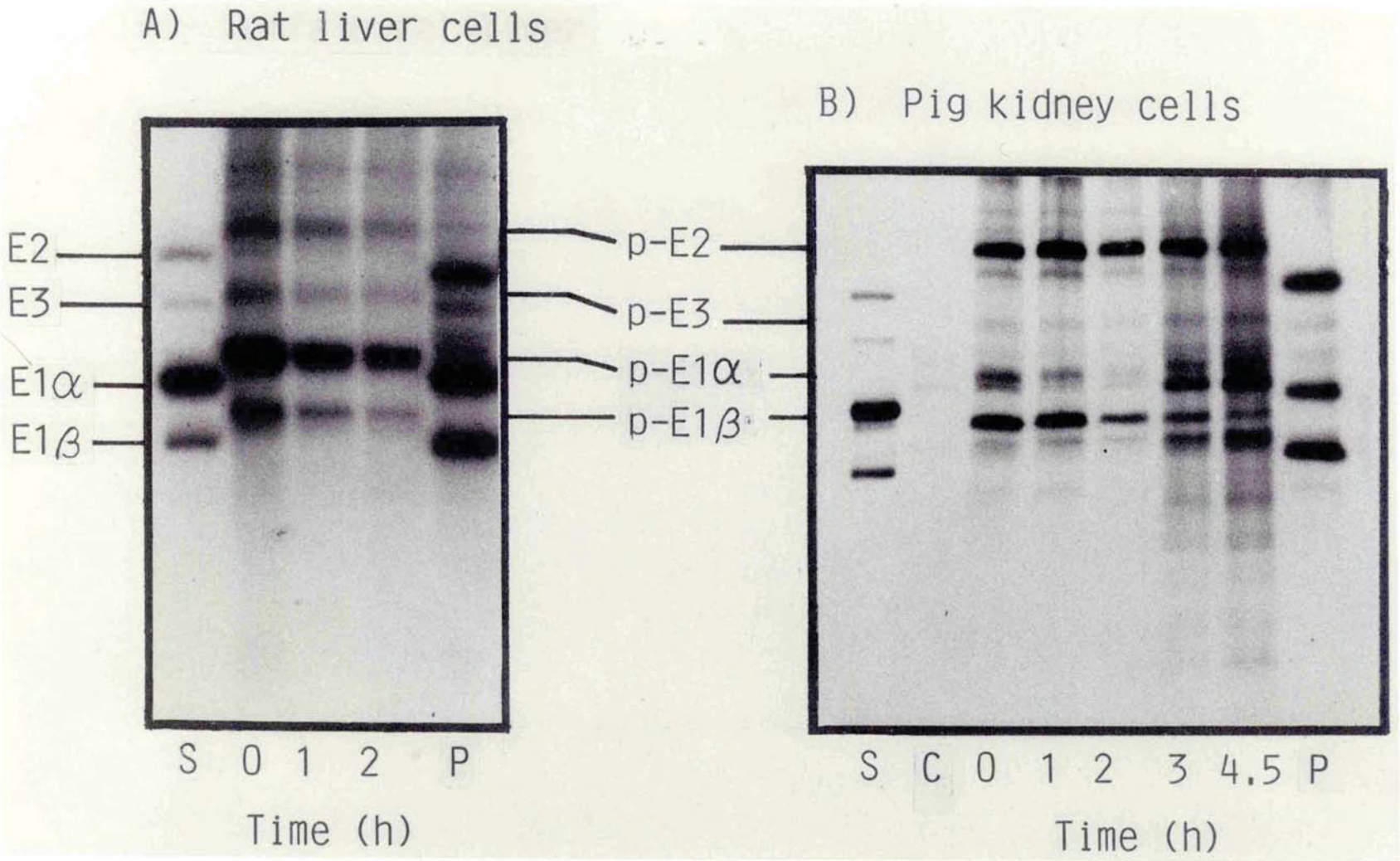
Data presented in Fig. 8.7 shows that precursor forms of PDC were very stable in rat liver and pig kidney cells when processing was inhibited by DNP. Even after a 4.5 h chase, a substantial amount of the pre-E2 could still be found, suggesting a half-life of several hours under these conditions. However, as shown in Fig. 8.7 B, after 2 h, appreciable degradation of the precursor to E1 α subunit had taken place. After 3 h, additional bands presumably corresponding to degradation products were also apparent.

These results are similar to those reported for pre-methyl-malonyl-CoA mutase in rat liver cells, but contrasts with several other studies on the stabilities of rat liver pre-carbamyl phosphate synthetase (Raymond & Shore, 1981) and pre-aspartate aminotransferase

Fig. 8.7 Stability of precursor forms to PDC components in the presence of DNP

Rat liver (A) or pig kidney (B) cultured cells were labelled with 200 µCi of [^{35}S] methionine in the presence of 2 mM-DNP. Cells were then "chased" for the indicated times (0-4.5 h) in a normal growth medium containing the same concentration of uncoupler. Cell extracts were immunoprecipitated with anti-PDC serum and analysed by fluorography after electrophoresis in SDS-polyacrylamide gels as described in sections 2.2.12.f. and 2.2.6.e.

Lane S, ^{3}H-labelled PDC; lane P, immunoprecipitates from cells labelled in the absence of DNP; lane C, control immunoprecipitates.


A) Rat liver cells
B) Pig kidney cells
E2
E3
E1α
E1β
p-E2
p-E3
p-E1α
p-E1β
S 0 1 2 P
Time (h)
S C 0 1 2 3 4.5 P
Time (h)

in chicken embryo fibroblasts (Jaussi et al., 1982). In those two cases, blockage of processing led to destruction of the precursors within 5-15 min. Several possibilties to explain this long term stability have been discussed by Fenton et al. (1984). One is that the precursors may be protected from degradation by endogenous proteases by their interaction with mitochondria or that precursors have conformations that avoid proteolytic attack from endogenous proteases.

8.8 Identification of precursor forms with subunit-specific antisera

Conclusive identification of the precursor forms corresponding to the individual subunits of the complex was achieved by performing immunoprecipitation studies with subunit-specific antisera.

Fig. 8.8 shows the immunospecific products obtained with antiserum to subunit E2 in bovine kidney (A) and pig kidney (B) cells. When the cells were labelled in the presence of 2 mM-DNP a component M_r 78,000 appears in the immunoprecipitated products, corresponding to the precursor to subunit E2 (lanes 2). The precursor has a similar size in these two cell lines. Its identity was confirmed as it was competed out from the final immunoprecipitates with 5 µg of subcomplex E2-X. The mature form was only competed effectively when 15 µg of sub-complex E2-X were added (lanes 3 and 3').

In immunoprecipitates with this antiserum an additional band of variable intensity appeared at M_r values of 48,000. Assuming that this is a contaminating protein that binds non-specifically to the immunoprecipitates, harsher conditions of washing or previous purification of the anti-E2 IgG from serum may prove to be effective.

Fig. 8.9 shows the results with antiserum to lipoamide dehydrogenase in pig kidney and rat liver cells. Gel (A) shows that anti-E3 antiserum is capable of immunoprecipitating the mature (lanes 1, 3 and 4) and precursor form (lanes 2) M_r 57,000 for E3 component in both

Fig. 8.8 Identification of precursor to subunit E2 by immunoprecipitation with anti-E2 serum

Bovine kidney cells (A) or pig kidney cells (B) were labelled for 4 h in a medium containing 200 μCi [^{35}S] methionine and 2 mM-DNP. Cell extracts were obtained and subjected to immunoprecipitation with anti-E2 serum. Immunocomplexes were dissociated and labelled proteins analysed by fluorography after electrophoresis on 10% (w/v) polyacrylamide slab gels.

Lane S, ^{3}H-labelled PDC; lane C, control immunoprecipitates; lane P, immunoprecipitates with anti-PDC serum from cells labelled in the absence of uncoupler; lane 1, immunoprecipitates with anti-E2 serum from cells labelled for 4 h in the absence of uncoupler; lane 2, immunoprecipitates with anti-E2 from cells labelled in the presence of DNP; lane 3 immunoprecipitates with anti-E2 from cells labelled for 18 h in the absence of uncoupler; lanes 1', 2' and 3', as lanes 1, 2 and 3, except that 5 μg, 5 μg or 15 μg of subcomplex E2-X were added before antiserum, respectively.

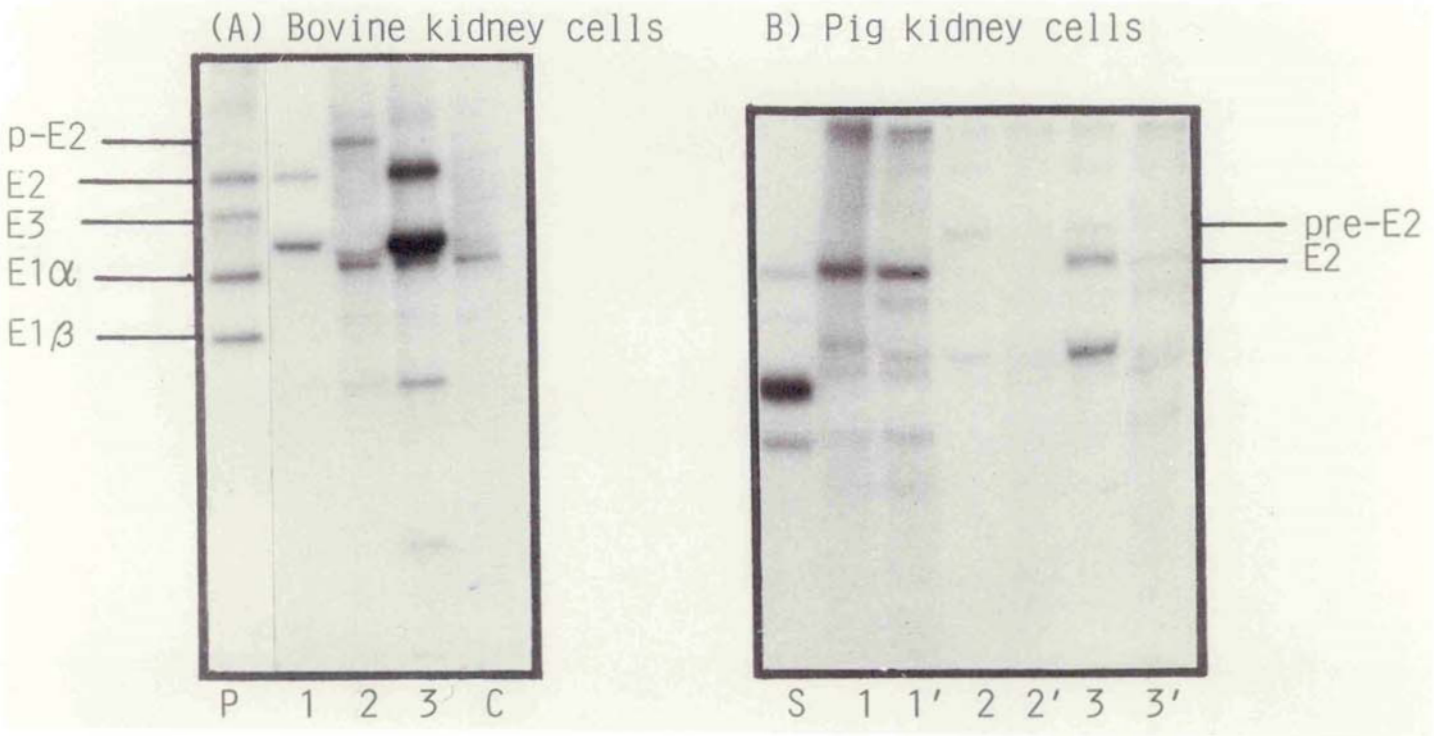
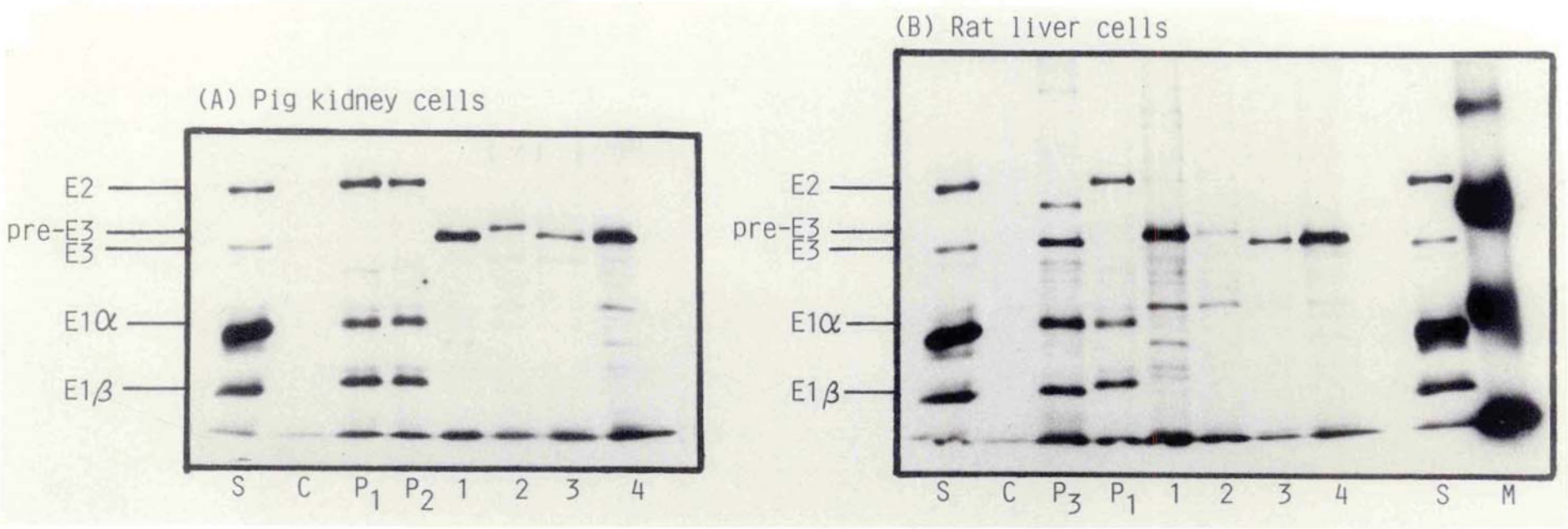

(A) Bovine kidney cells
B) Pig kidney cells
p-E2
E2
E3
E1α
E1β
pre-E2
E2
P 1 2 3 C
S 1 1′ 2 2′ 3 3′

Fig. 8.9 Identification of precursor form to lipoamide dehydrogenase (E3) using subunit-specific antiserum

Monolayers of pig kidney cells (A) or rat liver cells (B) were labelled in the presence of 2 mM-DNP and 200 μCi [^{35}S] methionine. After 4 h, the medium was removed from some dishes and replaced by normal growth medium for a chase period of 30 min. After immunoprecipitation, proteins were resolved in a 7% (w/v) SDS/polyacrylamide gel (16 cm x 17 cm) and the gel was processed for fluorography as described in Methods section 2.2.6.e.

Lane S, ^{3}H-labelled PDC; lane C, control immunoprecipitates; lanes P_1 (A and B) immunoprecipitates from cells labelled in the absence of uncoupler with antiserum to PDC; lane P_2 as P_1 except that 5 μg of pig heart E3 was added before antiserum; lane P_3 as lane P_1 except that a cell extract from rat liver was used instead; lanes 1, 2, 3 and 4 correspond to immunoprecipitates with anti-E3 serum from cells labelled in the absence of uncoupler, in the presence of 2 mM-DNP, DNP-treated after chase and in the absence of uncoupler for 20 h, respectively; M, ^{125}I-labelled M_r standards.


(A) Pig kidney cells
E2
pre-E3
E3
E1α
E1β
S
C
P1
P2
1
2
3
4
(B) Rat liver cells
E2
pre-E3
E3
E1α
E1β
S
C
P3
P1
1
2
3
4
S
M

types of cell. In rat liver as well as in bovine kidney cells (see Fig. 8.3), subunit E3 is detectable with the other components of the complex when anti-PDC serum is employed for immunoprecipitation, but this component is absent from the immunoprecipitation obtained from pig kidney cells (lanes P_1 and P_2). In Fig. 8.9 (B), a direct comparison between rat liver and pig kidney cells is possible (lanes P_3 and P_1). there was also a slight difference in the M_r values of the mature E2 subunit, as detected previously by immunoblotting experiments (Fig. 4.4). Fig. 8.10 shows the results obtained with antiserum to subunit E1 α in rat liver cells. The precursor form M_r 45,000 was detectable with antiserum to PDC (lane P_2) and also with anti-E1 α serum when the cells were labelled in the presence of 1-2 mM-DNP. After a chase period of 30 min, a band corresponding to mature E1 α subunit was clearly seen (lane ch).

The presence of a contaminating band at M_r 44,000 presented difficulties in assigning an accurate M_r value to the precursor of subunit E1 α (Fig. 8.10, B). The inclusion of non-radioactive actin (2.5 μg) in the immunoprecipitation assays, substantially reduced this contamination permitting a more reliable estimate (not shown).

Fig. 8.11 shows the results obtained with antiserum to subunit E1 β in bovine kidney cells. Precursor to this protein was identified as a band of M_r 40,000 (lane 1) which is converted to the mature component after a 30 min chase (lane 2).

Table 8.1 summarises data on the determination of M_r values for precursors to individual PDC polypeptides in the cultured cells studied.

Values of 59,000 for the pre-E3 polypeptide has been reported by Matuda et al. (1983) for the rat liver enzyme using an in vitro translation system. This estimate is slightly higher than the value determined from in vivo studies. The small difference in M_r value

Fig. 8.10 Immunoprecipitation of precursor and mature forms of E1 α in rat liver cells with subunit-specific antiserum

Semi-confluent monolayers of rat liver cells were labelled for 4 h with 150 μCi [^{35}S] methionine in the presence of the indicated concentrations of DNP. In (B) DNP-treated cells were "chased" for 30 min in the absence of uncoupler before harvesting. Immunoprecipitation, electrophoresis and fluorography were performed as described in sections 2.2.12.f and 2.2.6.e.

Lane S, ^{3}H-labelled PDC; lane C, control immunoprecipitates.

(A and B), Lanes 1 and 2, immunoprecipitates with anti-E1 α serum from cells labelled in the absence of uncoupler for 4 h or 18 h respectively. (B) Lane P_1, immunoprecipitates with antiserum to PDC; from cells labelled in the absence of uncoupler lane P_2 as P_1 but the cells were labelled in presence of 2 mM-DNP; lane ch, immunoprecipitates with anti-E1 α serum from DNP-treated cells after chase.

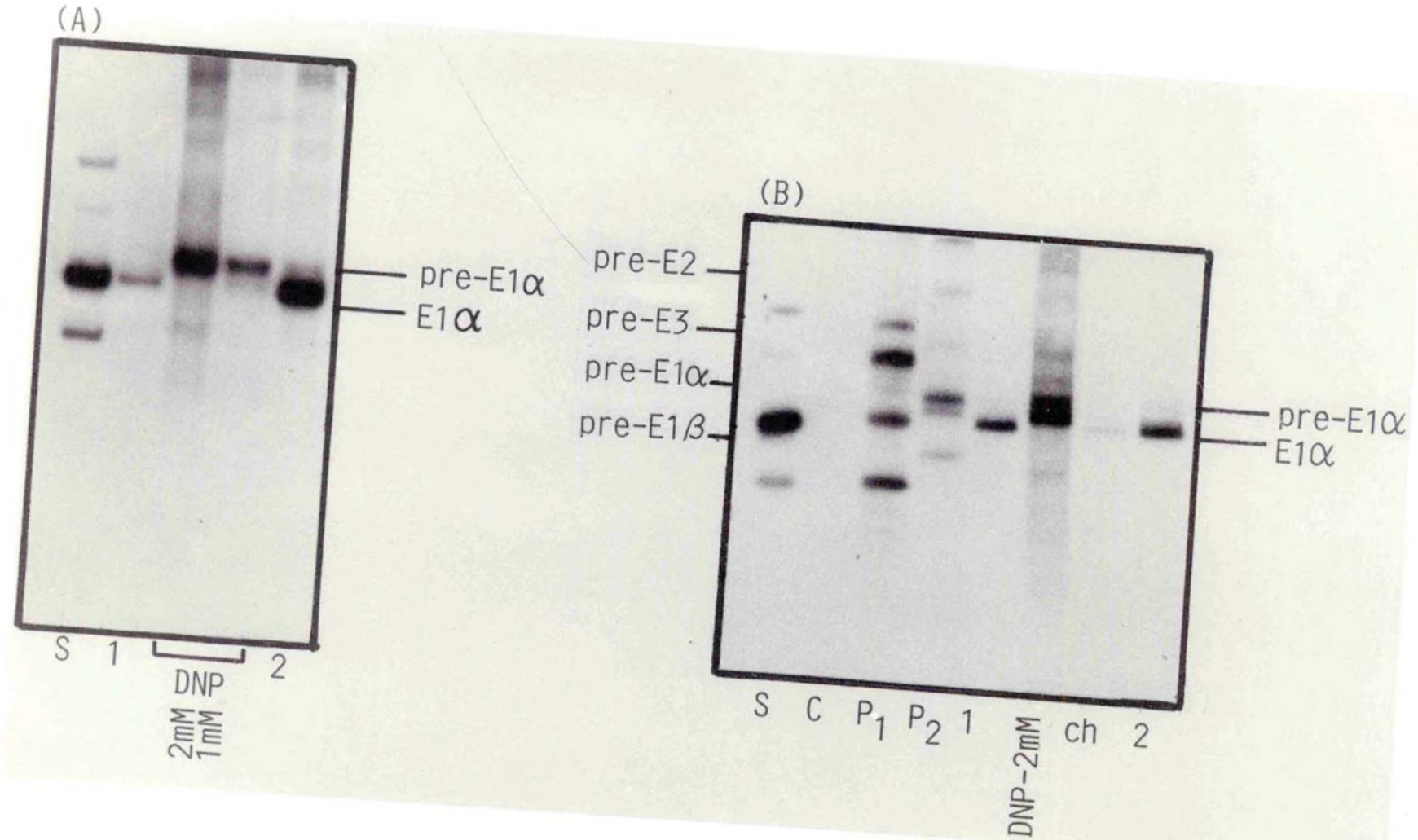

(A)
pre-E1α
E1α
S
1
DNP
2mM
1mM
2
(B)
pre-E2
pre-E3
pre-E1α
pre-E1β
pre-E1α
E1α
S
C
P1
P2
1
DNP-2mM
ch
2

Fig. 8.11 Identification of the precursor and mature forms of E1 β with subunit-specific antiserum

Monolayers of bovine kidney cells were incubated with 200 μCi [^{35}S] methionine in the presence of 2 mM-DNP. After 4 h labelling, cells were "chased" for 30 min before harvesting. Immunoprecipitation, electrophoresis and fluorography were performed as described in sections 2.2.12.f. and 2.2.6.e.

Lane S, ^{3}H-labelled PDC; lane C, control immunoprecipitates; lane P_1, immunoprecipitates with anti PDC serum from cells labelled in the absence of uncoupler; lane P_2 as P_1 except that 5 μg of purified E1 β protein were added before antiserum; lanes 1, 2 and 3, immunoprecipitates with anti-E1 β serum from cells labelled in the presence of 2 mM-DNP, DNP-treated cells after chase and cells labelled for 18 h in the absence of uncoupler, respectively.

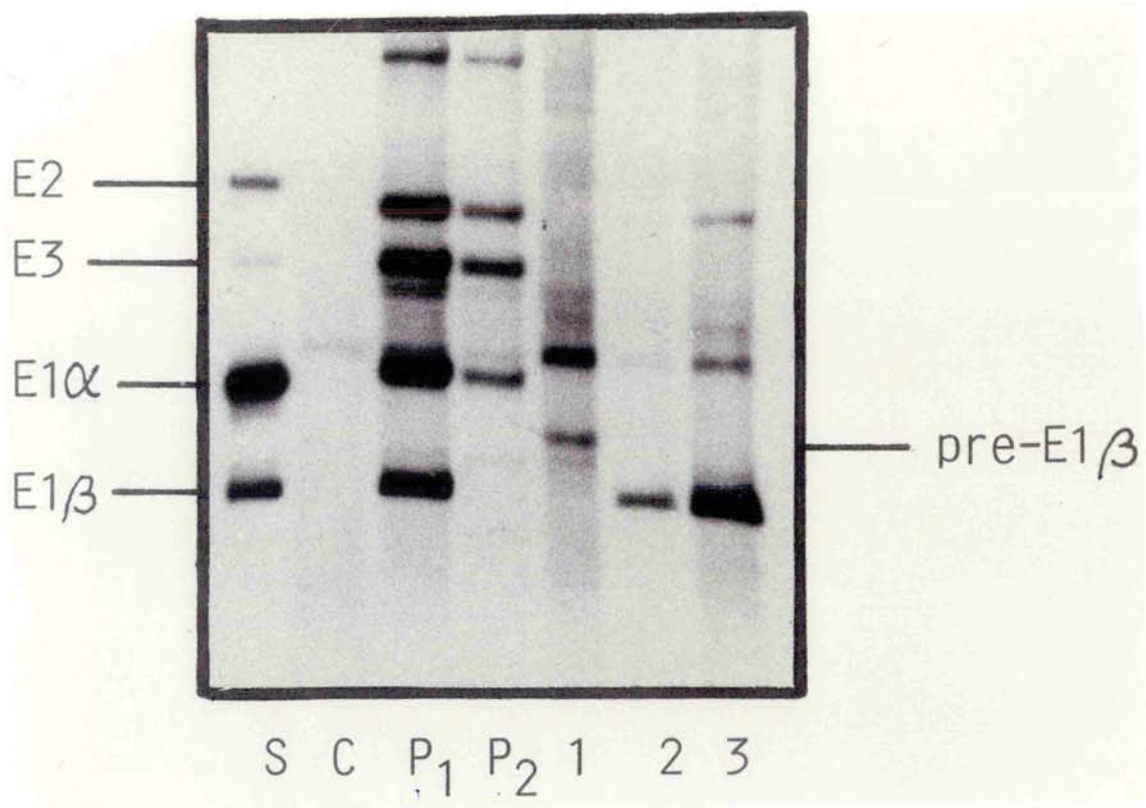

E2
E3
E1α
E1β
pre-E1β
S C P1 P2 1 2 3

Table 8.1 M_r of precursors to PDC polypeptides as estimated by SDS/polyacrylamide gel electrophoresis

Polypeptide	Apparent M_r	
	Mature	Precursor
Dihydrolipoyl acetyltransferase	70,000	78,000 ± 1,000 *
Lipoamide dehydrogenase	55,000	57,000 ± 1,000
Component X	52,000	?
Pyruvate dehydrogenase subunit E1 α	42,000	45,000 ± 1,000
Pyruvate dehydrogenase subunit E1 β	36,000	40,000 ± 1,000

* In rat liver cells, these values correspond to 68,000 and 76,000 ± 1,000 for the mature and precursor forms, respectively.

with respect to the mature enzyme is reflected in the difficulty in separating the two proteins in 7% (w/v) SDS/polyacrylamide gels (Fig. 8.9).

Component X remained undetected in the immunoprecipitated products using anti-PDC serum. Occasionally, a band at the expected M_r value was observed (as in Fig. 8.9, lane P_3). This could be related to the low abundance of this polypeptide in comparison with the other subunits of PDC and/or to its low content of methionine as indicated by preliminary amino acid composition data (J.A. Hodgson, unpublished results).

Chapter 9: GENERAL DISCUSSION

Mammalian PDC has been studied extensively during the last fifteen years. In particular its enzymatic and physicochemical characteristics have been the subject of detailed examination following its isolation from several tissues (Koike & Koike, 1976).

The regulation of this multienzyme complex by phosphorylation/dephosphorylation has also received considerable attention, as a result of its importance in the conservation of glucose in starvation and diabetes (Reed et al., 1980; Denton et al., 1981; Wieland, 1983). However, studies on the areas related to the biosynthesis, transport and assembly of the complex, as well as aspects of its long term regulation have been largely neglected (Hu et al.,1983).

The production of well-characterised specific antibodies against the native complex and its individual subunits was a necessary pre-requisite for monitoring the events involved in the biosynthesis of the complex. During the immunological analysis of the complex, additional important features were revealed, which were relevant to the structure, function and intracellular location of this enzyme.

9.1 Immunological studies on mammalian PDC

A major observation from our early immunological studies was that the lipoamide dehydrogenase subunit elicited a poor immunological response relative to the other major polypeptides of the complex. Evidence for this conclusion was obtained by immunoblotting analysis and direct immunoprecipitation studies of the dissociated multienzyme complex, in which E3 was excluded from the final immune complexes. However, immunoprecipitates with anti-PDC serum from [^{35}S] methionine-labelled cell lysates from cultured bovine and rat cells, but not from porcine kidney cells, showed a band corresponding to this subunit.

These results could be attributed to the use of later bleedings of the antiserum for these experiments, containing a higher titre for this protein and/or lower cross-reactivity with the pig kidney as compared to the bovine and rat enzyme. It is also possible that interaction with the pig kidney enzyme may involve primarily low affinity antibodies. Thus immune complex formation, in this case, may be disrupted by the stringent washing conditons used for immunoprecipitation.

The low immunogenicity of the E3 component was also observed after immuno-blotting analysis and immunoprecipitation of the ^{3}H-labelled 2-oxoglutarate dehydrogenase complex (De Marcucci et al., 1985a). This property of the E3 component may be interpreted as resulting from a high degree of conservation of amino acid sequence in this polypeptide.

The complete sequence of the E. coli enzyme has been determined by analysis of the cloned gene (Stephens et al., 1983). A remarkable degree of sequence homology was detected with tryptic peptides of the pig heart enzyme. Since E3 is a common component of the three 2-oxo-acid dehydrogenase complexes, it is feasible that this property has imposed constraints on amino acid substitution during evolution to preserve its differing and multiple interactions with the other subunits in these complexes.

Interactions between PDC and its specific antisera could also be useful for studies on structural/functional aspects of the enzyme, nature of lesions in inborn errors of metabolism and to assess evolutionary relationships between various organisms. The value of these studies would depend on the experimental approach on which they were based and on the heterogenous population of antibodies present in each particular antiserum. Application of immunological methodology to solve these problems has been discussed in excellent reviews by Cinader (1977) and Nagradova & Grozdova (1978).

Results described in this study demonstrate immunological relationships between the E1 β , E2 and E3 subunits of the S. cerevisiae PDC and the E1 subunit of the E. coli PDC with the ox heart complex.

The major method of examining evolutionary homologies between proteins is to investigate similarities in their amino acid sequence, either directly or by DNA sequencing. However, homologies can also be studied by immunological methods. The identification of common antigenic determinants is not necessarily indicative of a close relationship between species, as the same or related determinants could have arisen independently several times during evolution.

In this study, preservation of common antigenic determinants of PDC from prokaryotic to higher eukaryotic organisms was demonstrated by immunoreplica analysis. It may be interesting to extend these studies to several other species and to quantitate their immunoreactivity.

Quantification by immunoblotting analysis is difficult in view of the incomplete or variable transfer of proteins from polyacrylamide gels to nitrocellulose (Ghersone & Palade, 1983). Therefore, this could be attempted by other methods such as the nitrocellulose enzyme-linked immunoadsorbent assay developed recently for studies on immunological differences between cytochrome c oxidase isoenzymes (Kuhn-Nentwig & Kadenbach, 1985).

Of particular interest are the challenging opportunities for for application of immunological techniques to the study of inherited defects of PDC. A number of cases of children with persistent lactic acidosis and pyruvic acidosis, developmental retardation and neurological disease have been described. In several of these patients, deficiencies of PDC component enzymes (or pyruvate carboxylase) have been detected by activity measurements of the enzymes in tissue

extracts (Cederbaum et al., 1976; Koike et al., 1981; Borud & Pettersen, 1982; Matuda et al., 1984). It seems apparent that deficient oxidation of pyruvate leads to impaired production of AcSCoA and to accumulation of lactate in the blood.

9.2 Functional and structural studies on component X

An unexpected and interesting observation in this study was that ox heart PDC contained an extra polypeptide M_r 51,000±1,000 of unknown function (component X). Its immunological identity was established by employing highly sensitive immunoblotting techniques with several monospecific antisera (De Marcucci & Lindsay, 1985).

Component X may be a unique polypeptide of this multienzyme complex as no similar protein was detected in the analogous species of 2-oxo-acid dehydrogenase complexes: 2-oxoglutarate dehydrogenase complex, responsible for oxidative decarboxylation of 2-oxoglutarate in the citric acid cycle and the branched chain 2-oxo-acid dehydrogenase complex involved in the catabolism of leucine, isoleucine and valine. This may indicate a role for component X in the metabolism of two-carbon units.

A peptide subunit M_r 50,000 has also been detected in the yeast (S. cervisiae and S. carlsberguensis) complex (Kresze & Ronft, 1981a; Keha et al., 1982). It is still an attractive possibility that this polypeptide may be immunologically and functionally related to the mammalian component X.

On the structural aspects, protein X seems to be associated with the remaining components of the complex in reproducible amounts. The protein co-elutes with the peak fraction, M_r 8.5×10^6, in the final step of purification of the complex by gel filtration chromatography on Sepharose CL-2B columns. It also co-migrates with the peak of enzyme activity after sucrose-density gradient centrifugation in the presence

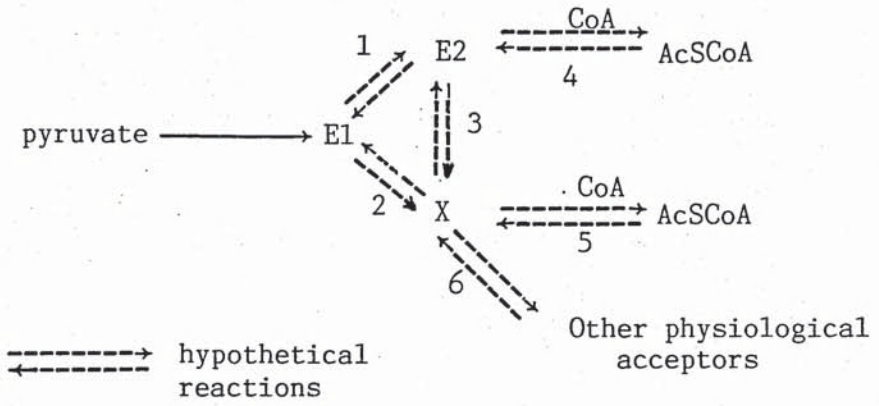
of 0.1 M-KCl and 1% (v/v) Triton X-100.

Based on quantitative scanning of Coomassie Blue-stained bands of the complex in SDS/polyacrylamide gels, it appears that 6% of the total absorbance, is represented by this component using the Laemmli gel system. On the other hand, component X retains 14-16% of the total radioactivity associated with the complex after labelling in the presence of [2-^{14}C] pyruvate. Assuming one "acetylatable" group per molecule of X, an estimate of 8-10 molecules of polypeptide/core is obtained which agrees with the value calculated on the basis of densitometric scans of 8-12 molecules of component X per core. This is a provisional conclusion, since more reliable stoicheiometric analysis is necessary by methods already available in the literature (Bates et al., 1975; Hale et al., 1979).

The accessibility of component X to proteolytic attack by papain or elastase in the native assembly and its binding to specific antibodies, suggests that at least part of its polypeptide chain is exposed on the surface of the complex.

Incubation of the complex in the presence of [2-^{14}C] pyruvate promotes acetylation of covalently-bound lipoyl groups in E2 while also acetylating component X. This may be the main reason for the previous suggestion that component X was a degradation product of E2 (Machicao & Wieland, 1980; Bleile et al., 1981). Inactivation of the complex by phosphorylation by the intrinsic protein kinase, causes a parallel decrease in the acetylation of E2 and X, indicating that this latter protein can participate in the acetylation/deacetylation reactions of the complex. This result suggests that loading of protein X must proceed through the formation of hydroxyethyl groups on the E1 component. As shown in the scheme below, it is not clear if X is acting as an intermediate in this process, i.e. if E1 can transfer acetyl groups

directly onto component X and then pass them onto E2, reactions 2, 3 and 4 in the scheme below, where X is an essential intermediate of the catalytic process. Alternatively, X may be an associated enzyme in equilibrium with E2 (reactions 1, 3 and 4).



Component X may be directly involved in transferring acetyl groups into CoA (reaction 5) and/or to other unknown physiological acceptors (reaction 6).

At present, several compounds have been tested for their capacity to promote deacetylation of the complex. Carnitine and acetoacetate have proven to be ineffective. Various thioesters were capable of facilitating release of radioactivity from the enzyme, but none were shown to promote preferential deacetylation of component X. This result could be due to their content of free CoA (0.5-5%; Ellman, 1959) or to the capacity of these compounds to acylate these proteins. Rahmatullah et al. (1985b) have reported that acylation of both the core component and protein X follows incubation of the enzyme in the presence of radioactive propionyl-CoA, malonyl-CoA and acetyl-CoA.

The relative abundance of component X in preparations of the complex (approx. 6%) argues against its possible role as a regulatory component of the complex. However, Roche and co-workers have advanced the idea that PDC may be regulated by the degree of acetylation of the complex. In support of this concept, they have shown that pyruvate dehydrogenase kinase activity is stimulated at low levels of acetylation of the complex. They have suggested the involvement of a few lipoyl groups in this effect or the participation of a protein containing lipoyl groups which functions as a regulatory subunit of the kinase (Cate & Roche, 1978; 1979).

The probable identity of this putative protein with component X is now being under experimental investigation by this research group (Roche & Rahmatullah, 1985; Rahmatullah et al., 1985a; Rahmatullah et al.,1985b).

It does not seem unreasonable to suppose that component X is an isoenzyme of E2, particularly since it is probable that it also contains lipoic acid (J.A. Hodgson, unpublished results). In this respect, there could be two subpopulations of PDC arising from complexes containing either E2 or X core subunits. However, at present, immunological inspection of PDC in several cell lines and rat tissues does not favour this idea, as component X shows no apparent preferential distribution between these types of cells.

An additional finding from studies on the acetylation of the complex in the presence of [2-^{14}C] pyruvate suggests that a slow accumulation of a diacetyl dihydrolipoamide derivative takes place after relatively long incubation times. Alternatively, acetylation of a secondary thiol group could be postulated.

Further studies carried out in our laboratory seem to favour the former mechanism (J.A. Hodgson, unpublished results). However, more

investigations of this reaction are necessary to assess its physiological importance for the mechanism of catalysis.

9.3 Subcellular localisation of mammalian PDC

The possible interaction of PDC with the inner mitochondrial membrane has been suspected for many years, partly stemming from difficulties encountered in trying to solubilise the enzyme from heart mitochondria (Addink et al., 1972; Pettit & Reed, 1982) or by histochemical studies (Nestorescu et al., 1973). More recently, this association has been shown by means of binding studies in which purified enzyme is found to interact with inverted inner membrane vesicles (Sümegi & Srere, 1984b).

Application of immunological techniques to study the subcompartmentalisation of mitochondrial enzymes has not been so far exploited. In this study, preliminary data using this approach have made it possible to obtain firm evidence for the strong interaction of PDC with inner mitochondrial membranes from heart. The possibility of quantitative estimation of the total amount of protein rather than the fraction of activity bound and its high sensitivity provide a valuable experimental tool for studying the nature of this association at the molecular level. This may prove to be a successful approach for understanding the organisation of the complex in the mitochondrion in the intact cell.

9.4 Biogenesis of mammalian PDC

Studies on the biogenesis of PDC polypeptides have revealed that the enzymes are synthesized as larger M_r precursors in various cultured cells. The precursors appear to have M_r values 2,000-8,000 larger than their mature counterparts.

The larger size for the extension of the E2 component of PDC is similar to that found for the transsuccinylase component of the 2-oxoglutarate dehydrogenase complex which has a M_r value of 7,000

larger than the mature enzyme (Hunter,A. & Lindsay, J.G., Eur. J. Biochem., submitted for publication).

Precursor to subunit E2 seems to remain stable in the cytoplasmic compartment in the presence of DNP for several hours. This may indicate that the precursor adopts a particular conformation which protects it from degradation by endogenous proteases.

There are reports that the precursor polypeptides of some mitochondrial matrix and inner membrane enzymes formed aggregates *in vitro*, exhibiting considerably larger M_r values than the oligomeric mature proteins. These include: carbamyl phosphate synthetase (Miura *et al.*, 1981), ornithine transcarbamylase (Kalousek *et al.*,1984) and fumarase (Ono *et al.*, 1985) in animal cells and the ADP/ATP carrier and subunit 9 of the F_1-ATPase complex in *Neurospora crassa* (Zimmermann & Neupert, 1980; Schmidt *et al.*,1983).

In contrast, precursor to malate dehydrogenase was found to be only slightly larger than the dimeric form of the mature protein (Chien & Freeman, 1984). It is not known whether this aggregation occurs *in vivo* also or there are other factors involved as part of the mechanism of import of these proteins (Argan *et al.*, 1983). Therefore, it would be of interest to examine the state of aggregation of precursors to PDC polypeptides in cells. This could conceivably be done after pulse-labelling cells in the presence of uncouplers followed by gel filtration chromatography of the cytosolic fractions coupled with immunoprecipitation of the eluted fractions with anti-PDC serum.

As discussed previously, component X has evaded detection in the immunospecific products obtained from several cell lines. It would also be interesting to further investigate whether this protein is synthesized as a larger M_r precursor and if it can be found associated

with the core before translocation. The low methionine content and abundance of component X may have hindered the identification of the precursor form of this protein. Therefore, it would be interesting to perform similar studies on cultured cells by including ^{14}C-labelled amino acids during the labelling period. The demonstration that many of the events involved in the biosynthesis of the main polypeptides of the complex can be studied in vivo makes one hopeful that this system will permit us to obtain an answer to these questions in the near future. This would allow to clarify definitively the identity of protein X and probably to establish whether it has a role in the assembly of the complex inside the mitochondrion.

Finally, the observation that some cross-reactivity exists between the antisera to ox heart PDC and the equivalent yeast enzyme also makes it possible to visualize accumulation of precursors in these cells by immunoblotting techniques. Preliminary data, not included in this study, suggests that rho$^-$ mutants of S. cerevisae are capable of accumulating substantial amounts of precursors to subunits E2 and E1 β in the present of CCCP. The M_r for the precursors detected are approx. 3,000 and 5,000 larger than their mature-sized counterparts, respectively. These studies will allow direct comparison of the biosynthetic pathways of the same enzyme between lower and higher eukaryotes.

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