STRUCTURAL AND FUNCTIONAL STUDIES ON TWO MITOCHONDRIAL PROTEINS: THE PYRUVATE DEHYDROGENASE COMPLEX AND THE PHOSPHATE CARRIER

A thesis submitted for the degree of

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by

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FOR MY PARENTS

ABBREVIATIONS

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

ANT	adenine nucleotide translocase
BCDC	branched chain 2-oxo acid dehydrogenase complex
BRL	Buffalo rat liver cells
BSA	bovine serum albumin
CHAPS	3- [(3-Cholamidopropyl)dimethyl-ammonio] -1-propanesulphonate
DMSI	dimethyl suberimidate
DMSO	dimethylsulphoxide
2,4 DNP	2,4-dinitrophenol
DOC	deoxycholate, sodium salt
DTNB	5-5'-dithiobis [2-nitrobenzoic acid]
DTT	dithiothreitol
FCCP	carbonyl cyanide p-trifluromethoxy phenyl hydrazone
HTP	Biogel-hydroxylapatite
Iodogen	1,3,4,6-tetrachloro-3α, 6α diphenyl glycoluril
leupeptin	acetyl-L-leucyl-L-leucyl-L-argininal
LMM	low methionine medium
MMM	minus methionine medium
NBL-1	bovine kidney cells
NEM	N-ethylmaleimide
NGM	normal growth medium
OAA	oxaloacetate
OGDC	2-oxoglutarate dehydrogenase complex

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PDC	pyruvate dehydrogenase complex
PDM	phenylene dimaleimide
PK-15	pig kidney cells
PMSF	phenylmethyl sulphonylfluoride
PPO	2,5 diphenyloxazole
PTP	phosphate transport protein
SDS	sodium dodecyl sulphate
SMP	submitochondrial particles
TEMED	N,N,N'N'-tetramethylethylenediamine
TLCK	N-α-p-tosyl-L-lysine-chloromethyl ketone
TPCK	L-1-tosylamide-2-phenylethylchloromethyl ketone
TPP	thiamine pyrophosphate
Tween 20	polyoxyethylenesorbitan monolaureate
URF	unidentified reading frame.

SDH	succinate dehydrogenase
pCMB	p-chloromercuriobenzoate
PTS	phosphate transport system

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SUMMARY

(A) Binding of bovine heart pyruvate dehydrogenase complex (PDC) to the inner mitochondrial membrane.

The interaction of bovine heart PDC with the inner mitochondrial membrane was investigated using a combined enzymological and immunological approach. Employment of high-titre monospecific antisera directed against intact PDC and its constituent subunits allowed the investigation of this association under conditions which inactivate the enzyme.

Initial studies demonstrated that the bulk of PDC activity (70-80%) remains bound to the membrane while soluble control enzymes (citrate synthase and fumarase) are readily released into the supernatant fraction, with only minimal activity remaining membrane associated.

Preferential association of PDC with the inner membrane was further investigated using the immune-replica technique.

PDC remains bound to the membrane following prolonged sonication and repeated washing in high or low ionic strength buffers (0.15M - 2M NaCl). Only under the stringent conditions employed as criteria for integral membrane proteins e.g. 6M urea and 100 mM Na_2CO_3 (pH 11) is the bulk of PDC removed from the membranes. Under these conditions it was found that only trace amounts of E2 and/or X (subunit of PDC) remained associated with the membrane.

The specific attachment of the multienzyme complex to the inner mitochondrial membrane has been confirmed by parallel studies on three Krebs cycle enzymes, namely succinate dehydrogenase (an integral membrane protein), fumarase and

citrate synthase (both soluble matrix enzymes).

Dissociation of the complex to its constitutive enzymes was achieved by washing submitochondrial particles in 0.25M $MgCl_2$ or 0.1M glycine buffer, pH 9.5. Immune-blot analysis demonstrated the preferential release of E1 and E3 while the E2-X 'core' assembly remains in association with the membrane. These results suggest that PDC is bound to the inner mitochondrial membrane exhibiting the characteristics of a tightly-bound peripherally located membrane protein. It is likely that this association is mediated via the E2 and/or X subunits of the complex.

Immune-blot analysis, probing purified NADH dehydrogenase with a variety of antisera demonstrated an [illegible] interaction of PDC with this complex array of polypeptides. It is postulated that this interaction of PDC with this region of the respiratory chain may be of functional significance in that it will allow the direct channeling of NADH into the electron chain, thereby coupling NADH production and oxidation in an efficient manner.

(B) Topographical and biosynthetic studies on phosphate transport protein (PTP).

For these studies high-titre monospecific antiserum was produced against the purified phosphate transport protein from rat liver. This antiserum was shown to elicit a strong [illegible] reaction against the parent antigen, i.e. the rat liver enzyme and also the corresponding protein from bovine heart when mitochondrial extracts were immunoblotted

and challenged with anti-PTP-serum. The 34 000 M_r polypeptide was detected in crude extracts of the cell lines chosen for the biosynthetic studies on this protein i.e. Buffalo rat liver (BRL), rat kidney (NBL) and pig kidney (PK 15) cells.

Initial studies to elucidate the mode of synthesis of PTP were carried out by labelling BRL and PK 15 cells overnight in the presence of [^{35}S] -methionine. Immunoprecipitation with anti-PTP-serum and subsequent detection of radiolabelled polypeptides by fluorography failed to identify the parent antigen, although several other mitochondrial proteins and their precursors were detected in this manner. However, immunoblotting of a non-radiolabelled immunoprecipitate from bovine heart mitochondria successfully demonstrated the 34 000 M_r polypeptide.

Topographical studies on the final membrane organisation of PTP were initiated by proteolytic digestion of bovine heart mitoplasts and sonicated particles. After resolving the protease-resistant membrane-bound polypeptides by SDS-PAGE, they were transferred to nitrocellulose and analysed by immunoblotting with anti-PTP-serum. It was found that PTP contains no protease-sensitive domains exposed at either the matrix or cytoplasmic surface of the inner mitochondrial membrane. Solubilisation of the protecting membrane by either Triton X-100 or SDS demonstrated that the protein was not inherently resistant to protease digestion while parallel incubations and subsequent analysis of the membranes using anti-SDH-serum provided an excellent control system for these studies.

Results from these studies, native M_r determination and crosslinking data have allowed the presentation of a model for the molecular arrangement of PTP within the inner mitochondrial membrane of bovine heart.

CHAPTER 1

INTRODUCTION

1.1 THE MITOCHONDRION

The average mitochondrion in a mammalian cell has approximately the same dimensions as the bacterium Escherichia coli. It is most commonly observed as an oval particle, 1-2 μm long and 0.5 μm - 1 μm wide. Mitochondria, therefore, are sufficiently large to be seen in the light microscope, and the earliest descriptions of this organelle were based on the observations of fixed and stained tissue. Although mitochondria were first observed microscopically in the mid-nineteenth century, it was not until 1947 that Pallade and coworkers successfully isolated these organelles in an intact state.

Electron micrographs of negatively stained mitochondria in transverse section clearly show two membranes: a limiting outer membrane and a highly invaginated inner membrane. The enzymes involved in oxidative phosphorylation are housed in the inner membrane, and thus those tissues, such as muscle, that demand the greatest output of oxidatively derived ATP will have mitochondria best adapted for this purpose - at the morphological level this is expressed in a highly invaginated inner membrane. These membranes divide the mitochondrion into two separate compartments creating a total of four locations, outer membrane, inner membrane, matrix and intermembrane space, where an enzyme can be situated in the organelle.

1.1.2 Composition of the inner and outer membranes

The outer mitochondrial membrane is 6-7 nm thick, smooth and unfolded. This membrane is enriched in phosphatidyl choline (see Table 1.1) and also contains a considerable amount of cholesterol (Parsons and Yano, 1967; Levy et al., 1969). The membrane structure is relatively inelastic but is freely permeable to molecules of M_r value below 10 000. Further analysis of this membrane indicates that it contains a heterogeneous group of enzymes which catalyse various aspects of lipid metabolism plus several important hydroxylation reactions. These enzymes do not constitute any integrated pathway and consequently compositional data do not indicate the function of this membrane. The most frequently used marker for this membrane is the enzyme monoamine oxidase.

Lipid	Mitochondria	Inner Membrane	Outer Membrane	Microsomes
Phospholipids (mg/mg protein)	0.16	0.30	0.88	0.30
Cholesterol (µg/mg protein)	2.28	5.26	30.1	30.2
	Percentage of total phospholipid			
Phosphatidyl choline	40.0	44.5	55.2	62.8
Phosphatidyl ethanolamine	28.4	27.7	25.3	18.6
Cardiolipin	22.5	21.5	3.2	0.5
Phosphatidyl inositol	7.0	4.2	13.5	13.4

Table 1.1: Lipid composition of mitochondrial membranes (Adapted from Parsons et al., 1967).

In contrast, the inner membrane shows some evidence of substructure on its matrix-facing surface and exhibits differential solute permeabilities. Small uncharged molecules (e.g. H_2O, O_2, CO_2, NH_3 and ethanol) are freely permeable but its permeability to hydrophilic ions (nicotinamide and adenine nucleotides, H^+) is extremely limited except via specific transport systems (see Section 1.1.4). Thus, this membrane represents the major permeability barrier between the cytoplasm and the matrix. This 6-8 nm thick membrane has a low phospholipid to protein ratio and offers a very large surface area by virtue of its highly invaginated surface. A characteristic feature of the inner mitochondrial membrane is its high content of the phospholipid cardiolipin, and low cholesterol levels which renders it relatively resistant to digitonin treatment (see Table 1.1). It houses the respiratory chain, ATP-synthesising apparatus and a variety of solute carriers.

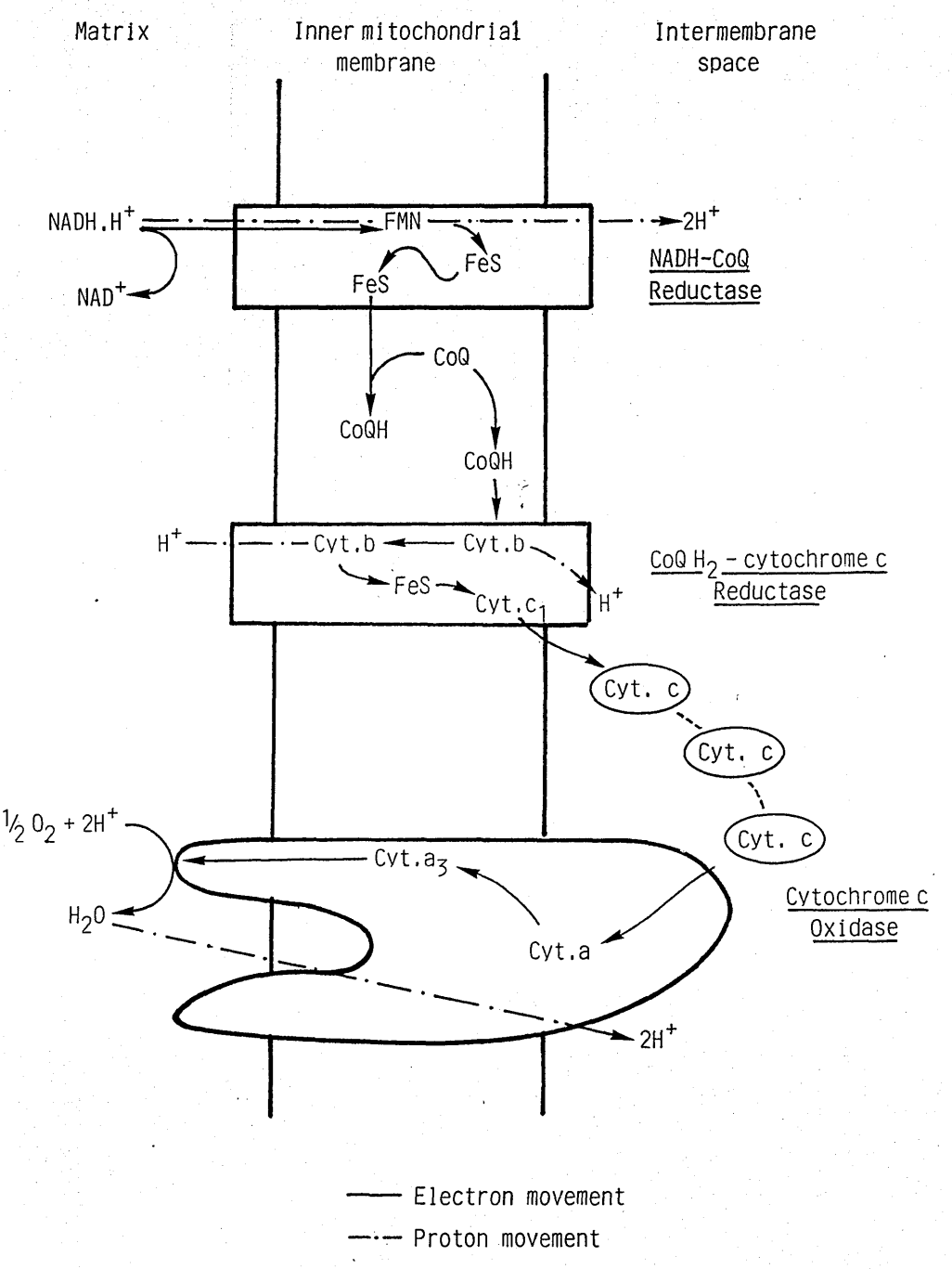
The intermembrane space is, excepting areas known as contact points, generally 6-8 nm wide and contains relatively few enzymes, most of which catalyse the interconversion of adenine nucleotides. The matrix, on the other hand, is extremely viscous and contains the vast array of enzymes that compose the tricarboxylic acid cycle (excepting succinate dehydrogenase which is a component of the inner mitochondrial membrane), fatty acid oxidation, part of the urea cycle in certain tissues, together with those that catalyse the synthesis of mitochondrial DNA and RNA. The first two catabolic pathways provide the adjacent respiratory chain with its major oxidisable substrate, NADH.

1.1.3 The electron transfer chain and oxidative phosphorylation

The most important function of mitochondria is that of oxidative phosphorylation. In this process the two electrons resulting from the oxidation of reduced coenzymes NADH or $FADH_2$ are ultimately transferred to oxygen with the concomitant generation of ATP.

Protons generated from this oxidation are soluble in aqueous solution as hydronium ions whilst free electrons are not. These are passed from NADH or $FADH_2$ to oxygen along a chain of electron carriers which are components of the inner mitochondrial membrane. Twelve or more electron carriers are grouped into four multiprotein intramembraneous particles. Each of the electron transport complexes exhibits lateral mobility in the plane of the membrane. There do not appear to be stable contacts between two or more enzymes. Transport of electrons from one complex to another may involve random collisions of the two. The lipid-soluble coenzyme Q shuttles electrons from the NADH-CoQ reductase and succinate dehydrogenase complexes to the $CoQH_2$-cytochrome c reductase complex. Cytochrome c interacts with specific sites both on the $CoQH_2$-cytochrome c reductase complex and on cytochrome c oxidase complex and thus transfers electrons from one to the other (see Fig. 1.1), acting as a mobile carrier. This random collision of the enzyme complexes involved in electron transport is sufficient to account for the rate of respiration in many tissues.

Fig.1.1 Electron transport in mitochondria.



The stoicheiometry of oxidative phosphorylation is not represented in this figure.

1.1.4 Mitochondrial transport systems

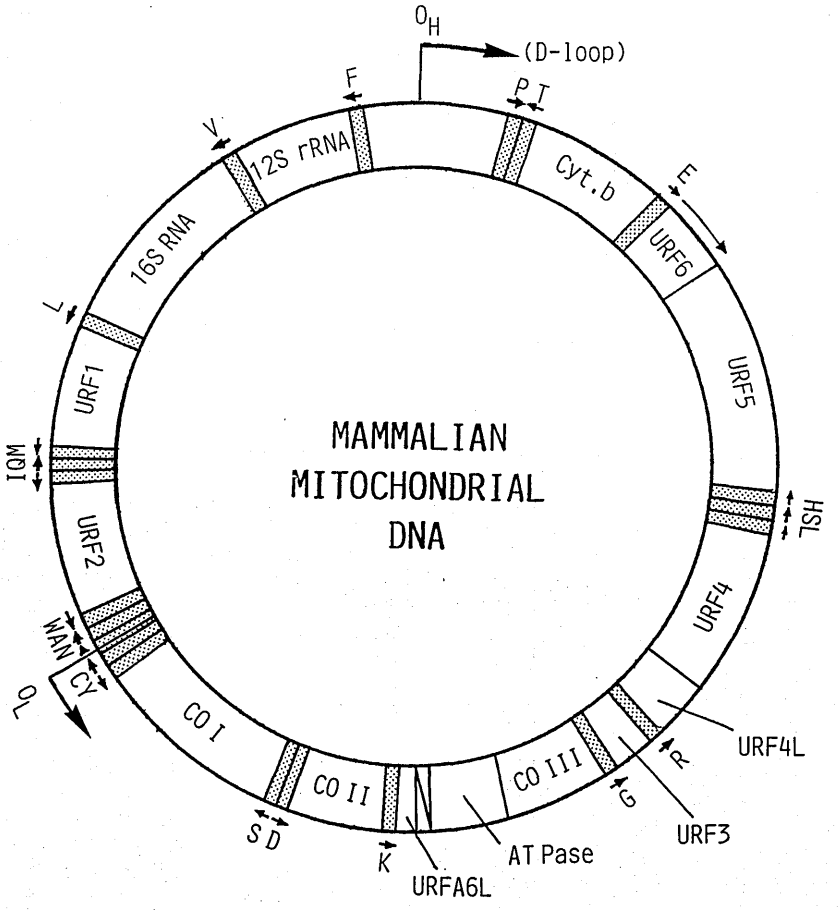
The specialised role of mitochondria in intermediary metabolism requires that only certain substrates, cofactors and metal ions must be accessible to the interior compartment. Of the substrates that must be capable of entering the matrix, the most important are O_2, H_2O, ADP, P_i (Section 1.3), pyruvate and fatty acids. At the same time products of mitochondrial oxidation must have means of exiting, these include citrate, CO_2 and ATP. Virtually all mitochondria, irrespective of source, are either freely permeable or have specific transport systems (see Table 1.2) that accommodate the efficient passage of these metabolites across the inner membrane.

From Table 1.2, it is apparent that mitochondria possess a broad spectrum of transport systems. Homogeneous preparations of mitochondria are easily obtainable providing convenient experimental material for transport studies. This shuttle of certain intermediates between the cytoplasm and mitochondria influences and regulates the overall metabolic balance of the cell. This aspect of transport will be discussed more fully in Section 1.3 (for review see Chappell, 1968).

1.1.5 Mitochondrial genome

The mitochondrial genome of higher eukaryotes is extremely well organised (Roe *et al.*, 1985; Anderson *et al.*, 1981; Anderson *et al.*, 1982). There are genes for two ribosomal RNAs, twenty two tRNAs and for no more than

Fig.1.2 Genomic map of mammalian mt.DNA.



Genomic map of mammalian mtDNA. The stippled areas represent tRNA genes designated by the single-letter amino acid code, with polarity given by the arrows. All protein-coding genes are encoded on the H strand (with counterclockwise polarity), with the exception of URF6, which is encoded on the L strand. COI, COII, and COIII: cytochrome oxidase subunits I, II, and III. Cyt.b.URF: unassinged reading frame. O_H and O_L: the origins of H and L strand replication respectively.

(Reproduction from Clayton 1984).

URF's have now all been assigned (Chomyn et al., 1986)

Transporter	Probable *in vivo* function	Inhibitors	Probable biological importance
Phosphate	OUT Phosphate → 0 ← OH^- IN	N-ethylmaleimide mersalyl	1. Mitochondrial ATP synthesis 2. Allowing flux of dicarboxylates and therefore tricarboxylates.
Adenine nucleotide	ADP^{3-} → 0 ← ATP^{4-}	Atractyloside carboxyatractyloside Bongkrekic acid	In mitochondrial ATP synthesis
Pyruvate	Pyruvate → 0 ← OH^-	α-cyano (4, OH) cinnamate	The link between glycolysis and the TCA cycle
Dicarboxylate	Malate → 0 ← Phosphate	n-butyl malonate 2-phenylsuccinate	1. Transferring reducing equivalents into and out of matrix 2. Provide C skeleton for PEP
Malate and succinate	Succinate → 0 ← malate	-	-
Tricarboxylate (citrate and isocitrate)	Citrate → 0 ← malate or isomalate Citrate → 0 ← isocitrate	Citrate analogues	1. Transfer of acetyl CoA into cytosol 2. Control of PFK 3. Provisions of NADPH into cytosol
Oxoglutarate	Oxoglutarate → 0 ← malate or malonate	Aspartate	1. Transfer of reducing equivalents across the membrane 2. Transport of amino groups into the matrix

Transporter	Probable *in vivo* function	Inhibitors	Probable biological importance
Glutamate	Glutamate → 0 ← OH^-	N-ethylmaleimide	Glutamate in the matrix gives rise to NH_2 for urea formation
Glu/Asp.	Glutamate H^+ → 0 ← Aspartate		1. Transfer of reducing equivalents 2. Aspartate involved in urea synthesis in cytosol 3. In gluconeogenesis
Glu/Gln	Glutamine → 0 ← Glutamate		NH_3 production in kidney
Ornithine/ citrate (liver only)			In operation of the urea cycle
Carnitine/ acyl carnitine (heart only)	Acyl Carnitine → 0 ← Carnitine	Carnitine derivatives	Movement of fatty acids across the membrane
Ca^{2+}	Ca^{2+} →	Ruthenium red	Storage of Ca^{2+} in the matrix
Ca^{2+}	Ca^{2+} → 0 ← $2Na^+$		

Table 1.2: Specific transport systems of the inner mitochondrial membrane.

thirteen proteins, most or all of which are involved in electron transport. Some of the protein-coding regions were initially only identified as unassigned reading frames or URF although recent studies have assigned components of the NADH dehydrogenase complex to these URF (Chomyn et al., 1985). There are no introns or spacers between the genes in higher eukaryotes, though some have been found in yeast, and a few genes even overlap slightly. It is now well documented that the overwhelming majority of mitochondrial proteins (> 90%) are encoded by nuclear genes and imported to the mitochondria in a post-translational fashion (for review, see Reid, 1985).

The only region of the mammalian mitochondrial DNA which is non-coding is the D-loop region which is involved in the initiation of replication. This is also the region at which the transcription of both strands is initiated (Montoya et al., 1982; Montoya et al., 1983). Transcription continues uninterrupted around the circular molecule and the transcripts are then processed to give individual messenger, ribosomal and transfer RNAs. The transfer RNA forms the punctuation between the various protein coding regions and provides the signal for the processing enzymes (see Fig. 1.2).

All of this information suggests that the mitochondrial genome in higher eukaryotes has been stripped of all elements that could be moved to the nucleus or dispensed with altogether. It also lends support to the popular, current hypothesis suggesting that mitochondria have arisen as

symbiotic prokaryotes providing oxidative metabolism to their pre-eukaryotic hosts. The application of the sensitive technique of Southern Blotting with cloned DNA fragments has allowed the identification of regions of mitochondrial-like DNA within the nuclear DNA of organisms as diverse as yeast (Farelly et al., 1983), insects (Gellissen et al., 1983) and rat (Hadles et al., 1983). This transfer of genes between the mitochondria and the nucleus is most readily recognised by the fact that the gene for the ATPase subunit IX is nuclear encoded in Neurospora crassa though a copy is found within mitochondrial DNA (Van den Boogaart, 1982). This copy is apparently silent and neither its origin nor physiological significance is known. Since the rate of production of base pair substitutions in mitochondrial DNA is about 10 times that in nuclear DNA, it provides a highly sensitive tool for studying short-time divergences amongst related species (Barton and Jones, 1983).

1.1.6 Mitochondrial biogenesis

Mitochondria were one of the first organelles to be isolated free of other cellular membranes and therefore served as a useful system for studying various aspects of structure, function, and, more recently, membrane biogenesis. There were several key discoveries which directed research in mitochondrial biogenesis, the most important of which was the observation that mitochondria possess their own protein synthesising machinery (Section 1.1.5).

Information to date suggests that cellular membranes do not form de novo but through the growth and division of pre-existing structures (for review see Palade, 1978). It therefore follows that the biogenesis of intracellular membranes must involve the addition of new constituents (proteins and lipid) to pre-existing compartments under conditions where the functional integrity of their membranes remains intact. With the existence of four mitochondrial sublocations where a given protein can be directed, this incorporation must be very specific (Sections 1.1.1 and 1.1.4) and be mediated via an intramitochondrial sorting process.

Considerable information on the biogenesis of mitochondria has been provided, in recent years, primarily through genetic and biochemical studies in yeast. The presence of cytosolic pools for many mitochondrial proteins, and the incorporation of these proteins into mitochondria can be divided conceptually into five steps (Fig. 1.3).

(i) synthesis of the polypeptide, usually as a larger M_r precursor on free cytoplasmic ribosomes.

(ii) recognition and binding of the precursor to the mitochondrial surface.

(iii) translocation of the precursor across or into one or both mitochondrial membranes, depending on its final destination. This step is dependent on the membrane potential.

(iv) processing of the polypeptide chain to the mature protein (may involve cleavage and/or other covalent modifications).

(v) assembly of the subunits to functional holo-enzymes (which in some cases may be associated with mitochondrial gene products).

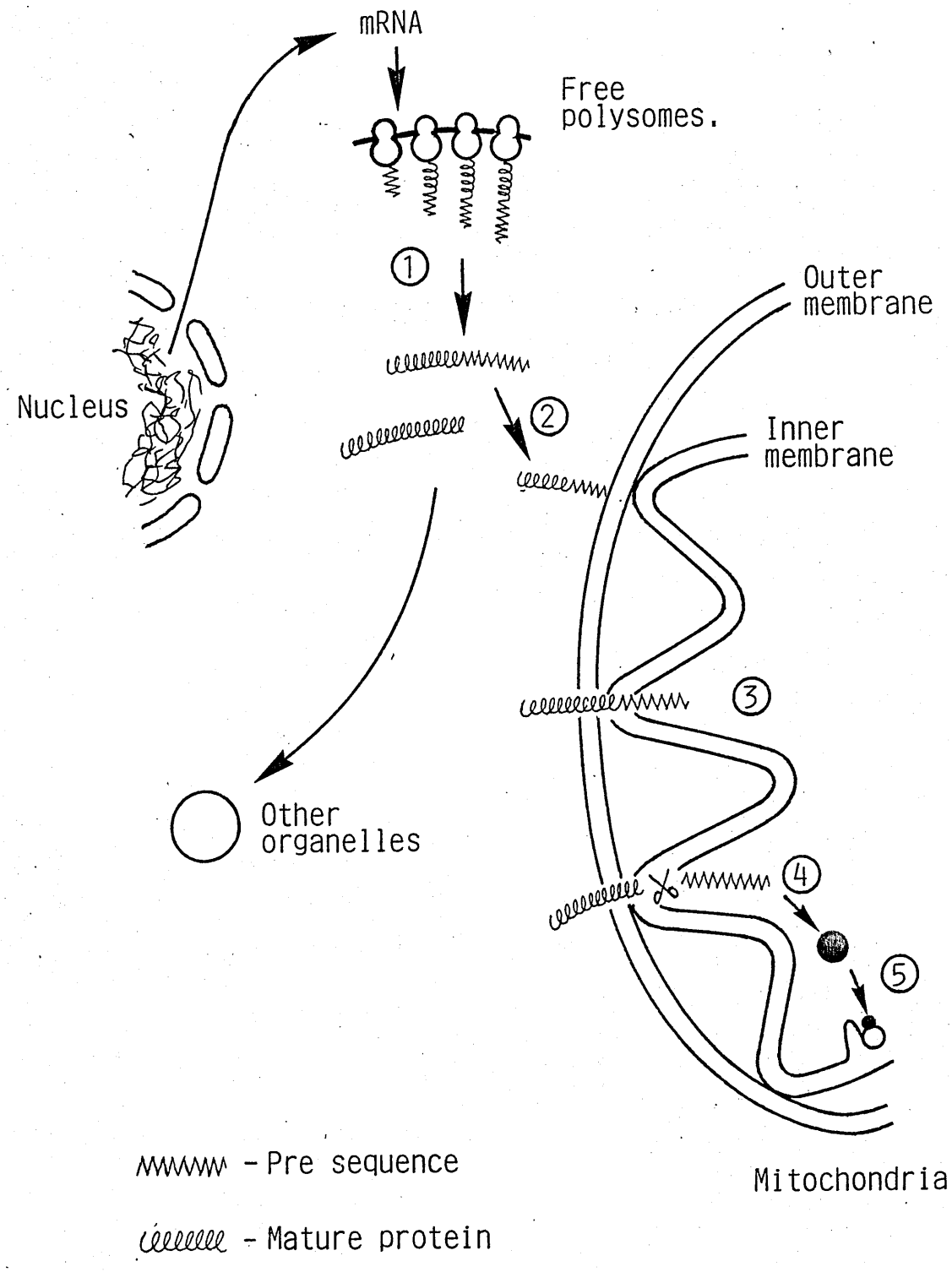
Evidence for a post-translational import mechanism, *in vivo*, has come primarily from the fractionation of pulse-labelled yeast sphaeroplasts. After a short pulse with [^{35}S] methionine, labelled precursor forms of mitochondrial polypeptides are found in the cytosolic fraction whilst mature forms are located within mitochondria and are thus protected from externally-added proteases (Janussi *et al.*, 1981; Mori *et al.*, 1981; Reid and Schatz, 1982). The mature polypeptide appears only after a lag period indicating the existence of cytosolic precursor pools.

Precursor molecules have also been identified and characterised using both *in vitro* translation, and *in vivo* pulse-chase labelling studies in mammalian cells.

The *in vitro* assay employs isolated mRNA which is translated in a cell-free system in the presence of radio-active amino acids. The precursor form of a mitochondrial polypeptide can then be immunoprecipitated from the translation mixture using antibodies directed against the mature polypeptide. In the majority of cases, the precursor molecule migrates with a higher apparent M_r value than the mature-sized subunit.

Figure 1.3: Steps in the biogenesis of an imported mitochondrial protein.

1. Precursor synthesis
2. Delivery and binding of precursors to mitochondrial surface
3. Translocation across membranes
4. Processing of precursor polypeptide
5. Refolding and assembly into functional units.


mRNA
Free
polysomes.
Nucleus
1
2
Outer
membrane
Inner
membrane
3
4
5
Other
organelles
- Pre sequence
- Mature protein
Mitochondria

The *in vivo* approach (see Chapter 5B) again employs highly specific antisera to precipitate selectively radiolabelled polypeptides from either cytosolic and mitochondrial fractions or from detergent-solubilised cell extracts. Resolution of precipitated polypeptides is achieved by SDS-PAGE and identification of radiolabelled bands by fluorography.

Both these methods and the fact that completed polypeptides from a cell-free translation system can be sequestered into isolated mitochondria strongly support a post-translational mechanism for import.

The N-terminal extension polypeptide is required to direct the precursor molecule to the organelle. Specific proteolytic processing enzymes exist within mitochondria for the maturation of the delivered precursor (Böhni *et al.*, 1980). This has been confirmed by gene fusion studies (see below). There are however, a number of precursor proteins which are targeted into each of the mitochondrial compartments without exhibiting transient signal sequences. For these proteins Gasser and Schatz (1983) have proposed an internal sequence which directs their mitochondrial delivery. The absence of a cleavable polypeptide extension has been documented for the inner mitochondrial membrane protein, adenine nucleotide translocase (Zimmerman and Neupert, 1980); a matrix protein, 2-isopropylmalate synthase (Gasser *et al.*, 1982) and an inter-membrane space component, cytochrome c, (Korb and Neupert, 1978).

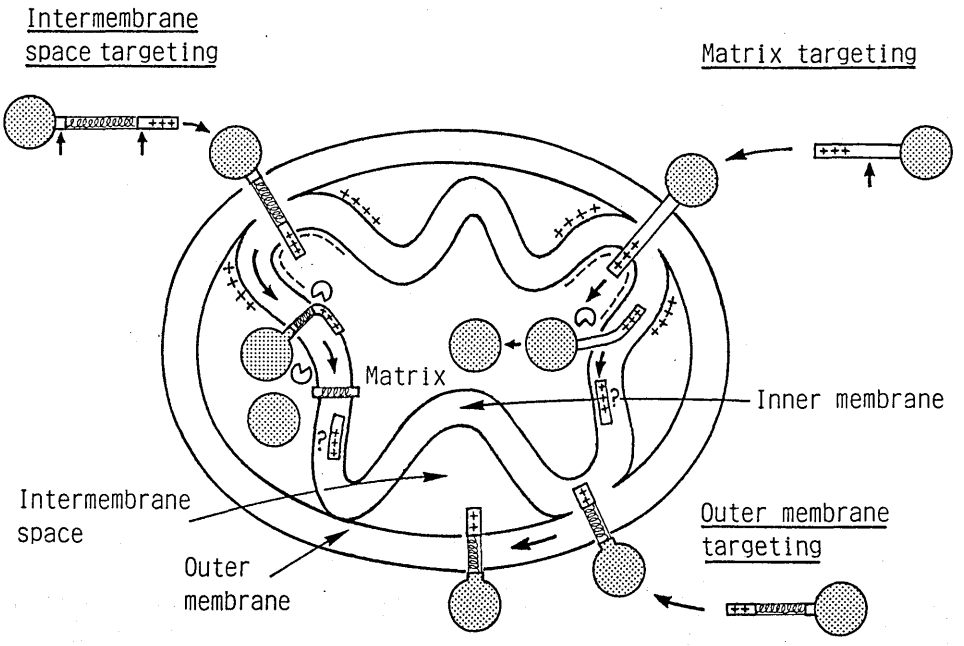
In some instances, this N-terminal extension might serve to maintain the precursor in a conformation that prevents premature assembly outside the mitochondrion. Also the mitochondrial precursor of N. crassa adenine nucleotide translocase binds to hydroxylapatite whilst the mature form not (Zimmerman and Neupert, 1980).

The specificity of the sorting process implies a receptor mediated process. Since the outer membrane of the mitochondrion is in direct contact with the cytoplasm, this bilayer has been the focus of studies to define components which specifically bind mitochondrial precursors. Different studies have demonstrated protease-sensitive regions on the mitochondrial surface (Hennig et al., 1983; Riezman et al., 1983; Zwizinski et al., 1983) which exhibit specific and saturable binding for mitochondrial precursors and not the mature forms. The potential number of protein candidates (about 20) available in the outer membrane suggests that a more involved mechanism may be required for the initial binding of precursor molecules to the outer mitochondrial membrane, rather than a receptor-mediated process. Indeed, on recent evidence showing that soluble factors present in a homologous cell free lysate can be substituted by a yeast cytosolic fraction (Ohta and Schatz, 1984) and be shown to stimulate the import of a precursor to mitochondria, it is tempting to speculate that cytoplasmic components may directly participate in the sorting of precursor proteins for mitochondrial delivery.

Although it is generally believed that import of precursors occurs at 'points of contact' between the inner and outer membrane, no compelling experimental evidence has been provided to document these 'points' as import sites for precursor protein uptake.

The well described cell biology of the mitochondrial import pathway in yeast provides the foundation for applying various genetic techniques to the molecular aspects of import. Studies have demonstrated that the information required to direct the cytoplasmically synthesised precursors to the mitochondria is contained within its primary sequence. Following the isolation of nuclear genes encoding mitochondrial components, gene fusion studies allowed the definition of the sequences which participate in targeting . This was established by the observation that gene constructs encoding a mitochondrial amino terminus could import hybrid proteins consisting of a non-mitochondrial protein at the carboxy-terminus (Douglas et al., 1984; Hase et al., 1984). Hurt et al. (1985) extended these studies and demonstrated that the first twelve amino acids were sufficient to direct import to the correct mitochondrial compartment. The dodecapeptide retained the properties of the complete cleavable mitochondrial sequence in being hydrophilic, rich in basic and hydroxylated amino acids and devoid of acidic amino acids. Indeed, Roise et al. (1986) have proposed a model in which mitochondrial presequences can form an amphilic α helix which enables the presequence to interact directly with energised mitochondrial membranes.

Fig.1.4 A model for intramitochondrial protein sorting
(Adapted from Hurt and Van Loon, 1986).



A model for intramitochondrial protein sorting . Imported molecules are composed of a transported 'passenger' protein (stippled circle) with an attached matrix - targeting presequence (open box with positive charges) or outer membrane targeting (open box with positive charges followed by a coil). The sites where the presequences are cleaved are indicated by arrows. Precursors enter the mitochondrion at 'contact sites' between the mitochondrial outer and inner membranes.

(Adapted from Hurt and Van Loon, 1986)

Based on elegant gene fusion studies, Hurt and van Loon (1986) have proposed a model for intracellular targeting and intramitochondrial sorting of imported proteins. In this model they propose that each precursor molecule contains essentially a matrix-targeting sequence (as described above). If this domain is followed immediately by a stop-transport sequence (i.e. long interrupted stretch of uncharged amino acids followed by charged residues) protein transport across the outer or inner membrane is prevented. This precursor molecule becomes either an outer membrane protein or an intermembrane space protein. Cleavage sites may be present for proteolytic removal of all or part of the presequence (see Fig. 1.4).

The final stage of the import pathway remains to be elucidated, i.e. the assembly of these proteins to functional units. The interaction between the nuclear and mitochondrial genome requires that a mechanism must exist not only to regulate cytoplasmic precursor protein synthesis but also to control the rate of mitochondrial growth with that of the cell. This coordination has been shown to exist, in that haem (a mitochondrial product) regulates the transcription of cytochrome c (Guarente and Mason, 1983). Further analysis of the mechanism of mitochondrial communication with the nucleus will elucidate much of the remaining mystery of mitochondrial biogenesis.

1.1.7 The future of mitochondrial research

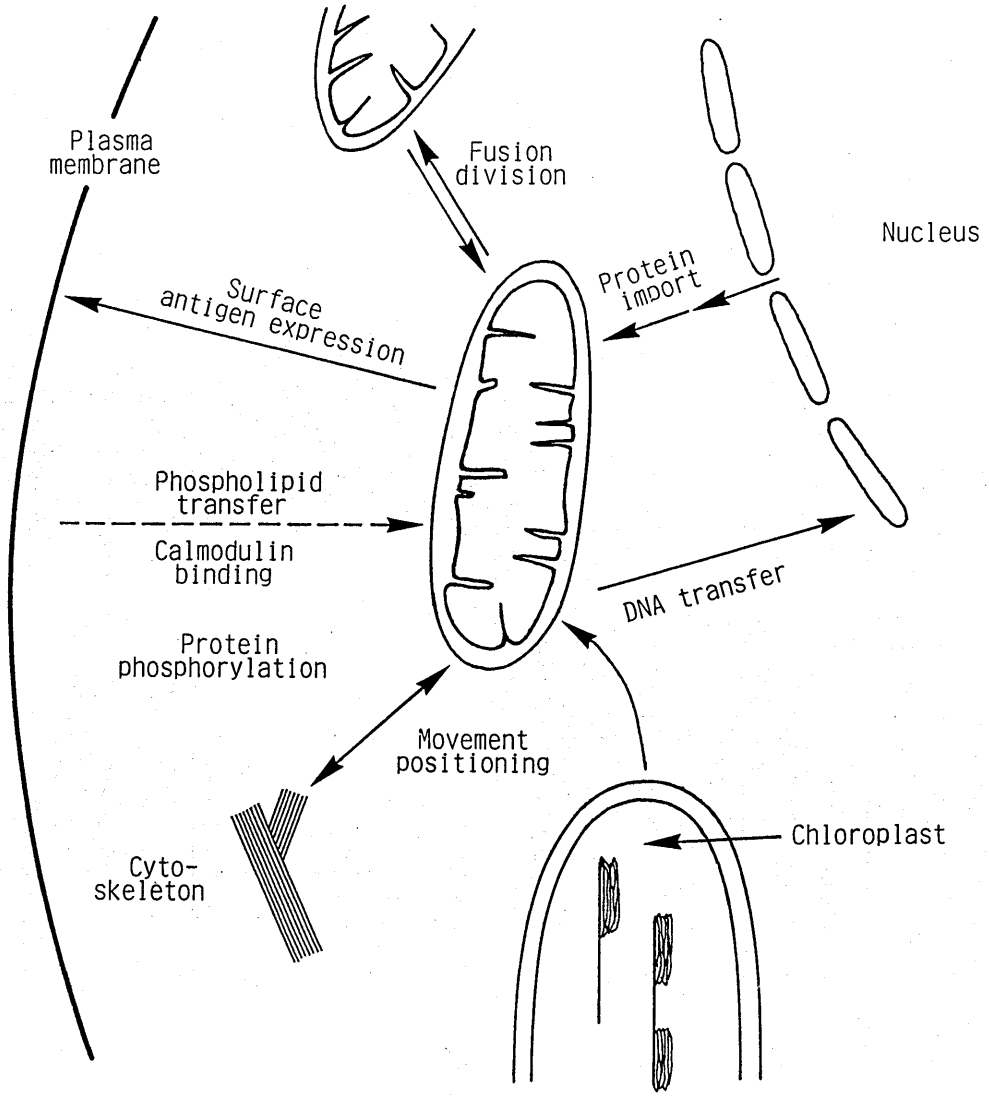
This introductory section (Section 1.1) serves to illustrate the major advances in mitochondrial research during the past thirty years.

In the early stages mitochondria were generally assumed to be self-contained organelles 'fed' by small molecules from the cytoplasm. This classical model of mitochondria originated from studies which demonstrated that mitochondria could carry out all their major functions in the absence of proteins and co-factors from the cytoplasm (Hogeboom et al., 1948). The discovery that mitochondria also contained their own genome (Section 1.1.5) further emphasised this autonomy.

A view of the eukaryotic cell is now emerging in which mitochondria interact structurally, functionally and biosynthetically with other cellular components (see Fig. 1.5). The complexity of many cellular functions and their regulation emphasises the need for communication and co-ordination between these organelles and the other cellular components.

The autonomy of mitochondria was first challenged in 1962 by Wildman and coworkers who observed mitochondria to be continually breaking up and fusing with each other. More recent observations, including the discovery that more than 90% of mitochondrial proteins are encoded by the nuclear genome, suggest that mitochondria interact extensively with other cellular structures. Also the high level of genetic recombination between mitochondrial genome populations in yeast cells during somatic growth (Birky, 1978) suggests that fusion of mitochondrial inner membranes, and hence the mixing of DNA-containing matrix compartments, may be a frequent event.

Fig.1.5 Interactions between mitochondria and other subcellular structures.



(Adapted from Yaffe and Schatz (1984a).

The demonstration that mitochondria have the ability to transfer DNA to the host nucleus (Section 1.1.5) further supports the endosymbiotic hypothesis of mitochondrial evolution. Indeed it has been argued that the cell's need for a mitochondrial genome is only because nuclearly encoded mitochondrial proteins have to be compatible with a pre-existing co-translational export machinery and those mitochondrially encoded proteins are confined to this genome by virtue of their hydrophobic segments (von Heijne, 1986). This hypothesis can be tested by applying the recent advances in gene fusion studies i.e. by fusing a mitochondrial targeting sequence to mitochondrially encoded proteins and transferring the resulting gene to the nucleus.

Interactions between mitochondria and the nucleus are not limited to occasional exchanges of DNA. As mentioned previously, mitochondrial biogenesis must require a continuously operating regulatory system linking the two organelles. Very little is known about this regulating system and thus it must represent the next major area of mitochondrial research to be investigated.

It was previously believed that mitochondria were static organelles or that any movement was via random diffusion rather than in any specific or guided fashion. The observation that mitochondria exhibit saltatory motion (Adams, 1982) and that they often occupy specific intracellular positions that correlate with the microtubular network (Ball and Singer, 1982) suggest that mitochondria are tethered to the cytoskeleton and that this association controls mitochondria

movement. These interactions have escaped chemical detection, so far, but they may explain why, upon cell fractionation, a large percentage of mitochondria are lost in the rapidly sedimenting fraction (Shore and Tata, 1977).

Smith and coworkers (1983) found that expression of a class I component of the major histocompatibility complex is maternally controlled and correlated with a sequence difference in mitochondrial DNAs. Despite the nuclear location of this gene and the inability of mitochondria to export proteins it is difficult to explain how the mitochondrial genome can exert this influence on a protein component of the plasma membrane. This observation illustrates dramatically the limits of our knowledge on how mitochondria interact within the eukaryotic cell.

These interactions are difficult to study by conventional biochemical methods since, by and large, they are lost upon cell fractionation. As many of these interactions are probably mediated by the outer mitochondrial membrane, the isolation of sealed outer membrane vesicles and the cloning of the polypeptides within these vesicles may help clarify these ambiguities.

By exploiting the advances in genetic techniques and applying these principles to the well characterised yeast system, Yaffe and Schatz (1984) have isolated two mutants in which mitochondrial assembly has been rendered temperature sensitive. These mutants can be used to isolate known proteins of the import machinery, to clone the genes coding

for these proteins, and to overproduce these components. Moreover they might help to identify additional cellular components that are necessary for mitochondrial assembly.

The future of mitochondrial research will concentrate increasingly on attempting to understand more fully the phenomena described above.

The classical view of mitochondria has almost completely been replaced, despite sufficiently explaining such important functions as oxidative phosphorylation and electron transport, and mitochondrial research will focus primarily on understanding the complex interactions between mitochondria and other cellular components.

1.2 PYRUVATE DEHYDROGENASE MULTIENZYME COMPLEX

1.2.1 Multienzyme complexes

The eukaryotic cell exhibits a high degree of organisational complexity, being subdivided into a number of organelles and compartments, each of which has a set of unique functions and different protein composition. These spatially distinct functions are interdependent and the basic cellular processes from DNA synthesis to ATP generation depend upon the interaction of proteins and cofactors from these compartments.

Much of the metabolism within the cell can be explained by the action of a few thousand enzymes promoting the specific reactions of their substrates. These enzymes channel their products into a network of metabolic pathways, often organised as cycles. It has become apparent that a major part of

metabolism is concerned with the creation of complex molecules that aggregate spontaneously in highly specific ways to generate structure. Thus when a number of enzymes are physically associated together to form a defined particle which can catalyse a series of linked biochemical conversions, such an aggregate is termed a multienzyme complex. The physical interaction of the enzymes in these complexes results in a distinct morphology and the most highly organised multimeric complexes in the cell are probably the arrays of membrane bound enzymes of oxidative phosphorylation (Section 1.1.4) in mitochondria.

There are several well characterised examples of multienzyme complexes e.g. tryptophan synthetase from E. coli (Yanofsky and Crawford, 1972); fatty acid synthetase from eukaryotic sources (yeast: Lynen, 1972; mammalian: Stoops et al., 1978) and the 2-oxo acid dehydrogenases from all organisms so far studied (Reed, 1974; Perham, 1975).

This organisation of individual enzymes into multienzyme complexes can provide a number of functional advantages e.g. it may facilitate the opportunity for increased specificity and modes of control not possible with structurally independent enzymes. For example, tryptophan synthetase from E. coli, (Yanofsky and Crawford, 1972) is a tetrameric enzyme with subunit structure $\alpha_2\beta_2$. Resolution of the complex results in α_2 and β_2 dimeric species, both of which are catalytically active, being involved in two partial reactions. In the assembled complex, however, the overall reaction is catalysed with greater efficiency than either of the constituent

activities and potentially competitive reactions involving subunit-specific activities are suppressed e.g. serine deaminase.

The channeling of substrates and co-factors which may be retained on the surface of the complex may be considered as a form of compartmentalisation and may exclude competition from enzymes of another pathway. Examples of this may be found in the arom complex of N. crassa (Giles et al., 1967) or any of the 2-oxo acid dehydrogenase complexes so far studied. The compartmentalisation within multienzyme complexes may also serve to prevent degradation of unstable intermediates in an aqueous solution.

Within multienzyme complexes the association of components is stable. This stability allows their isolation/purification as discrete entities, thus providing the opportunity for detailed in vitro study of their assembly and the interaction of subunits. Indeed, in some cases the individual components have been resolved and reconstituted to form functional aggregates indistinguishable from the native complex.

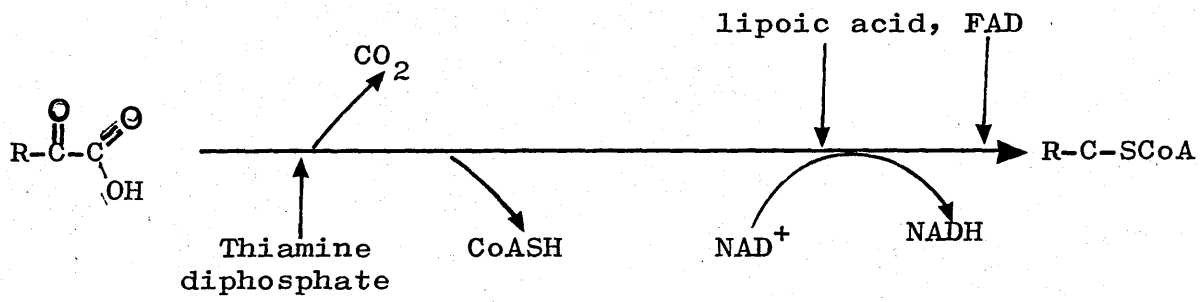
The association of enzymes within cells may be far more extensive than was originally imagined. There are indications that the enzymes of the glycolytic pathway form functional particles in trypanosomes which are known as glycosomes (Gorringer and Moses, 1978) and also that certain enzymes of the citric acid cycle are physically linked (D'Souza and Srere, 1983; Sumegi and Srere, 1984).

Apart from an interest in each multienzyme complex as an object in itself, its study will result in a deeper understanding of the mechanisms whereby proteins influence

the action of other proteins and the integration of protein function. This knowledge can thus be applied to the principles governing cellular organisation.

1.2.2 2-oxo acid dehydrogenase complexes

The three major 2-oxo acid dehydrogenase complexes in mitochondria consist of three enzymic activities that function successively to catalyse the following overall reaction:



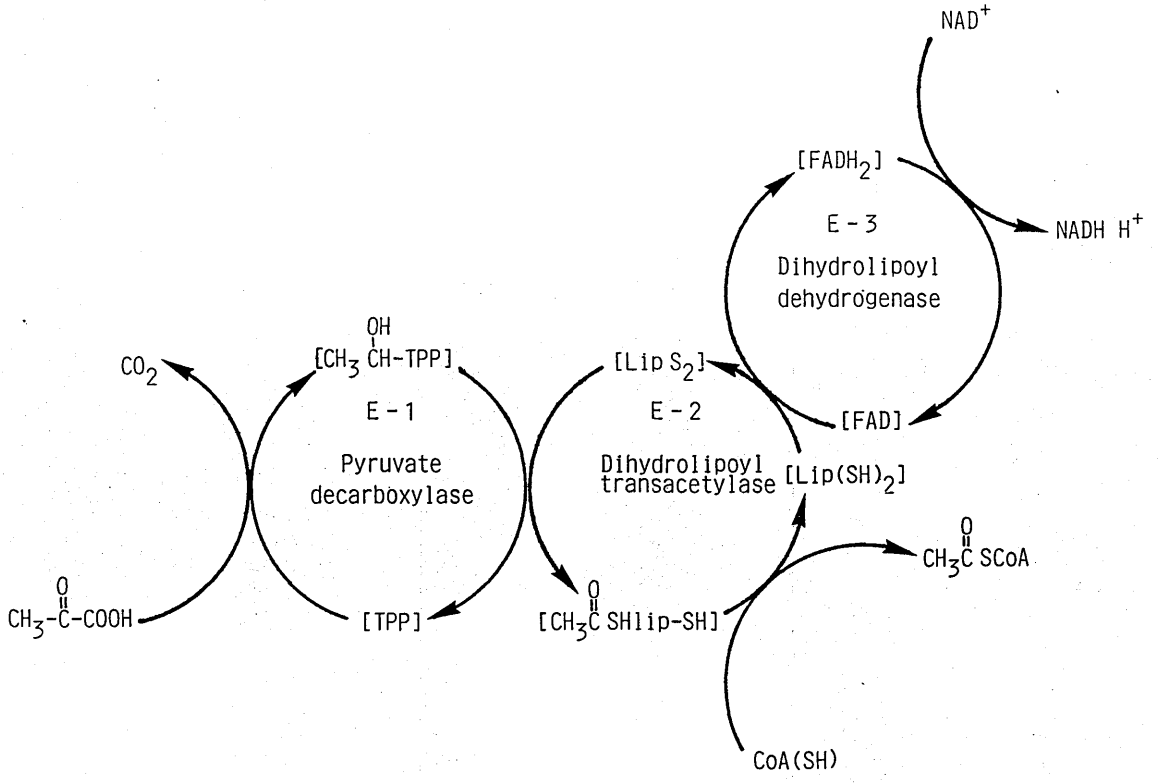
The specific reactions catalysed by the pyruvate dehydrogenase complex (PDC); the 2-oxo glutarate complex (OGDC) and the branched-chain 2-oxo acid complex (BCDC) are shown in Table 1.3. There are no fewer than five coenzymes and prosthetic groups involved in the sequence of reactions catalysed by the complexes; thiamine diphosphate (TPP); lipoic acid; coenzyme A; flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD^+). The complex also has an absolute requirement for Mg^{2+}.

The PDC catalyses the oxidative decarboxylation of pyruvate to acetyl CoA (Fig. 1.6). *In vivo* this is an irreversible process, and the regulation of this complex is of crucial importance, particularly in mammals which lack the ability to synthesise glucose from acetyl CoA. As will be illustrated in Section 1.2.6, PDC is subject to control

Complex	R	Substrate	Product
Pyruvate dehydrogenase	CH_3	Pyruvate	Acetyl-CoA
2-oxoglutarate dehydrogenase	$CO_2 \cdot CH_2 \cdot CH_2$	2-oxoglutarate	Succinyl CoA
Branched chain-	$(CH_3)_2 \cdot CH \cdot CH_2$	2-oxoisocaproate	Isovaleryl CoA
2-oxoacid dehydrogenase	$(CH_3 \cdot CH_2) \cdot CH_3 \cdot CH$	2-oxo-methyl valerate	2-methyl butyrylCoA
	$(CH_3)_2 \cdot CH$	2-oxo-iso valerate	Isobutyryl CoA

Table 1.3: Specific reactions catalysed by the 2-oxo acid dehydrogenase complexes.

Figure 1.6 Reaction sequence of PDC



E1 $CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}CO_2H + E_1\ [TPP] \longrightarrow E_1\ [CH_3CHOH\text{-}TPP] + CO_2$

E2 $E1[CH_3\text{-}CHOH\text{-}TPP] + E_2\ [lip\ S_2] \rightleftharpoons E_1\ [TPP] + E_2\ [CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}S\text{-}lip\text{-}SH]$

$E_2\ [CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}S\text{-}lipSH] + CoA\text{-}SH \rightleftharpoons E_2\ [lip(SH)_2] + CH_3\text{-}\overset{O}{\overset{\|}{C}}\text{-}SCoA$

E3 $E_2\ [lip(SH)_2] + E_3\ [FAD] \rightleftharpoons E_3\ [lip\ S_2] + E_3\ [FADH_2]$

$E_3\ [FADH_2] + NAD^+ \rightleftharpoons E_3\ [FAD] + NADH + H^+$

by feed-back inhibition (NADH, acetyl CoA) and by a phosphorylation-dephosphorylation mechanism involving covalent modification of specific serine residues on the E1 α subunit (Yeaman *et al.*, 1978).

PDC provides acetyl CoA, an important biosynthetic metabolite utilised in the synthesis of fatty acids, steroids, and, in most aerobic organisms, the oxidation of acetyl CoA via the tricarboxylic acid cycle is the source of metabolically utilisable energy in the form of ATP.

PDC from ox heart (M_r 8.5×10^6) is a large catalytic array of three enzymes: pyruvate decarboxylase (EC 1.2.4.1); dihydrolipoamide acetyltransferase (EC 2.3.1.12) and dihydrolipoamide dehydrogenase (EC 1.6.4.3) (Linn *et al.*, 1972). These and the corresponding activities in the analogous 2-oxo acid dehydrogenase complexes are frequently termed E1, E2 and E3, respectively. While the E3 component is common to all three complexes, each contains a distinct carboxylase and acetyltransferase subunit. The general sequence of reactions catalysed is shown schematically in Figure 1.6.

These subunits are self-assembling and form an ordered and symmetrical structure as determined by evidence from reconstitution and electron microscopic studies.

The BCDC and OGDC have also been characterised in mammalian tissues. The branched-chain complex, like the PDC, is regulated by the reversible phosphorylation of the α subunit of its decarboxylase component (Fatania *et al.*, 1983;

Lau et al., 1983; Cooke et al., 1983). No evidence exists for the regulation of OGDC activity by a similar mechanism.

The 2-oxo glutarate complex is an enzyme of the tricarboxylic acid cycle producing succinyl CoA which is required in the biosynthesis of porphyrins, lysine and methionine. The branched-chain 2-oxo acid complex catalyses the committed step in the degradation of the branched-chain amino acids. In mammals this is of nutritional importance in dealing with excess valine, leucine and isoleucine in the diet (Goldberg and Chang, 1978). These amino acids are ultimately converted to the corresponding derivatives of CoA. A deficiency of this enzyme activity in humans results in the elevation of the 2-oxo acid in blood and urine. This elevation ultimately causes Maple Syrup Urine Disease (Chuang et al., 1981) which unless patients are restricted to a diet with low levels of these amino acids from an early age, is fatal.

Each of the 2-oxo acid dehydrogenase complexes has been purified from several mammalian sources: PDC has been isolated from ox kidney (Kresze and Steber, 1979; Cate and Roche, 1979); porcine liver (Roche and Cate, 1977) and bovine heart (Stanley and Perham, 1980). 2-oxo glutarate complex has been purified from ox kidney (Reed and Oliver, 1968) and porcine heart (Koike and Koike, 1976). The branched-chain enzyme has been isolated from bovine liver and heart mitochondria (Danner et al., 1979; Petit et al., 1978).

Although PDC has been well characterised from other sources, in particular E. coli (for review see Reed and Petit, 1981) and yeast (Kresze and Ronft, 1981a,b), the remainder of this study will concentrate on the mammalian enzyme.

1.2.3. Composition of mammalian PDC

The mammalian pyruvate dehydrogenase complex can be resolved into four major polypeptides by SDS-PAGE (Reed, 1974; Koike and Koike, 1976; Stanley and Perham, 1980) although on substitution of the phosphate-buffered system (Weber and Osborne, 1969) by the Tris-glycine system of Laemmli, a fifth polypeptide (component X) M_r 50 000 is readily resolved from the E3 component (Kresze and Steber, 1979; Stanley and Perham, 1980; de Marcucci et al., 1985; de Marcucci and Lindsay, 1985). The identity and function of this band has not yet been fully elucidated.

The E1 component migrates as two bands of M_r 42 000 ($E1_\alpha$) and M_r 37 000 ($E1_\beta$) following resolution by SDS-PAGE (ox heart: Barrera et al., 1972; porcine heart: Koike and Koike, 1976). The isolated E1 enzyme of mammalian PDC has an $\alpha_2\beta_2$ tetrameric subunit composition (M_r 154 000) rather than the larger (M_r 100 000) dimer forming the E1 of the E. coli PDC (Reed, 1974). In ox heart, the E3 enzyme is composed of two identical FAD-containing subunits, migrating with an apparent M_r value of 55 000. The E2 component consists of a single polypeptide chain which assembles to the high M_r core structure. Conflicting reports on the M_r values

of the core subunits have been attributed to methodological differences: electrophoresis in SDS yields an apparent M_r in the range of 70 - 74 000 (Barrera et al., 1972; Machicao and Wieland, 1980) whereas Barrera et al. (1972) estimated, by means of sedimentation equilibrium, an M_r of 52 000, a value confirmed by Kresze et al. (1980) on determining the M_r value by gel electrophoresis in 6M guanidine.

The assembled complex has a high M_r value 8.5×10^6 from ox heart and is composed of multiple copies of the E1 and E3 subunits which are tightly but non-covalently bound to the E2 core assembly (M_r 3.1×10^6). Electron microscopic studies (Reed and Oliver, 1968) revealed that the E2 'core complex' has the appearance of a pentagonal dodecahedron. The inferred icosahedral symmetry (532), together with molecular mass considerations, predicted the presence of 60 subunits in the intact E2 component.

The mammalian complex also contains small amounts of two regulatory enzymes, a kinase and a phosphatase, that modulate the activity by phosphorylation (inactivation) and dephosphorylation (activation) respectively (Fig. 1.7).

The PDC kinase activity is apparently associated with both the E1 and E2 components of the complex (Jilka et al., 1986). Although its precise stoicheiometry with respect to the other components is unknown, it has been shown to vary in a tissue-specific manner with estimations of 1-2 moles per mole of heart complex and 3-5 moles for the kidney enzyme. The ATP-dependent kinase activity copurifies with the complex, a key factor in its isolation and subsequent characterisation (Stepp et al., 1983).

Inactivation of the complex is achieved by the phosphorylation of one of three possible serine residues in the α subunit (Yeaman et al., 1978). PDC kinase contains subunits, α and β, of M_r 48 000 and 45 000, respectively. Stepps et al. (1983) have identified the α subunit as the catalytic subunit from proteolysis studies and have proposed that the β subunit may function in a regulatory capacity.

The phosphatase, in contrast to the kinase, is only loosely bound to the complex. Reed and Petit (1981) obtained a highly-purified phosphatase from bovine kidney and subsequent characterisation revealed that it consisted of two non-identical subunits of M_r 97 000 and 50 000. The phosphatase reaction requires Mg^{2+} and in addition the binding of the phosphatase has an absolute requirement for Ca^{2+} (Denton et al., 1972).

These two 'converter' enzymes are integrated into the complex and comprise a monocyclic interconvertible enzyme cascade (Stadtman and Chock, 1977) (Fig. 1.7).

1.2.4 Structural and functional studies on mammalian PDC

Despite the requirement for the participation of all three constituent enzymes in the overall reaction, it is clear that the active sites are not necessarily represented in equal numbers in the enzyme complex (Table 1.4). This suggests that PDC and the analogous 2-oxo acid dehydrogenases do not function by simple direct transfer of substrate among three active sites arranged on a 1:1:1 basis and therefore that the structure and organisation of the multienzyme

Enzyme	M_r	Subunit No.	Subunit M_r	Subunits per molecule complex
Native complex	8.5×10^6			
PDH	154 000	2 2	42 000 37 000	60
Dihydrolipoyl acetyltransferase	$3\ 1 \times 10^6$	60	72 000	60
Dihydrolipoyl dehydrogenase	110 000	2	55 000	12
PDC kinase	100 000	1 1	48 000 45 000	2
PDC phosphatase	150 000	1 1	97 000 50 000	0

Table 1.4: Subunit composition of bovine heart PDC.

complexes must relate to the enzyme mechanism. Indeed, early electron microscopic studies (Hayakawa et al., 1969) indicate that the molecules of the E1 and E3 components are apparently bound to the lipoate acetyltransferase core and are distributed in such a manner as to allow the efficient interaction of subunits and cofactors. Latterly, this interaction has been probed using both biochemical and biophysical techniques in an attempt to clarify the relationship between the structure and function of PDC.

1.2.4.1 Use of limited proteolysis to probe subunit structure

PDC activity in crude extracts from rat liver decreases rapidly and irreversibly (Wieland, 1975). This inactivation was attributed to a lysosomal protease which Kresze and Steber (1979) have termed INACTIVASE. This leupeptin-sensitive protease was shown to act on the E2 core without affecting its quaternary structure butto leadto the release of the other subunits (Kresze and Steber, 1979). Each of the individual enzymes retained its activity while that of complex was almost completely lost. This evidence suggests that there are probably no gross conformational changes at the active site of each constituent enzyme on assembly to form the high M_r value multienzyme complex.

The lysosomal protease, and similarly trypsin and papain (Kresze et al., 1980) cleave the core component, lipoate acetyltransferase, to yield two principal fragments which have been isolated following separation on SDS-PAGE.

It is now established from this and further studies on the native enzyme by Bleile *et al.*, (1981), that the acetyltransferase subunit consists of two non-identical domains: a compact domain of approximately 26 000 M_r, identified as the subunit binding domain which also contains the catalytic site for transacetylation; attached to this is a flexible extended domain of approximately 28 000 M_r carrying the covalently-bound lipoyl moiety.

Immune mapping studies following proteolysis with elastase of the native enzyme gave similar results (de Marcucci *et al.*, 1986). Employing subunit-specific antisera directed against the E2 component, the authors demonstrated the generation of two major fragments. The larger 45 000 M_r fragment was shown to be particularly immunoreactive. This enhanced reactivity, relative to the second M_r fragment, is primarily thought to be as a consequence of its unusual structural features i.e. an extended domain structure protruding from the inner core of the acetyltransferase assembly (Bleile *et al.*, 1981). The high antigenic reactivity of epitopes located in flexible or mobile regions of protein has been reported previously (Westhof *et al.*, 1984).

Release of the lipoylated domain from native PDC following limited proteolysis with trypsin has also been reported for the *E. coli* enzyme (Bleile *et al.*, 1979; Hale and Perham, 1979). The large C-terminal domain of the E2 chain that remains aggregated to form the inner core of the complex retains both the acetyl transferase active site and the binding site for

the E1 and E3 subunits. Indeed, it has proven possible to isolate these lipoyl domains as three distinct functional entities after limited proteolysis of the complex with Staphylococcus aureus V8 proteinase.

The susceptibility of this region of polypeptide chain to various proteases is thought to be as a result of its protrusion from the aggregated core complex, a structural domain believed to be an integral feature of the enzyme mechanism.

1.2.4.2 Mobility of the E2 polypeptide

Proton NMR studies have often been used to demonstrate significant internal mobility in the structure of several small molecules. This has been shown to involve not only specific amino acid side chains but also larger regions of polypeptide chain.

Wawrzynczak et al., (1981) have demonstrated that ox heart PDC contains a highly mobile region, which, when coupled to the limited proteolysis and electron microscopic data, was assigned to the E2 component. More elaborate studies were carried out by Perham et al., (1981) who reported on similar observations for the E. coli complex. Perham and coworkers resolved E. coli PDC into two subcomplexes: E1-E2 and E2-E3. Both these subcomplexes were shown to enjoy the prominent features of the proton NMR spectra of the intact complex. This mobility was attributed to the core complex since the lipoate acetyltransferase was the only common polypeptide (Roberts et al., 1983).

This mobility is an intrinsic property of the protein and is unrelated to any substrate-induced conformational change and may explain some features of the enzyme complex. It may permit the lipoyl-lysine swinging arm structure to interact over more of the complex surface than the length of the lysine lipoate arm (1.4 nm) would suggest at first. Spin label experiments (Ambrose and Perham, 1976) are consistent with this idea, although fluorescent energy transfer measurements (Angelides and Hammes, 1979) have indicated that the distance between active sites is almost twice the span of the swinging arm.

1.2.4.3 Number of lipoic acid residues per E2 subunit

The lipoic acid cofactor is reductively acetylated via a hydroxyethyl-TPP intermediate formed by decarboxylation of pyruvate on the E1 component of the complex. This acetyl group is transferred to CoA(SH) at an active site on enzyme E2, forming the product acetyl CoA. This transfer generates the reduced form of lipoic acid and its subsequent re-oxidation is achieved by lipoamide dehydrogenase (Fig. 1.6). Thus lipoic acid plays the key role in the mechanism of the pyruvate dehydrogenase complex, visiting three active sites on E1, E2 and E3, respectively, and mediating the transfer of acetyl groups to CoA (SH).

There is still some controversy concerning the number of lipoyl residues bound per E2 chain. Most evidence suggests that only a single lipoyl moiety is present on each E2 polypeptide (White et al., 1980; Hamada et al., 1975;

Stanley et al., 1981). These authors studied the extent of incorporation of radiolabelinto PDC in the absence of acetyl CoA, using [2-^{14}C] pyruvate. In contrast, Cate and Roche (1979), in a similar study, estimated the number of lipoyl groups per E2 chain to be 2. However, Hodgson et al. (1986) have suggested that this second, slower phase of acetylation may not be the reductive acetylation of a second lipoyl group as it results in protection against N-ethylmaleimide modification of lipoyl groups on E2, but perhaps a reactive cysteine residue which may be of catalytic significance.

Extensive studies have been carried out on the lipoyl domains of the E. coli enzyme. The primary structure of the E2 polypeptide inferred from the DNA sequence (Stephens et al., 1983) has revealed three highly homologous sequences containing a lysine residue which is capable of lipoylation (Hale and Perham, 1980). The lipoyl group of each domain, like the mammalian counterpart, becomes reductively acetylated, in the intact complex, in the presence of substrate (Packman et al., 1984a). Genetic reconstructions (Graham et al., 1986) of E. coli PDC have demonstrated that lowering the number of lipoyl segments from 3→2 or 2→1 per E2 chain still permits the assembly of functional PDC. This is consistent with the view that the three lipoyl domains are folded as independent units. These truncated polypeptides have been shown to be unimpaired in catalytic activity and in the intramolecular coupling of active sites. This genetically-reconstructed E. coli PDC thus closely resembles the PDC (icosahedral symmetry) of B. stearothermophilus (Packman et al., 1984b);

B. subtilis (Hodgson et al., 1983) and of ox heart mitochondria (Reed, 1974; Bleile et al., 1981; Stanley et al., 1981b) and suggests that the two 'extra lipoyl' groups are catalytically redundant with no role attributable to them.

1.2.4.4 Conclusion relating PDC structure to catalytic function

During the catalytic cycle of the PDC, the substrate is transferred between the physically separate active sites by the lipoyl-lysine 'swinging arms' on which it is retained in thioester linkage (Reed, 1974; Ambrose and Perham, 1976; Grande et al., 1976). Proteolysis studies and proton NMR spectroscopy have located these flexible regions on the E2 polypeptide, in segments which protrude from the aggregated core assembly which binds the E1 and E3 components and which contain the lipoyl group. Acetylation studies, using radiolabelled pyruvate, have demonstrated that an extensive network of coupling reactions exists, which permits the intramolecular transfer of acetyl groups between lipoic acid residues of different E2 subunits in the same enzyme core (Bates et al., 1977; Collins and Reed, 1977; Packman et al., 1983). Also, a lipoyl group can visit the active site of several E1 subunits in the same complex (Cate and Roche, 1979; Stanley et al., 1981; Stepp et al., 1981; Hackert et al., 1983).

The elegant genetic studies of Graham et al. (1986) when coupled to existing data from tryptic digests (Bleile et al., 1979) or NEM inactivation experiments (Ambrose-Griffin et al., 1980) demonstrate that more than half of

the E. coli PDC lipoyl groups can be removed without impairing the enzyme's activity. These extra lipoyl domains can be regarded as being redundant with respect to the catalytic cycle of the enzyme-complex though perhaps more stringent kinetic analysis may elucidate a role for them.

1.2.5 Structural and functional studies on component X

Recently a previously-unrecognised fifth polypeptide, component X, has been detected in mammalian PDC. This polypeptide is readily resolved from the E3 subunit by SDS-PAGE in Tris-glycine buffers (de Marcucci and Lindsay, 1985; de Marcucci et al., 1985). This component has been demonstrated subsequently to form a close physical and functional union with the E2 core assembly (de Marcucci and Lindsay, 1985). A similar component may be tightly bound to the E2 assembly of Saccharomyces cerevisiae where an additional 50 000 M_r component is also present in comparable amounts to that of the mammalian complex.

While component X in mammalian PDC has been observed previously, reports demonstrating that it is capable of becoming acetylated suggested that it was a proteolytic fragment of the E2 polypeptide. It was also thought to be the intrinsic kinase activity prior to its isolation. However by employing refined immunological techniques and standard protein chemistry (de Marcucci et al., 1985, 1986; Jilka et al., 1986) it has been shown that component X is a distinct polypeptide.

1.2.5.1 Physical association of component X with the E2 assembly

Immunoblotting analysis of SDS extracts of bovine, rat and pig cell lines have indicated that protein X is a normal cellular component with a specific mitochondrial location. The presence of protein X in mammalian PDC may be a unique feature of this multienzyme complex, since analysis of the two analogous complexes, OGDC and BCDC, has failed to detect a similar polypeptide. This may, then, indicate that component X is involved in the metabolism of two carbon units.

Component X remains bound to E2 during purification of this subunit and is not removed by p-hydroxymercuriphenyl sulphonate, a reagent that promotes the release of the intrinsic kinase from the assembly (Stepp *et al.*, 1983). Under conditions that are known to dissociate the constituent enzymes of the complex i.e. treatment with 0.25M $MgCl_2$ or high pH, component X remains tightly associated with the core enzyme. It has proved impossible to destroy this specific interaction without employing denaturing conditions e.g. 6M urea or SDS.

1.2.5.2 Structural studies on component X

Employing the immune replica technique de Marcucci *et al.* (1986) were unable to detect any cross reactivity of anti-X-serum with E2 or any other component of the complex. In this context, Jilka *et al.* (1986) reported that their anti-X-serum also reacts with the lipoyl-bearing domain of the acetyltransferase. This conflicting observation may simply reflect differences in the method of antigen preparation or in the nature of the polyclonal response in mice and rabbits.

One-dimensional peptide mapping studies with ^{125}I-labelled lipoyl acetyltransferase and component X subunits indicate, by the absence of any proteolytic fragment common to each digest, that these two proteins are structurally distinct entities. Similar analysis of purified subunits, initially radiolabelled with [^{14}C]-pyruvate and [^{14}C]-NEM confirms that distinct [^{14}C]-labelled fragments are generated from these two subunits. This result suggests, therefore, that despite both being acetylated, the domains containing the acetyl groups are structurally dissimilar. These data also support the immunological findings, which demonstrate that component X is not a proteolytic fragment of the E2 subunit.

1.2.5.3 Functional studies on component X

Component X has been shown to incorporate N-ethylmaleimide only in the presence of pyruvate or NADH (Hodgson et al., 1986). Incubation of the native complex in the presence of [2-^{14}C] pyruvate also results in the rapid uptake of radiolabel into both E2 and X. Deacetylation of both components is achieved by the addition of CoASH but whether both proteins interact independently with the cofactor is not known.

Hodgson et al. (1986) investigated the nature of the group on component X which became modified with [^{14}C]-NEM only in the presence of pyruvate. Identification of the [^{14}C] acetylated groups on component X as a lipoyl moiety was achieved by comparative elution of the [^{14}C]-labelled acid hydrolysis products generated from the E2 and X peptides. These peptides were readily distinguishable from the hydrolysis

products of NEM-derivatised cysteine. This eliminated the possibility that the site of N-ethylmaleimide incorporation on protein X was a cysteine thiol exposed by conformational change on addition of substrate.

Hodgson and Lindsay (1986) have subsequently demonstrated, via cross-linking experiments with the bifunctional agent phenylene dimaleimide, that the lipoyl groups on E2 and component X can interact spatially. Subunit-specific antisera were employed to distinguish between E2/E2 and X/X homodimers and also E2/X hetereodimers. These results also suggest that E2 and component X are present on the same core assembly.

1.2.5.4 Possible functions of component X

The relative abundance of component X, approaching levels of E3 in the complex, argues against its possible role as a regulatory component. However, it has been suggested (Cate and Roche, 1978) that PDC activity may be influenced by the degree of acetylation of the lipoyl transacetylase. In a more recent study Jilka *et al.* (1986) have shown that the intrinsic PDC kinase can be stimulated two-fold to three-fold when as few as six lipoyl groups are acetylated per E2 core. It is possible that this effect is mediated via component X.

The parallel behaviour of E2 and protein X in the acetylation/deacetylation reactions may reflect the close physical and functional relationship between these two proteins; it may also suggest that component X is an isoenzyme of E2.

Alternatively, component X may represent an associated enzyme engaged in the transfer of two carbon units, derived from pyruvate, to other physiological substrates e.g. carnitine.

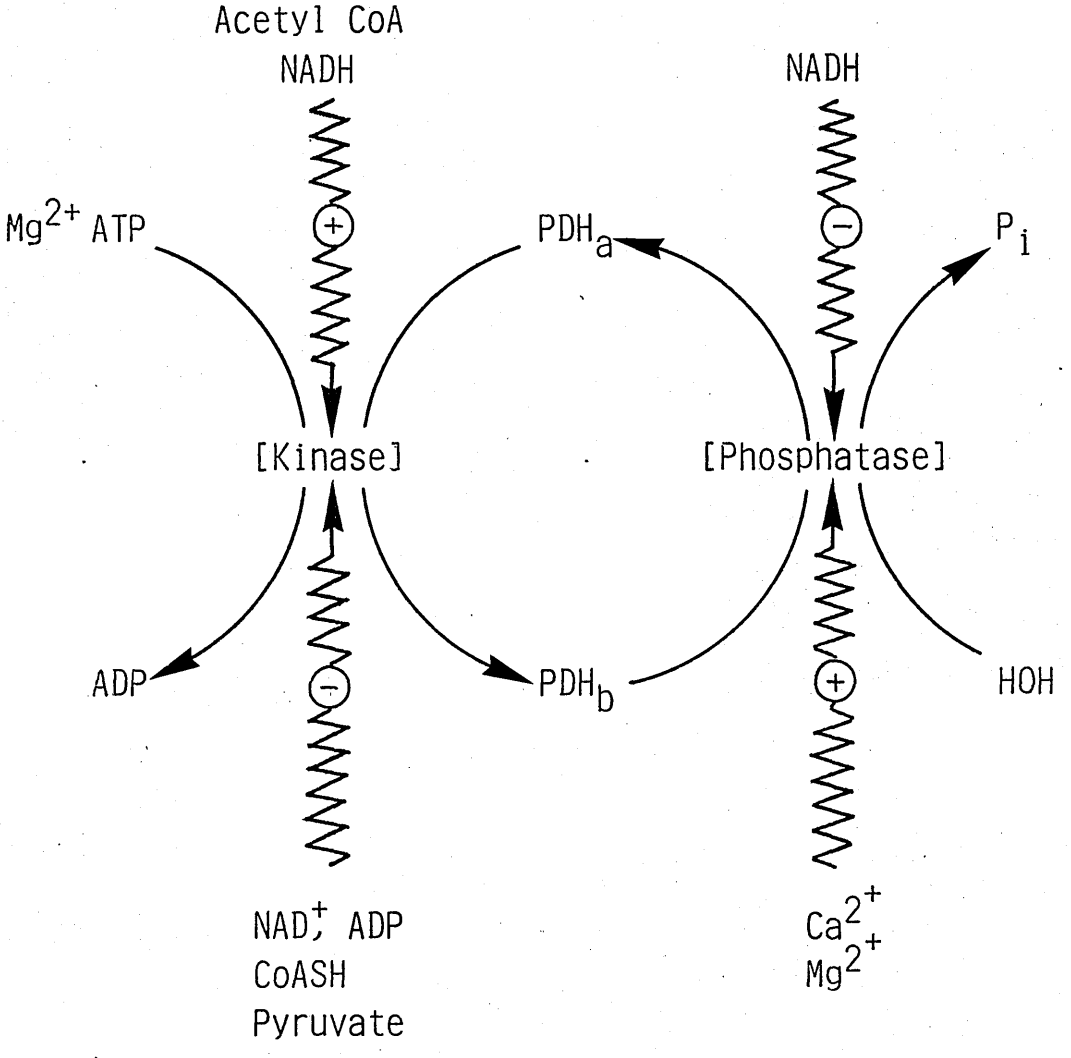
The protein may also provide an anchoring function i.e. in allowing the PDC to bind to the inner mitochondrial membrane (see Chapter 3). Prior to Halestrap's (1978) preliminary identification of a 15 000 M_r polypeptide as a candidate for the pyruvate transporter, the possibility existed for component X to fulfil this function.

1.2.6 Regulation of mammalian PDC

The central role of mammalian PDC in metabolism has prompted an extensive investigation of its regulatory properties. PDC provides acetyl CoA, an important biosynthetic metabolite utilised in the synthesis of fatty acids, leucine and steroids.

In animals this conversion of pyruvate to acetyl CoA is irreversible and thus represents a net loss of carbohydrate reserves to the cell. Garland and Randle (1964) first suggested that the activity of the complex was regulated by feedback inhibition of the products, NADH and acetyl CoA. It was found that if PDC activity was measured in a system which allowed the accumulation of products, the rate of reaction declined rapidly to less than 10% of the original value. When the removal of acetyl CoA was achieved by the addition of carnitine and carnitine acetyltransferase, this caused the reaction rate to return to 70% of the original value. Tsai *et al.* (1973) demonstrated subsequently that acetyl CoA and NADH are effective competitive inhibitors of

Fig. 1.7 Phosphorylation - dephosphorylation cycle of mammalian PDC.



PDH_a : dephosphorylated, active form.

PDH_b : phosphorylated, inactive form.

CoA and NAD^+, respectively. This mode of control of PDC activity is of particular importance in heart and diaphragm tissue (Denton *et al.*, 1975).

Linn *et al.*, (1969a, 1969b) demonstrated that the complex existed in an inactive phosphorylated form and an active dephosphorylated form. Covalent modification of the complex is mediated by an intrinsic ATP-dependent kinase and a phosphatase which is loosely associated with the complex. The activity of PDC from several mammalian and avian tissues, *Neurospora crassa* and plants is controlled in a similar manner. BCDC is also modulated by an analogous phosphorylation-dephosphorylation cycle. No evidence exists for similar control of PDC in prokaryotes or yeast or for OGDC in eukaryotic or prokaryotic cells.

The characteristics and composition of the kinase and phosphatase are discussed in Section 1.2.3.

A novel mechanism has been postulated whereby lipoate may act as the transmitter of regulatory interactions of PDC kinase (Kerbey *et al.*, 1976). It has been suggested (Cate and Roche, 1975) that the regulatory effects on the E1 component are mediated by the extent of acetylation or degree of oxidation/reduction of E2 (and possibly component X, i.e. Section 1.2.6). This controversy has not yet been resolved.

In diabetes and starvation, there is a marked decrease in the activity of the complex in heart, liver and kidney (Wieland *et al.*, 1971). This effect is reversed upon feeding, the administration of insulin or inhibitors of fatty acid oxidation. This modulation of activity has been attributed

to stimulation of the pyruvate dehydrogenase kinase or an increased synthesis of a protein activator of the kinase or the kinase itself (Hutson and Randle, 1978; Kerbey and Randle, 1982). For reviews on this subject see Wieland (1983) and Reed and Yeaman (1985).

1.2.7 Localisation of PDC within the mitochondria

PDC can be prepared from disrupted mitochondria, generally by employing a freeze-thaw procedure to destroy the integrity of the membrane (Linn et al., 1972; Cooper et al., 1974). Both, the above methods rely on the preliminary preparation and accumulation of large stocks of mitochondria. A more recent method (Stanley and Perham, 1980) dispensed with stock-piling of mitochondria and achieved a much greater yield of the 2-oxo acid dehydrogenase by the inclusion of Triton X-100 in the buffer used for the initial disruption of the heart tissue. These authors found that all of the assayable activity was soluble and represented a three-fold increase in the activity recovered in the absence of Triton X-100. This large increase in yield, which accompanies the addition of detergent, is consistent with the hypothesis that much, if not all, of the PDC activity is associated with the inner mitochondrial membrane. This observation will be discussed more fully in Chapter Three.

1.3 THE PHOSPHATE TRANSPORT SYSTEM

In the aerobic cells of eukaryotic organisms almost all the ATP is synthesised in mitochondria, with the remainder being generated via substrate-level phosphorylation reactions which occur largely in the cytoplasm. ATP, once synthesised, is transported into the cytoplasm via the adenine nucleotide translocase (ANT) to be used in the many endergonic reactions of cellular metabolism, generating ADP, AMP and P_i. Thus two mitochondrial uptake systems are required which must be directly linked to ATP synthesis: namely adenine nucleotide transport (ADP) and the transport of inorganic phosphate (P_i).

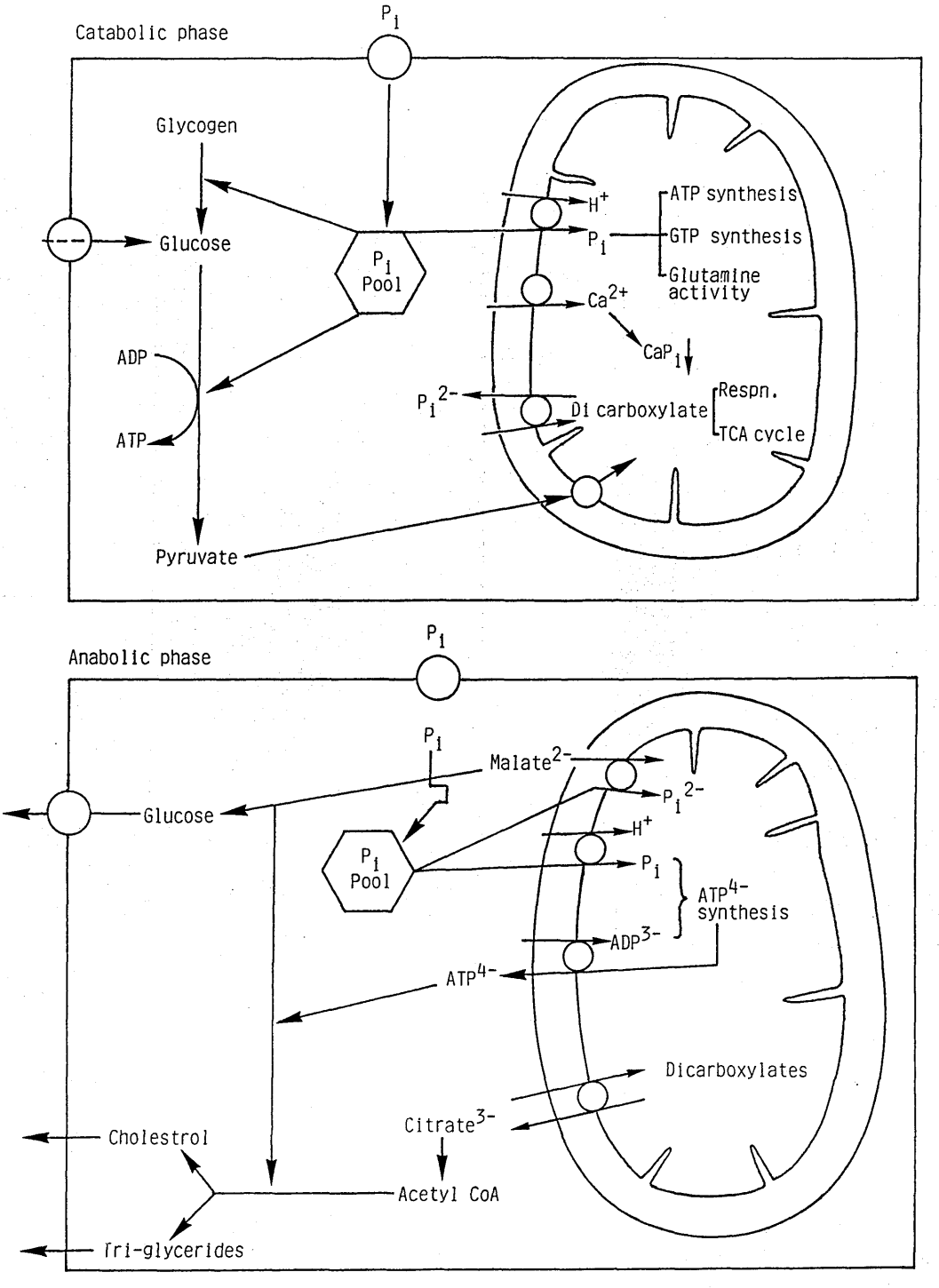
As a consequence of the pioneering work of Chappell (1965) and Fonyo (1966) on phosphate efflux and its inhibition by thiol group reagents, respectively, it is now well established that two P_i systems exist in the inner mitochondrial membrane:

(i) an electroneutral dicarboxylate/P_i antiporter which is mainly involved in the exchange of substrates between the matrix and the cytoplasm (McGivan *et al.*, 1971; Palmieri *et al.*, 1971).

and (ii) an electroneutral phosphate/proton symporter or phosphate/hydroxyl antiporter (for review see Fonyo, 1978) which catalyses the influx of 90% of the mitochondrial P_i (Coty *et al.*, 1975).

The latter system is dependent on the transmembrane pH gradient and is inhibited by sulphydryl group reagents such as NEM, mersalyl and pC-MB (Fonyo and Bessman, 1968;

Fig. 1.8 The importance of P_i and P_i transport processes to cell metabolism.



Tyler, 1969; Coty and Pedersen, 1974, 1975; Wohlrab and Flowers, 1982). The other carrier, the P_i/dicarboxylate antiporter is insensitive to NEM but is inhibited by mersalyl, pC-MB or substrate analogues such as n-butyl malonate or 2 phenyl-succinate (Meyer and Tager, 1969; Meijer et al., 1970; Coty and Pedersen, 1974).

1.3.1 Importance of phosphate transport in cell physiology

The importance of phosphate transport in cell physiology is evident from the numerous regulatory processes to which it is linked (Fig. 1.8).

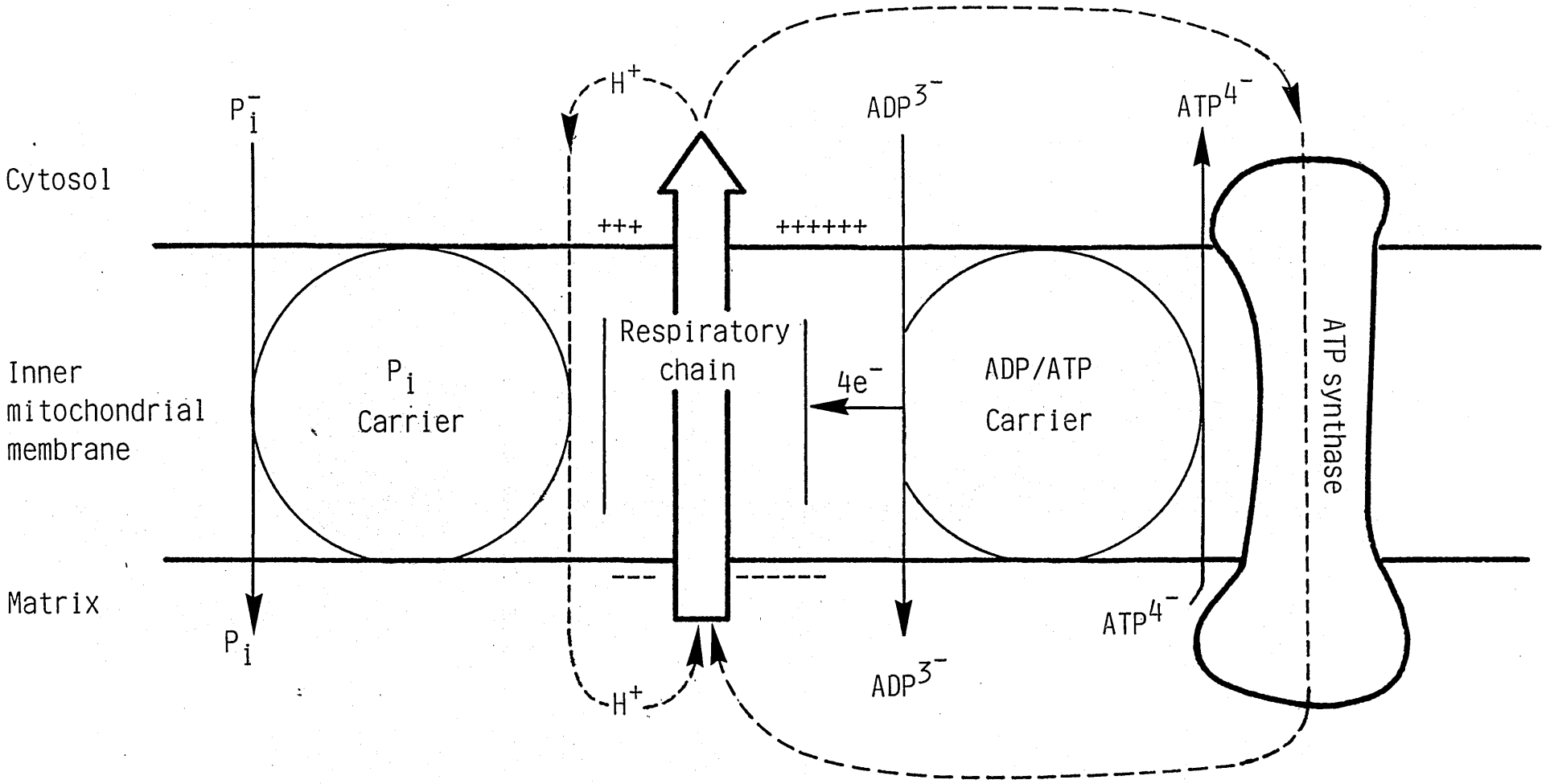
(a) it controls the influx of P_i required for ATP synthesis and consequently maintains the phosphate potential in the cell.

(b) it is directly involved in the H^+ cycle which takes place in the inner mitochondrial membrane and thus lends support to Mitchell's Chemiosmotic Theory which states that ATP synthesis and electron transport are coupled to a proton gradient (Fig. 1.9) (Mitchell, 1961).

(c) through its link with the P_i/dicarboxylate exchange, it controls the distribution of a certain number of Kreb's cycle intermediates e.g. malate.

On the basis of these observations any protein(s) which is (are) responsible for the bulk of mitochondrial phosphate transport must have an affinity for its substrate, P_i; it must react with sulphydryl group reagents which inhibit transport and must catalyse P_i transport when incorporated into liposomes in the correct orientation.

Fig. 1.9 Relationship between phosphate transport, ATP synthesis, H^+ cycle and the electron transport chain.



To this end, the key factor in elucidating the mechanism of phosphate transport in mitochondria would be the identification of the protein(s) responsible and its characterisation on the basis of the above criteria.

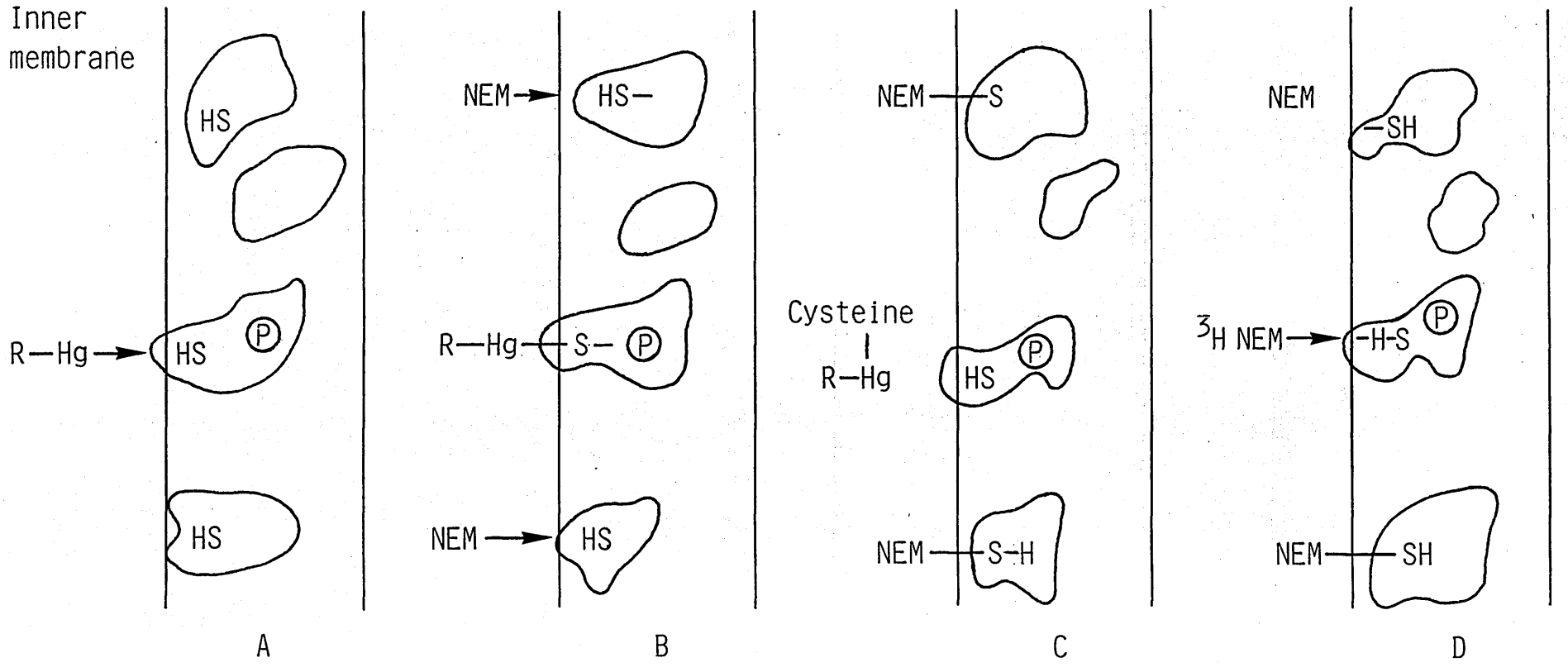
1.3.2 Identification of the phosphate transport protein

Kadenbach and Hadvary (1973) were first to demonstrate that chloroform extracts from rat liver mitochondria exhibited an affinity for P_i. These extracts were found to contain proteins with free sulphydryl groups and the addition of sulphydryl group reagents was observed to reduce their affinity for P_i.

The same approach was used by Guerin (1978) who succeeded in isolating a 10 000 M_r proteolipid from yeast which was associated with cardiolipin. This proteolipid contained sulphydryl groups and also exhibited an affinity for $[^{32}P]$ P_i. Subsequent studies with yeast grown in the presence of chloramphenicol and with mitochondria from DNA-deficient 'petite' mutants suggested that the isolated proteolipid was translated from mitochondrial ribosomes (Rigoulet et al., 1977).

Coty and Pedersen (1975) found that if limiting amounts of $[^{3}H]$ NEM were added to rat liver mitochondria such that phosphate transport was inhibited, analysis by SDS-PAGE revealed the presence of at least ten different radiolabelled polypeptides, demonstrating that NEM reacts non-specifically with a variety of proteins in the mitochondrial inner membrane. By exploiting the reversibility and specificity of the inhibition by mercurials of the phosphate/H^+ symporter, Coty

Fig. 1.10 Protecting action of mersalyl against NEM and labelling with [^{3}H] NEM of proteins protected by mersalyl.



and Pedersen (1975) developed an elegant procedure for labelling the sulphydryl groups involved in phosphate transport in a more specific manner. The phosphate transport system (PTS) was initially protected by inhibition with mersalyl, and then all free sulphydryl groups were blocked unassociated with transport by the addition of non-radiolabelled NEM. Mersalyl inhibition of the PTP was reversed by addition of DTT, restoring about 65-75% of the original activity of the carrier. Subsequent inhibition of transport with $[^{3}H]$ NEM resulted in a marked increase in specificity, modifying only five polypeptides in the M_r range of 30 000 - 32 000 (Fig. 1.10).

These prominent NEM-modified bands were localised in the inner membrane of various types of mitochondria (Briand et al., 1976; Hadvary and Kadenbach, 1976; Touraille et al., 1977; Wohlrab and Greaney, 1978; Wohlrab, 1979). However, no protein of M_r value between 8 000 - 10 000 was protected by the mercurial (Kadenbach and Hadvary, 1973).

After this treatment phosphate transport, as measured by the rate of uptake of $[^{32}P]$ P_i, could be shown to be controlled by the transmembrane pH gradient since transport was strongly stimulated by the ionophore, valinomycin. Thus it was concluded that mersalyl protects those proteins which are essential for phosphate transport (Coty and Pedersen, 1975).

Of these five protein bands, studies on the flight muscle of the adult blowfly, Sacrophaga bullata, labelled two bands of M_r 32 000 and 45 000 with $[^{3}H]$ NEM. This

membrane system is particularly well suited to these studies since it is highly specialised containing only the NEM-sensitive carrier and not the dicarboxylate carrier. On comparison of this labelling pattern to that obtained in the rat liver system, Coty and Pedersen (1975) suggested that the 32 000 M_r peptide was the most likely NEM binding component of the PTS (Wohlrab and Greaney, 1978).

Further studies on the blowfly flight muscle and on rat heart SMP confirmed this result by identifying a 32 000 M_r polypeptide via high resolution SDS-PAGE as the strongest candidate for the alkylated component of the PTS (Wohlrab, 1979).

1.3.3 Purification of the protein(s) involved in the phosphate transport system

The NEM-sensitive carrier of bovine and pig heart mitochondria has been isolated and reconstituted into liposomes (Kolbe et al., 1981; Touraille et al., 1981). This protein was identified as a single band (M_r 34 000) by conventional SDS-PAGE analysis which could be labelled with [^{3}H] NEM (Wohlrab, 1980; Kolbe et al., 1981; Touraille et al., 1981). The adenine nucleotide translocase which copurified in the preparation of Wohlrab (1980) and Wohlrab and Flowers (1982) was removed by chromatography on Celite (Kolbe et al., 1981) or on mersalyl-ultrogel (Touraille et al., 1981).

One of the criteria to be fulfilled by the purified component of the PTS is that it should catalyse a P_i-P_i exchange when reconstituted into liposomes. The M_r 34 000

species as isolated by Kolbe et al. (1981), and Touraille et al. (1981) could only maintain 2% of the activity achieved in isolated mitochondria by Coty and Pedersen (1975), although the exchange was found to be insensitive to carboxyatractyloside and n-butyl malonate (the ANT inhibitors) but sensitive to NEM and mercurials.

Gradient SDS-PAGE revealed that the purified PTP described above could be resolved into five bands of similar M_r value. It has been suggested (Kolbe et al., 1981) that the mitochondrial phosphate transporter occurs as a complex of different polypeptides and more recently that at least two of these bands represent proteolytic fragments of the parent polypeptide, although protease inhibitors had no effect on the apparent heterogeneity of the purified carrier (Kolbe et al., 1982). Recently, Gibb et al. (1986) reported an improved scheme for the purification of the PTP from rat liver which involves a modification of the method of Kolbe et al. (1981). A single polypeptide, subunit M_r 34 000, can be visualised after Coomassie Blue staining at the final stage of the purification, in general agreement with the value quoted from other mammalian sources (Wohlrab, 1980; Kolbe et al., 1981).

1.3.4 Effect of cardiolipin on the reconstituted activity of the phosphate transport protein

Bisaccia and Palmieri (1984) have obtained evidence that cardiolipin can be used to improve the purification of the phosphate transport protein to such an extent that only one major protein band is detectable in gradient SDS gel

electrophoresis. Previous studies have demonstrated that the reconstituted transport activity could be increased several fold by the inclusion of cardiolipin in the solubilisation buffer (Kadenbach et al., 1982; Mende et al., 1982). The specific effect of cardiolipin was corroborated by results showing a reversal of the enhanced activity by the presence of doxorubcin, which forms a specific complex with cardiolipin (Cheneval et al., 1983). This apparent affinity for cardiolipin strongly supports the idea of a specific interaction of the carrier protein with cardiolipin, which, in reconstitution studies, may maintain the phosphate carrier in its native conformation. This specific effect was not observed with other phospholipids tested.

1.3.5 Further characterisation of the phosphate transport protein

Kolbe et al. (1984) attempted to characterise the PTP further by purifying the protein from bovine heart on a large scale. Silver-staining of the SDS polyacrylamide gel resulted in two bands, designated putatively as the α and β subunits of the phosphate transport protein. These 'subunits' were shown to exist in the ratio of 1:1. $PTP_{\alpha + \beta}$ migrated with similar mobilities (M_r 34 000), also exhibiting identical peptide maps if fragmented with either CNBr or HCl/dimethyl sulphoxide/HBr. The amino acid composition was determined and comparative studies with the adenine nucleotide translocase (Aquila et al., 1982) and the phosphate carrier demonstrates that both proteins have markedly hydrophobic characteristics.

Edman degradation shows that both bands of the PTP display the same amino terminus. Both proteins lack either a methionine or a formylated methionine in the first position, suggesting that, unlike the yeast phosphate carrier (Guerin and Napias 1978; Rigoulet et al., 1977) and the cytochrome c oxidase subunits I, II and III (Schatz and Mason, 1974), the PTP is not of mitochondrial origin but is coded for by a nuclear gene and synthesised cytoplasmically.

Comparison of the amino terminal sequence generated from Edman degradation with the reported reading frames of the bovine heart mitochondrial genome (Anderson et al., 1982) further supported the nuclear origin of the carrier protein.

Whether the differences observed between PTP_{α} and PTP_{β} are of any functional significance remains to be elucidated. Kolbe, in the above publication, has suggested that these proteins may constitute the subunits of the phosphate carrier - an hypothesis prompted by functional existence of the PTP's sister protein, ANT, as a dimer (Klingenberg et al., 1978). This apparent subunit heterogeneity may be explained by transient disulphide bond formation during electrophoresis as carboxymethylation prior to SDS-PAGE results in the detection of a single polypeptide. To this end further characterisation of α and β is required, with possible studies on the native membrane system to further discriminate between the two proteins.

1.3.6 Location of the NEM binding site of the phosphate transport protein

As a consequence of the hydrophobic nature of this protein, amino acid sequencing by conventional methods has proven difficult, with complications arising as a result of the purified protein aggregating, thereby rendering it inaccessible to Edman degradation reactions.

To overcome these technical problems Kolbe and Wohlrab (1985) developed an elaborate protocol to isolate a formic acid fragment generated from (^{3}H) NEM labelled bovine mitochondrial PTP. By thermolysin digestion of the radioactive formic acid fragment and high performance liquid chromatography isolation of the radioactive subfragment, Leu^{39}-Arg^{43}, the sole NEM binding residue has been identified as cysteine (residue 42).

Further characterisation of this formic acid fragment (FAI) revealed significant homology in the triplicated gene regions of the mitochondrial adenine nucleotide translocase from bovine heart and Neurospora crassa. The fragment is predicted to contain a transmembrane segment (Phe^{15}-Val^{43}). The nuclear origin of this carrier protein has again been suggested (Section 1.3.5) from the lack of significant homology between the reverse-translated FAI (utilising mitochondrial codons) and the bovine mitochondrial genome.

1.3.7 Identifying the substrate-binding site of the phosphate transport protein

Despite the identification of cysteine at residue 42 as the binding site of the inhibitor N-ethylmaleimide, very little information is available on the substrate binding site

of the phosphate transport protein. Indeed, it has not yet been established whether the particular -SH group responsible for the inhibition by N-ethyl maleimide is located at or near the phosphate binding site, although the presence of phosphate decreases, to some extent, the reactivity of -SH blocking reagents with the phosphate transport protein (Klingenberg et al., 1974; Fonyo and Vignais, 1980; Ligeti and Fonyo, 1984).

Utilising a photoreactive phosphate analogue (Lauquin et al., 1980), Tommasino and coworkers (1987) sought to investigate the effect of 4-azido-2-nitrophenyl phosphate (ANPP) on the activity of the phosphate carrier of pig heart mitochondria. They have successfully demonstrated that [^{32}P] ANPP inhibits phosphate transport in the dark in a competitive manner, and that upon photoirradiation the probe becomes irreversibly bound to the carrier. Under these conditions inhibition is dependent on both the pH of the medium and concentration of the probe.

Therefore ANPP appears to be a suitable reagent for characterising the phosphate binding site of the mitochondrial carrier although further investigations should concentrate on locating the modified amino acid(s) in the primary structure of the protein.

1.3.8 Concluding remarks

The identification, purification and subsequent characterisation of the protein(s) involved in P_i transport has (have) proven much more difficult than that of the protein(s) responsible for adenine nucleotide transport.

The main reasons for this are:

(a) its affinity for its substrate, P_i, is neither very high (Km 1-2 mM) nor very specific since other proteins and phospholipids can also exhibit an affinity for P_i.

(b) -SH group reagents are not very specific; however, the strong affinity of the transporter for mercurials is noteworthy.

isfaction of the third criterion, i.e. the reconstitution of the protein(s) responsible for phosphate transport in mitochondria is clearly difficult in view of these uncertainties.

A complete understanding of the phosphate carrier as well as the adenine nucleotide translocase, which in terms of amount probably constitute two of the main membrane transport systems in aerobic eukaryotic organisms, would provide an excellent model system for the investigation of ion transport across biological membranes.

Successful reconstitution experiments involving the purified protein may provide an insight into the mechanism whereby the free energy, supplied through the respiratory chain, is used for ATP synthesis, and what chemical groups are involved in the fixation and translocation of hydrogen ions released by the respiratory chain.

The data generated from amino acid composition and sequencing studies may provide a molecular link between phosphate transport and ATP synthesis, which Houstek et al. (1981) have already suggested by demonstrating that the DCCD-reactive protein often isolated in association with the mitochondrial ATP synthase (M_r 33 000) is identical with the phosphate carrier.

AIMS AND SCOPE OF THIS THESIS:

Two important mitochondrial proteins were chosen, namely the pyruvate dehydrogenase multienzyme complex and the phosphate transport protein, to investigate the relationship between protein structure and function. Each aspect of this investigation necessitated the production of high-titre monospecific antisera directed against each protein.

(a) Pyruvate dehydrogenase complex:

(i) to investigate the distribution of PDC activity within the matrix-inner membrane compartment.

(ii) to probe the nature of the enzyme's association with the inner mitochondrial membrane.

(iii) to determine if PDC is associated exclusively with a specific region of the respiratory chain.

(b) Phosphate transport protein:

(i) to investigate the biosynthesis of nuclearly-encoded protein.

(ii) to probe the intramembrane organisation of PTP, which, in view of its role in P_i/OH^- exchange would be expected to be transmembraneous and contain accessible domains on both matrix and cytoplasmic faces of the inner membrane.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and biochemicals

Acetyl CoA, p-amino benzamidine, benzamidine-HCl, Coomassie Brilliant Blue R-250, CHAPS, digitonin, DTNB, 2,4 DNP, DOC, EGTA, guanidine-HCl, iodoacetamide, leupeptin, malic acid, mannitol, MOPS, NAD^+, NEM, oxaloacetic acid, 2-oxoglutaric acid (monosodium salt), pyruvic acid (monosodium salt), PMSF, 1,10 phenanthroline, TEMED, and Tween 20 were purchased from Sigma Chemical Co., Poole, Dorset, U.K.

DMSO, DTT, PPO, sucrose and toluene were obtained from Koch Light Laboratories Ltd., Colnbrook, Berks., U.K..

ATP, CoA (trilithium salt) and Tris were the products of Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K..

Acrylamide, L-cysteine-HCl, EDTA, Folin Ciocalteu's Phenol Reagent, N,N-methylenebisacrylamide and SDS were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.

DMSI was generously donated by Professor J.R. Coggins of this Department. FCCP was the gift of Dr P.G. Heytler, E.I. Du Pont De Nemours and Co. (Inc), Delaware, U.S.A. Iodogen™ was purchased from Pierce and Warriner, Chester, (U.K.) Ltd., 2-mercaptoethanol was supplied by Riedel-de-Haën, via A. & J. Beveridge Ltd., Edinburgh, U.K.. Pyronin Y was obtained from George T. Gurr Ltd., London SW6, U.K.. Salicylate (sodium salt or free acid) came from Aldrich Chemical Co., Gillingham, Dorset, U.K.. Triton X-100 was bought from Robson & Haas (U.K.) Ltd., Croydon, U.K.

All other chemicals were of analytical grade or of the highest available purity.

2.1.2 Radiochemicals

N-[Et-2-^{3}H] Ethylmaleimide (50 Ci/mmol) was purchased from New England Nucleur, Southampton, U.K.. N-[Et-2,3-^{14}C] Ethylmaleimide, (10 mCi/mmol), L-[^{35}S] methionine (approx. 1200 Ci/mmol) and [9,10(n)-^{3}H] palmitic acid (55 Ci/mmol) were supplied by Amersham International p.l.c., Bucks., U.K.. Na I^{125}, carrier free, was obtained from the Western Infirmary, Glasgow, U.K..

2.1.3 Chromatography materials

Celite (Type 535) was from Serva via Uniscience, Cambridge, U.K.. Biogel Hydroxyapatite was supplied by Bio-Rad Laboratories, Holywell Industrial Estate, Watford, Herts., U.K.. Sephadex G-25 and Superose 12 were the products of Pharmacia (G.B.) Ltd., Milton Keynes, Bucks., U.K..

2.1.4 Proteins and enzymes

The following commercial enzyme and protein preparations were purchased from Sigma Chemical Co., Poole, Dorset, U.K.:-

Aldolase (EC 4.1.2.13) from rabbit muscle, BSA (essentially fatty acid and globulin free), TLCK-treated α-chymotrypsin (EC 3.4.21.4) from bovine pancreas, clostripain (EC 3.4.22.8) from Clostridium welchi, citrate synthase (EC 4.1.3.7) from porcine heart, elastase (EC 3.4.21.11) from porcine pancreas, fumarase (EC 4.2.1.2) from porcine

heart, papain (EC 3.4.22.2) type (111) from Papaya latex, Pronase E type (XIV) from Streptomyces griseus, protein A-Sepharose CL-4B, protein A from Staphylococcus aureus, Cowan I strain, TPCK-treated trypsin (EC 3.4.21.4) type (XII) from bovine pancreas.

Diaphorase (lipoamide dehydrogenase) (EC 1.8.1.4) from porcine heart and protease K (EC 3.4.21.4) type (XI) from Tritirachium album was obtained from Boehringer Corporation (London) Ltd., Sussex, U.K..

Electrophoresis calibration kit for low M_r proteins was purchased from Pharmacia (G.B.) Ltd., Milton Keynes, Bucks., U.K..

2.1.5 Cell-culture materials

Glasgow-modified Eagle's medium and L-glutamine (200 mM) were purchased from Flow Laboratories Ltd., Irvine, Ayrshire, U.K..

Glasgow-modified Eagle's medium without L-methionine and L-glutamine, myo-clone foetal calf serum, new born calf serum, MEM non-essential amino acids (100x), sodium bicarbonate solution (7.5% (w/v)), trypsin ((10x, 2.5% (w/v)) and Nunc, sterile Roux flasks and petri-dishes were obtained from Gibco Europe Ltd., Paisley, Renfrewshire, U.K..

Sterile glassware and the following sterile solutions were provided by the staff of the Wellcome Tissue Culture Unit associated with this department:-

Penicillin/Streptomycin

penicillin	10 000 000.00 U
streptomycin	10 g

distilled water to 1 litre

Sterilise via Millipore filtration. Store at -20°C in 10 ml aliquots.

Versene

NaCl	80.00 g
KCl	2.00 g
Na_2HPO_4	11.50 g
KH_2PO_4	2.00 g
Versene (EDTA)	2.00 g
phenol red (1% (w/v))	15.00 ml

distilled water to 10 litres.

Sterilise by autoclaving. Store, in small batches, at room temperature.

2.1.6 Biological materials

BRL (rat liver), NBL-1 (bovine kidney) and PK-15 (pig kidney) cell lines were supplied by Flow Laboratories Ltd., Irvine, Ayrshire, Scotland. Bovine hearts were obtained from Paisley Abbatoir, Sandyford Road, Paisley. Bovine hearts were removed from the animals within 1 h of slaughter, chilled immediately on ice and used in the laboratory within 2 h of slaughter. New Zealand White Rabbits and 150-350 g female rats (Albino Wistar strain) were provided by the departmental animal house.

2.1.7 Photographic materials

Hyper-Film™ -MP was supplied by Amersham International p.l.c., Bucks., U.K. X-Omat S and XAR-5 X-ray films, Kodak, FX-40 liquid fixer and Kodak LX-24 X-ray developer were purchased from Kodak Ltd., Manchester, U.K..

2.1.8 Miscellaneous

Cronex 'Lightening-Plus' intensifying screens were purchased from Du Pont (U.K.) Ltd., Stevenage, Herts., U.K.. Ecoscint Scintillation Fluid is the product of National Diagnostics, Somerville, New Jersey, U.S.A. Freund's Adjuvants (complete and incomplete) were supplied by Difco Laboratories, West Moseley, Sussex, U.K.. Heat inactivated horse-serum was obtained from Gibco Europe Ltd., Paisley, Renfrewshire, U.K.. Nalgene sterilisation filter units, type S (115 ml, 0.2 µm pore size) were from Nalge Company, Rochester, New York, U.S.A. Nitrocellulose paper (0.45 µm pore size) was the product of Schlercher and Schüll, Dassel, West Germany. Normal rabbit serum came from the Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire, U.K.. Pansorbin (standardised 10% (w/v) formalinised Staphylococcus aureus cells) was supplied by Calbiochem-Behring Corp., Bishop Stortford, Herts., U.K.. Plast-X cassettes, for exposure of X-ray films were obtained from Anthony Monk (Eng.) Ltd., Sutton-in-Ashfield, U.K..

2.2 METHODS

2.2.1 Protein determination

Protein concentration was determined by using the method of Lowry *et al.* (1951) as described by Markwell *et al.* (1978) using BSA as a standard.

The following stock solutions were prepared:-

Solution A: 2% (w/v) Na_2CO_3, 0.4% (w/v) NaOH, 0.16% (w/v) sodium tartrate and 1% (w/v) SDS.

Solution B: 4% (w/v) $CuSO_4 \cdot 5H_2O$

Solution C: 100 parts A mixed with 1 part B.

BSA Stock: 1 mg/ml BSA stock stored at -20°C.

Folin Ciocalteu's phenol reagent: diluted 1:1 (v/v) with distilled water on day of use.

A sample volume of 1.0 ml containing 10 - 100 µg protein was mixed with 3.0 ml of solution C and at room temperature for 10-60 min. Samples were then mixed vigorously with 0.3 ml of diluted phenol reagent and incubated at room temperature for a further 45 min. before the absorbances at 660 nm were read.

Alternatively, where appropriate, the Biuret method (Gornall *et al.*, 1949) was employed. Stock solutions were prepared as follows:-

Biuret Reagent: 3 mM $CuSO_4$, $5H_2O$, 10 mM NaK $C_4H_4O_6 \cdot 4H_2O$, 0.6% (v/v) NaOH, 6 mM KI.

BSA Stock: 10 mg/ml BSA stored at -20°C.

A sample volume of 0.5 ml containing 100 µg - 5 mg protein was mixed with 2.0 ml of Buiret reagent and incubated for 15 min. at room temperature before the absorbance was read at 540 nm.

2.2.2 Enzyme assays

(a) Citrate Synthase

Citrate synthase activity was assayed as described by Srere (1969). The assay involves measuring the appearance of the free sulphydryl group of the released CoA using DTNB. The reaction is monitored at 412 nm where the resultant mercaptide absorbs strongly (E^{M}_{412} 13 600).

Assays were followed at 25°C by adding in this order, 200 µl of 0.5 M. Tris-HCl, pH 8.0; 200 µl of 0.5 mM DTNB; 100 µl of 3 mM acetyl-CoA; distilled water and enzyme to 0.9 ml. The initial change in absorbance at 412 nm is measured to check acetyl CoA deacylase activity. 100 µl of 5 mM oxaloacetate was added to start the reaction and the change in absorbance at 412 nm was followed once more.

1 unit of activity is defined as the amount of enzyme required to release 1 µmole CoA per min. at 25°C.

(b) Fumarase

Fumarase was assayed using a modification of the method of Racker (1950).

The reaction mixture was made up as follows:-
336 mg of L-malic acid was dissolved in approximately 40 ml of 0.1M potassium phosphate buffer, pH 7.6. The pH was readjusted to 7.6 with approximately 5 ml of 2N NaOH before the volume was finally adjusted to 50 ml with 0.1M potassium phosphate buffer, pH 7.6.

0.97 ml of the reaction mixture was placed in a cuvette and the reaction initiated by following the appearance of fumarate, which absorbs at 240 nm due to the presence of a carbon-carbon double bond ($E^{1\mu M}_{1\ cm\ 240\ nm}$ 2.44).

(c) 2-Oxo acid dehydrogenase complexes

The overall activities of pyruvate dehydrogenase and the 2-oxoglutarate dehydrogenase complexes were assayed according to the method of Danson et al. (1978). The following stock solutions were employed in the assay:-

Solution A: 50 mM potassium phosphate buffer, pH 7.6 containing 3 mM NAD^+, 2 mM $MgCl_2$, and 0.2 mM TPP. The pH was adjusted to 7.6 with KOH as required. Stored, in 10 ml batches, at -20°C.

Solution B: 130 mM L-cysteine-HCl containing 6.8 mM Na_2 CoAsH.

Solution C: 100 mM 2 oxo-acid.

The reaction mixture included 670 µl of solution A, 14 µl of solution B and 14 µl of solution C. This mixture was pre-incubated at 30°C for 2 min. and the reaction was initiated by the addition of approximately 2 µg of enzyme. The production of NADH was followed at 340 nm.

1 unit of activity (katal) is defined as the amount of enzyme required to catalyse the conversion of one mole of substrate per second under the conditions of the assay.

2.2.3 Polyacrylamide gel electrophoresis (SDS-PAGE)

2.2.3.1 Tris-Glycine buffered system

(a) Analytical gels

SDS-PAGE was carried out using the discontinuous buffer system of Laemmli (1970). The following stock solutions were prepared:-

Solution A: 3.0M Tris-HCl, pH 8.8, containing 0.25% (v/v) TEMED.

Solution B: 28% (w/v) acrylamide containing 0.735% (w/v) N,N'-methylenebisacrylamide, deionised using Amberlite IRA-400.

Solution C: 0.1M Tris-HCl, pH 6.8 containing 0.8% (w/v) SDS and 0.25% (v/v) TEMED.

Reservoir Buffer: 25 mM Tris base containing 192 mM glycine and 0.1% (w/v) SDS. The pH of this buffer should be 8.3.

Gels were cast in our own, home-made apparatus in glass slabs of 19.0 cm x 9.5 cm. Spacers were made of teflon and were 0.15 cm thick. The Bio-rad Protean 16 cm apparatus was used if longer gels were required. The dimensions of these gels were 18.0 cm x 14.0 cm x 0.15 cm.

Resolving gels of the required percentage were prepared, from the above stock solutions, according to the following table:-

	Percentage acrylamide		
	7.5%	10%	12.5%
Solution A	25.0	25.0	25.0
Solution B	52.9	71.5	89.3
Solution C	1.0	1.0	1.0
Distilled water	118.6	100	82.2

(all volumes in the table are in ml.)

These solutions were mixed in a Buchner flask, degassed and 150 mg of ammonium persulphate was added to initiate polymerisation. The mixture was degassed again before being poured into the casting apparatus. The resolving gels were overlaid with isopropanol.

Stacking gels were generally of 5.3% (v/v) acrylamide and made from the stock solutions as follows: 17.5 ml of solution B, 10.0 ml of solution C and 55.0 ml of distilled water. As before, polymerisation was initiated with the addition of 150 mg of ammonium persulphate following degassing. To facilitate well formation, polymerisation was allowed to occur around a teflon template.

(b) Preparation of samples for SDS-PAGE and conditions for electrophoresis

Samples of protein were prepared for electrophoresis by boiling for 3 min. in Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 2% (w/v) SDS, 10% (w/v) sucrose and 0.1% (w/v) pyronine Y) containing either 5% (v/v) 2-mercaptoethanol or 10 mM DTT. Following boiling, iodoacetamide was added to 0.1M. Electrophoresis was carried out at 50 mA until the dye front was within 0.5 cm of the bottom of the gel.

(c) Staining and scanning of polyacrylamide gels

Protein bands were detected by immersing the gels in a solution of:-

(i) 0.04% (w/v) Coomassie Brilliant Blue R.250 in 25% (v/v) isopropanol, 10% (v/v) acetic acid for 16-20h followed by destaining in 10% (v/v) acetic acid.

or (ii) 0.1% (w/v) Coomassie Brilliant Blue R.250 in 50% (v/v) isopropanol, 10% (v/v) acetic acid for 1h followed by destaining in 20% (v/v) methanol, 10% (v/v) acetic acid.

Destained polyacrylamide gels were scanned using an LKB 2203 Ultrascan Laser Densitometer.

(d) Determination of M_r values from SDS-polyacrylamide gels

Apparent M_r values were calculated with reference to the mobility of a set of standard marker proteins which included:- phosphorylase b, 92 000; BSA, 67 000; ovalbumin, 45 000; carbonic anhydrase, 30 000; trypsin inhibitor, 21 000 and α-lactalbumin, 14 000.

2.2.3.2 Phosphate buffered system

(a) Preparation of gels (5.0% (w/v) acrylamide)

The following stock solutions were used.

Solution A: 1M sodium phosphate buffer, pH 6.5. This was prepared by dissolving 81.0g NaH_2PO_4/l and 59.3g NaH_2PO_4/l. The pH was checked and adjusted as required.

Solution B: 20% (w/v) SDS.

Solution C: Acrylamide: bisacrylamide solution: 28% (w/v) acrylamide containing 0.735% (w/v) N'-N'-methylenebisacrylamide.

Solution D: Running buffer: a solution of 0.1M sodium phosphate buffer, pH 6.5, containing 0.1% (w/v) SDS.

A solution was prepared containing 35 ml acrylamide: bisacrylamide stock (C); 141 ml distilled water; 20 ml 1M sodium phosphate buffer, pH 6.5, (a); 1 ml 20% (w/v) SDS solution (b); 100 µl TEMED. This solution was degassed prior to the addition of 150 mg of ammonium persulphate to initiate polymerisation. The degassing procedure was followed, as before. The gels were cast in slabs using the apparatus described in Section 2.2.3.1a. Again, teflon templates were utilised to allow well formation. As before, the gels were stored at $4^{o}C$ and generally used within one week.

(b) Preparation of samples for SDS-PAGE and conditions for electrophoresis

Protein solution was mixed in the following buffer:- 0.25 ml 20% (w/v) SDS; 50 µl 2-mercaptoethanol; 1.0 ml glycerol, 50 µl 1M sodium phosphate buffer, pH 6.5; 0.1 ml of 0.5% (w/v) pyronin Y in a final volume made to 5 ml with distilled water. Protein samples were mixed in at least an equal volume of the dissolving buffer. Samples were prepared for electrophoresis by boiling in the above sample buffer for 3 min. Gel electrophoresis was performed at 80 mA until the dye-front was within 0.5 cm of the bottom of the gel.

Conditions for staining and destaining of gels were as detailed in Section 2.2.3.1c. Apparent M_r values were determined as Section 2.2.3.1d.

2.2.4 Gel filtration

Gel filtration was performed on the Pharmacia FPLC System and using a pre-packed Superose 12 column supplied by Pharmacia. I am grateful for the expert assistance of Mr J. Neagle in operating this system.

Apparent M_r determination was carried out by comparing the retention time of standard M_r proteins supplied by Boehringer-Mannheim and including:
BSA, 67 000; ovalbumin, 44 000; soya bean trypsin inhibitor, 21 000 and cytochrome C, 12 500.

2.2.5 Radiolabelling techniques

(a) Iodination of protein A and M_r standards

Protein A and M_r standards were iodinated using the solid phase oxidising agent, Iodogen™, as described by Salacinski et al. (1981).

1 mg of Iodogen™ was dissolved in 0.5 ml of chloroform and coated onto the surface of a vial by blowing off the chloroform under an air current. Protein A (1 mg) or M_r standards (578 µg) dissolved in 0.5 ml of 20 mM Tris-HCl, pH 7.2, containing 0.15M NaCl was added to the vial coated in Iodogen™ together with 300-500 µCi of NaI^{125}. Iodination was allowed to proceed for 15 min before the reaction was stopped by removing the sample and applying it to a 8.0 cm x 1.3 cm Sephadex G-25 column equilibrated in 20 mM Tris-HCl, pH 7.2, containing 0.15M NaCl. Void volume fractions, of approximately 1.0 ml, containing I^{125}-labelled protein A were collected, pooled and divided into small aliquots containing 3×10^6 c.p.m.. Aliquots were stored at -20°C.

(b) Modifying PDC with N-(Et-2,3-^{14}C)ethylmaleimide

Thiol groups of PDC were labelled with N-(Et-2,3^{14}C) ethylmaleimide as follows:-

100 µg of purified PDC was precipitated in 4 volumes of acetone for several hours at -20°C. The resultant pellet was resuspended in 200 µl of 20 mM Tris-HCl, pH 7.2, containing 2% (w/v) SDS. To facilitate the incorporation of label into each of the five constituent bands, the complex was pre-incubated for 2 min with a final concentration of 2 mM pyruvate. 2 µCi of N-(Et-2,3^{14}C)ethylmaleimide was then added to the reaction mixture. The reaction was allowed to proceed for 30 min at 30°C before being terminated by the addition of 2-mercaptoethanol to a final concentration of 5% (v/v). Labelled protein was precipitated in acetone as before. The pellets were washed once in 80% (v/v) cold acetone, dried and resuspended in 20 mM Tris-HCl, pH 7.2, containing 2% (w/v) SDS. A 2 µl aliquot of this solution was counted and the radiolabelled protein was stored in small aliquots at -20°C.

2.2.6 Detection of radioactivity

(a) Measurement in samples radiolabelled with ^{125}I was determined by counting in an LKB Wallac 1275 mini gamma counter.

(b) Liquid scintillation spectrometry

Aqueous samples of total volume 0.5 ml were either (i) made up to 5.0 ml with 0.5% (w/v) PPO and 35% (v/v) Triton X-100 in toluene or (b) made up to 5.0 ml with Ecoscint Liquid Scintillation Fluid and counted in a Beckman LS6800 Liquid Scintillation Counter.

(c) Autoradiography

Autoradiography was employed to visualise ^{125}I-labelled proteins in polyacrylamide gels or on nitrocellulose paper. The nitrocellulose paper was dried at room temperature and the gels dried under vacuum before being exposed to either X-Omat-S-X ray film or Hyper-Film^{TM}-MP at -70^{o}C with a Cronex 'Lightening Plus' intensifying screen to enhance autoradiographic detection (Laskey & Mills, 1977).

(d) Fluorography

Gels for analysis using fluorography were stained in Coomassie-Blue as outlined in Section 2.2.3.1c, or fixed in 25% (v/v) methanol, 10% (v/v) acetic acid before processing with DMSO-PPO, as detailed by Bonner and Laskey (1974). Gels were incubated for 3 x 30 min in DMSO, then for 3-4 h with 20% (w/v) PPO in DMSO and finally for 1-2 h under running water before being dried under vacuum. Gels were thereafter exposed to Hyper-Film^{TM} - MP at -70^{o}C.

This method was latterly superceded by that of Chamberlain (1979). Gels were stained or fixed (as above) then washed for 30 min in 2-3 changes of distilled water to remove all traces of acid. Prior to drying the gel and exposure to X-ray film, the gels were incubated for 30 min at room temperature in 1M salicylate.

2.2.7 Tissue culture

(a) Medium

PK-15 cells were routinely grown in Glasgow-modified Eagle's medium supplemented with 5% (v/v) foetal calf serum,

1% (v/v) non-essential amino acids, 10^5 U/l penicillin, 100 mg/l streptomycin and 7.5% (w/v) $NaHCO_3$ (normal growth medium).

BRL and NBL-1 cells were grown in the above medium containing 10% (v/v) new born calf serum and 10% (v/v) foetal calf serum, respectively.

Minus methionine medium (MMM) consists of Glasgow modified Eagle's medium (without L-met or L-glu) supplemented with 1% (v/v) non-essential amino acids, 2 mM glutamine, 10^5 U/l penicillin, 100 mg/l streptomycin and the appropriate serum type at the concentration described above.

Low methionine medium (LMM), used during the incorporation of $[^{35}S]$ methionine with cellular protein, was 5% (v/v) NGM in minus methionine medium.

(b) Routine culture of cells

Cells were grown in normal growth medium in 175 cm^2 plastic Roux batches at 37°C in an atmosphere of 95% (v/v) air/5% (v/v) CO_2. Cells were subcultured on reaching confluency i.e. every 3-4 days.

Monolayers were rinsed twice with versene solution (Section 2.1.5), once with trypsin prior to an incubation at 37°C with sufficient trypsin to cover the monolayer. On release of the cells from the plastic surface 20 ml of NGM was added and the cells transferred to a sterile universal. The cells were disaggregated to a single cell suspension by gentle passage up and down sterile pipettes of decreasing bore size. Cells were counted in a haemocytometer and 3-5 x 10^6 cells/ml were returned to the Roux flask in approximately 50 ml NGM.

(c) Labelling of cells with $[^{35}S]$ methionine

For the incorporation of $[^{35}S]$ methionine into protein, cells were seeded into 10 cm diameter sterile petri-dishes and a cover of 3 x 10^5 cells/ml in 10 ml of NGM. The cells were then incubated at 37°C in a humidified incubator containing an atmosphere of 95% (v/v) air/5% (v/v) CO_2 for 24-48 h i.e. until the cells achieved semi-confluency. NGM was replaced with 4 ml of LMM following an initial rinse in this medium. To deplete the intracellular methionine pools, the cells were incubated in this medium for 90 min. prior to the addition of 100-200 µCi of $[^{35}S]$ methionine. Petri-dishes were incubated at 37°C for either 4 h or overnight prior to lysis and the preparation of extracts as described in Section 2.2.8b.

(d) Labelling of cells with $[^{35}S]$ methionine in the presence of uncouplers of oxidative phosphorylation

Replicate dishes of cells cultured in NGM as before, including the pre-incubation with LMM for 90 min prior to the addition of 4.0 ml of LMM containing either uncoupler added from freshly-made stocks of 200 mM DNP or 1 mM FCCP (final concentration of 2 mM and 10 µM respectively). Incubation was for 5-10 min at 37°C prior to the addition of 200 µCi of $[^{35}S]$ methionine. Cell lysates were prepared (Section 2.2.8b) following a 4 h incubation at 37°C as described previously.

2.2.8 Preparation of cell extracts and subfractions of cells

(a) Isolation of mitochondria from tissue culture cells

Mitochondria were prepared from cultured cells following the procedure of Attardi and Ching (1979) starting from approximately 2 x 10^8 cells. All procedures were carried out at 4°C.

Cell monolayers were rinsed, harvested into approximately 40 ml of ice cold PBS (20 mM potassium phosphate buffer, pH 7.4, containing 0.15M NaCl) and centrifuged for 10 min at 800 x g.The cells were washed twice in 0.13M NaCl containing 5 mM KCl and 1 mM $MgCl_2$ before being resuspended in 6.0 ml of 10 mM Tris-HCl, pH 6.7, containing 10 mM KCl and 0.15 mM $MgCl_2$. Homogenisation was carried out in a Potter-Elvenhjem teflon-glass homogeniser at half maximal speed for 3 strokes. Cell breakage was monitored under a light microscope. On addition of 0.25M sucrose the solution was centrifuged at 1500 x g to remove nuclei. Mitochondria were pelleted from the supernatant fluid by centrifugation at 5000 x g for 10 min. The resultant pellets were resuspended in 2.0 ml 10 mM Tris-HCl, pH 6.7, containing 0.15 mM $MgCl_2$ and 0.25M sucrose. Mitochondria were washed by repeating the last two centrifugation steps. Prior to determining the protein concentration by the modified Lowry procedure (Section 2.2.1) the remaining mitochondria were resuspended in 1.0 ml of 10 mM Tris-acetate, pH 7.0, containing 0.25M sucrose.

(b) Preparation of cell lysates from [^{35}S] methionine labelled cells

The following standard solutions were prepared and sterilised by passage through a Nalgene filter unit (pore size 0.2 μm):-

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

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

3D-Lysis Buffer: 100 mM Tris-HCl, pH 8.2, containing 100 mM KCl, 5 mM $MgCl_2$, 1% (v/v) Triton X-100, 1% (w/v) SDS and 2% (w/v) DoC.

All solutions were supplemented with 1 mM benzamidine-HCl, 1 mM 1,10 phenanthroline and 1 mM PMSF immediately before use. All procedures were carried out at 4°C. Cell monolayers were washed three times in ice-cold PBS before being harvested into 1.0 ml of Triton-TKM buffer. Low speed centrifugation (1000 x g for 5 min) removed the nuclei. The supernatant fraction was diluted 1:1 (v/v) with 3D-lysis buffer prior to centrifugation at 24 000 x g for 30 min to remove any remaining cellular debris. Lysates were used immediately for immune precipitation and stored at -70°C until required.

2.2.9 Preparation and subfractionation of mitochondria

(a) Preparation of rat liver mitochondria

Mitochondria were prepared from rat liver according to the method of Chance and Hagihara (1963). The following isolation medium was used throughout the preparation:-

20 mM MOPS, pH 7.2, containing 0.225M mannitol, 0.075M sucrose and 0.05 mM EGTA. All procedures were carried out at 4°C.

Starved female rats of the Wistar strain (150-350 g) were killed by cervical dislocation. Livers were removed and washed free of blood using cold isolation medium before being chopped finely using scissors. Initial homogenisation was carried out in Potter-Elvenhjem homogeniser with a loose-fitting teflon pestle. (2 passes at maximal speed) to facilitate tissue breakdown. Cell disruption was caused by homogenisation using a tight-fitting teflon pestle (5 passes at setting 5, clearance 0.006-0.008 in. If mitochondria were being prepared in bulk, i.e. 35-40 rats, cell breakage was effected by blending for 15 secs at high speed in a Waring Blender. The homogenate was centrifuged at 800 x g for 7 min to remove nuclei, red blood cells and cellular debris. Mitochondria were collected from the supernatant fraction by centrifugation at 6,500 x g for 15 min. The resultant pellets were washed twice in half the initial volume of isolation medium and again the mitochondria were collected by centrifugation as previously described. A low speed centrifugation step (800 x g for 7 min) may be included prior to the final wash if red blood cells remain with the mitochondrial fraction. The final pellet was stored at -20°C until required or if further purification was required the mitochondria were resuspended in isolation medium at a final concentration of 20 mg/ml.

Isolated mitochondria were further purified on a discontinuous sucrose density gradient prepared as follows:-

4.0 ml	54% (w/w) sucrose in 10 mM Tris-HCl, pH 7.5
10.0 ml	45% (w/w) sucrose in 10 mM Tris-HCl, pH 7.5
10.0 ml	39% (w/w) sucrose in 10 mM Tris-HCl, pH 7.5
6.0 ml	20% (w/w) sucrose in 10 mM Tris-HCl, pH 7.5.

The gradients were centrifuged at 49 000 g for 2 h in a Beckman SW27 rotor.

Following centrifugation 3 bands were visible: the upper band of density 1.16 g/cm^3 consists principally of microsomes and plasma membrane; the intermediate band of density 1.19 g/cm^2 is composed of purified mitochondria, the lower band of density 1.22 g/cm^3 is composed mainly of lysosomes with some mitochondrial contamination. Mitochondria harvested from the gradient were diluted slowly in isolation medium and centrifuged at 6 500 x g for 10 min to remove the sucrose and collect the mitochondria.

(b) Preparation of bovine heart mitochondria

Bovine heart mitochondria were prepared according to the method of Smith (1967).

Bovine hearts were removed from the animals within an hour of slaughter, chilled immediately on ice and used within two hours of slaughter. After trimming off fat and connective tissue, the heart was cut into 5 cm cubes, 300 g of which was passed through a mincer. Minced tissue was placed in 400 ml of 10 mM Tris-HCl, pH 7.8, containing 0.25M sucrose and the pH was adjusted to 7.5 $\pm$ 0.1 using 2M Tris base.

After neutralisation, the ground tissue was placed in cheese-cloth, squeezed free of blood, then resuspended in 400 ml of 10 mM Tris-HCl, pH 7.8, containing 0.25M sucrose, 1 mM Tris-succinate, 0.2 mM EDTA (sucrose solution). 3 ml of 2M Tris base was added to maintain the correct pH and the material was blended at high speed for 15 sec in a Waring Blender. A further 3 ml of 2M Tris base was added and blending continued for a further 5 sec. The pH of the solution was further adjusted to 7.8 with 2M Tris base and the suspension homogenised using a Potter-Elvenhjem homogeniser with a loose-fitting teflon pestle (3 passes at high speed). The homogenate was centrifuged at 1000 x g for 15 min. The supernatant fluid was centrifuged at 5 000 x g for 30 min. The resultant pellet was washed once or twice more in the sucrose solution. Unless required immediately, the mitochondria were frozen at -20°C as pellets.

(c) Preparation of bovine heart submitochondrial particles

Bovine heart mitochondria were resuspended in sucrose solution (Section 2.2.9b) before being sonicated for 3 x 15 sec using an MSE sonicator (high amplitude and setting 3). Unbroken mitochondria were removed by centrifugation at 6 500 x g for 7 min, the supernatant fluid removed and the pellet resuspended in 3 ml of sucrose solution before being sonicated, as before. This procedure was repeated until none of the mitochondria remained intact. The broken mitochondria were distributed equally to Beckman Ti 50 centrifuge tubes and centrifuged at 100 000 x g for 1 h. The resultant extract, which is composed primarily of matrix components, was discarded

unless required and the pellet, essentially the inner mitochondrial membrane, was washed several times in 20 mM potassium phosphate buffer, pH 7.4. This fraction (bovine heart submitochondrial particles) was frozen at -70°C in small aliquots and a protein concentration of 16 mg/ml until required.

2.2.10 Purification of the phosphate-hydroxyl ion antiport protein from bovine heart and rat liver mitochondria

This method is essentially that of Kolbe et al. (1981) employing the modification of Gibb et al. (1986).

Mitochondria, prepared as described in Sections 2.2.9a and 2.2.9b, were allowed to thaw slowly at room temperature before being resuspended in 0.5% (w/v) Triton X-100 buffer (20 mM LiCl, 0.1 mM EDTA, 0.5 mM DTT, 0.5% (w/v) Triton X-100 and 20 mM H_3PO_4 adjusted to pH 7.0 with LiOH) at a protein concentration of 10 mg/ml. This pre-extraction effectively removes around 90% of mitochondrial proteins. After centrifugation for 1 hr at 100 000 x g, the resulting pellet was solubilised by incubating for 15 min on ice with an 8% (w/v) Triton X-100 buffer (as above) at a protein concentration of 10 mg/ml and diluted 1:1 with ice-cold distilled water. Insoluble material was removed by centrifugatic and the supernatant fraction was subjected to adsorptive chromatography with Bio-gel hydroxyapatite (0.5 g HTP/ml of solubilised membrane). The sample was incubated on ice for 15 min before being centrifuged at 27 000 x g for 20 min. This step was repeated by re-extracting the pellet in 4%

(w/v) Triton X-100 buffer. The supernatant fractions were pooled and residual HTP removed by low speed centrifugation (800 x g for 10 min) prior to adsorption chromatography on washed Celite 535 (0.5 g Celite/ml supernatant). The supernatant fluid was collected and the pellet washed as for HTP. The pooled supernatant fractions, again, were clarified by low speed centrifugation. The purified protein was frozen at -20°C until required.

2.2.11 Immunological Techniques

(a) Immune replica technique

This protocol, which follows a modification (Batteiger et al., 1982) of the method of Towbin et al. (1979), allows the immunological detection of proteins following their electrophoretic transfer from polyacrylamide gels to nitrocellulose paper.

Proteins were resolved in polyacrylamide gels (generally 10% (w/v) acrylamide). They were then electrophoretically transferred to nitrocellulose sheets, as described by Towbin et al. (1979), in 25 mM Tris base containing 192 mM glycine, 0.02% (w/v) SDS and 20% (v/v) methanol for either 3 h at 400 mA or 16-20 h at 40 mA. Excess binding sites on the nitrocellulose were saturated by incubating for 1 h at room temperature in 20 mM Tris-HCl, pH 7.2, containing 0.15M NaCl, 0.5% (v/v) Tween 20 and 5 mg/ml NaN_3 (wash buffer). This buffer was discarded and the nitrocellulose incubated for 90 min at room temperature in wash buffer supplemented with 0.5% (v/v) heat-inactivated horse serum and the

appropriate dilution of antiserum (generally 1:100). The nitrocellulose membrane was then washed five times in wash buffer over 30 min before being incubated for 60 min in wash buffer supplemented with ^{125}I-labelled Protein A (approximately 3 x 10^6 c.p.m.). The nitrocellulose was washed another five times in wash buffer before being dried and the antigen located by autoradiography (Section 2.2.6c).

The nitrocellulose membrane may be stained for protein before being processed i.e. immediately following electrophoretic transfer, or following autoradiography. For this the nitrocellulose should be immersed in 0.1% (w/v) Amido Black, 50% (v/v) methanol, 10% (v/v) acetic acid for 2-3 min followed by destaining in 50% (v/v) methanol, 10% (v/v) acetic acid for 2-5 min (Schaffner & Weissman, 1973).

(b) Purification of IgG from antisera

Approximately 5 ml antisera was extensively dialysed against 20 mM Tris-HCl, pH 8.0, containing 28 mM NaCl and 0.02% (w/v) NaN_3. The dialysed antiserum was applied to a DEAE-Affigel Blue column (18 cm x 1 cm) and eluted with the above buffer. The fractions containing IgG were collected, pooled and concentrated by freeze-drying. The resultant material was resuspended to 65% of the original volume and dialysed against 10 mM Tris-HCl, pH 7.4, containing 0.02% (w/v) NaN_3 at 4°C. Samples of IgG were aliquoted and frozen at -20°C until required.

(c) Immune precipitation from [^{35}S] methionine-labelled cellular extracts

The composition of the buffer containing the cell extracts was adjusted to that of 3D-TKM by diluting 1:1 with 3D-lysis

buffer (Section 2.2.8a). Aliquots (100 - 1000 µl) of cell extract containing 10 x 10^6 c.p.m. were incubated with 10-20 µl of the appropriate antiserum for 2 h at room temperature. At this stage 2 volumes of 10% (v/v) Pansorbin was added and the incubation proceeded for 1 h to precipitate the antibody-antigen complexes. The resultant pellets were collected by centrifugation (14 000 x g for 2 min). The pellets were washed 3 times in 3D-TKM buffer and once in 10 mM Tris-HCl, pH 7.4, by resuspending the pellets in 1 ml of the appropriate buffer and then collecting the immune complexes by centrifugation as above. Immune complexes were eluted from the surface of the S. aureus cells by boiling for 5 min in 50 µl of Laemmli sample buffer containing 10 mM DTT (Section 2.2.3b). A 5 µl aliquot was counted for radioactivity and the remainder of the sample was analysed by SDS-polyacrylamide gel electrophoresis followed by fluorography (Section 2.2.6d).

(d) Immune precipitation of non-radiolabelled cell lysates

This method was adapted from that of Anderson and Blobel (1983).

The antigen, in a volume of 10-100 µl, was solubilised by boiling for 2 min in a final concentration of 1% (w/v) SDS. The suspension was then diluted with four volumes of dilution buffer (60 mM Tris-HCl, pH 7.6, containing 190 mM NaCl, 6 mM EDTA and 1.25% (w/v) Triton X-100. This buffer was supplemented with 1 mM PMSF, 0.2 mM leupeptin and 1 mM p-amino benzamidine). 10 µl of non-immune rabbit serum

was added and the mixture was incubated overnight at 4°C.

100 µl of 10% (w/v) Pansorbin was added and the suspension was incubated for 1 h at room temperature with end-over-end mixing. The cells were collected by centrifugation (14 000 x g for 2 min) and 10-100µl of specific antiserum was added to the supernate. After an incubation for 1 h at room temperature, adsorption with Pansorbin was carried out as before. The immune complexes were collected as previously described. The resultant pellet was washed three times with 1 ml of 50 mM Tris-HCl, pH 7.5 containing 150 mM NaCl, 5 mM EDTA, 0.1% (w/v) Triton X-100 and 0.02% (w/v) SDS. A final wash was carried out in the above buffer in the absence of detergents. The antigen-antibody complex was dissociated from the S. aureus cells by boiling in 50 µl of Laemmli sample buffer containing 10 mM DTT (Section 2.2.3b). The supernatant fraction was analysed by the immune replica technique and autoradiography.

2.2.12 Cross-linking of proteins using dimethyl suberimidate

Crosslinking was carried out essentially by the method of Coggins (1978).

The following stock solutions were used:-

(i) TNM buffer: 0.05M Triethanolamine buffer, pH 8.0, containing 0.1M NaCl and 0.01M $MgCl_2$

(ii) 0.4M NaOH

(iii) 2 x TNM buffer: 0.1M Triethanolamine buffer, pH 8.0, containing 0.2M NaCl and 0.02M $MgCl_2$.

(iv) Dissolving buffer: 1 volume NaOH (ii) and 1 volume 2 x TNM buffer (iii).

(v) 0.1M Stock DMSI solution.

Solid DMSI was stored at -20°C. Since this material is very hygroscopic, stock bottles were allowed to reach room temperature for 1 h and were not opened for longer than necessary. A small amount of DMSI was transferred to a stoppered bottle, weighed accurately and dissolved in the appropriate volume of dissolving buffer (iv) to give 0.1M DMSI. DMSI prepared in this manner had a pH of 8.0. This stock solution was used within 5 min of preparation.

(a) Crosslinking of aldolase using DMSI

A sufficient volume of DMSI was added to a solution of aldolase (1-2 mg/ml) in TNM buffer, pH 8.0 to give a final DMSI concentration of 6 mM. The mixture was incubated for 60 min. at room temperature (23-25°C) with gentle swirling. Crosslinked proteins were examined by SDS-PAGE using the phosphate buffered system (Section 2.2.3.2).

CHAPTER 3

BINDING OF BOVINE HEART PDC TO THE INNER MITOCHONDRIAL MEMBRANE

3.1 INTRODUCTION

There are several levels of organisation within the eukaryotic cell. At the structural level this is evident by the presence of a complex internal network of membranes and the existence of several types of membrane-bound organelles which exhibit a characteristic morphology e.g. the mitochondrion or chloroplast. The existence of multi-enzyme complexes e.g. pyruvate dehydrogenase complex (PDC) offers the cell yet another level of compartmentation, the advantages of which are discussed in Section 1.2.

Until recently it was generally believed that most enzyme components of different metabolic pathways located within compartments e.g. within the cytoplasm or mitochondrial matrix, were randomly dispersed. Although interactions have been observed previously these were thought to be an artefact of the isolation procedure, primarily caused by the redistribution of structural components e.g. membranes. The situation within the eukaryotic cell has been further complicated by the existence of the microtubular lattice, an extensive network of inter-connecting filaments (Wolosewik and Porter, 1979; Porter and Tucker, 1981). Recent studies have suggested that the classical soluble enzymes of the cytoplasm are not freely diffusable because they are associated with this structure (Clegg, 1984a,b). Indeed, it has been postulated that the subcellular distribution of some enzymes may not be invariant, but alter in response to the changing requirements of

the cell (Masters, 1981). For example, it has been reported that most glycolytic enzymes are associated with actin filaments within skeletal muscle (Clarke and Masters, 1975; Walsh *et al.*, 1981). The extent of binding of these enzymes and their catalytic properties are influenced by a variety of factors including substrates, pH, ATP, Ca^{2+} and Mg^{2+}. This suggests that the enzymes' function *in vivo* may be regulated by an interaction with structural proteins.

Studies on the organisation of enzymes within compartments have been extended to those located within the mitochondrial matrix. In particular, Srere and coworkers (1974, 1978, 1980, 1982) have proposed that enzymes of the Krebs cycle interact, spatially, in the mitochondrial matrix as a multienzyme complex. This complex is proposed to operate functionally near, or on, the matrix side of the inner mitochondrial membrane.

There have been a number of conflicting reports concerning the association of matrix Krebs cycle enzymes with the mitochondrial inner membrane. Wit-Peeters *et al.* (1971) concluded that no clear distinction could be made between membrane-bound and soluble enzymes but that the enzymes differed only in the affinity of their binding to this membrane. Other workers (Landriscina *et al.*, 1976) have reported that aspartate aminotransferase and glutamate dehydrogenase were isolated in a membrane-free fraction. Thus, early studies so far have not provided unequivocal evidence for or against specific interactions of matrix

proteins with the inner mitochondrial membrane, although these differences are thought to reflect more the variety of methods used than to be of any functional significance.

Early studies which attempted to demonstrate the existence of a high M_r aggregate of Krebs cycle enzymes by either sucrose density gradient centrifugation or gel filtration of mitochondrial extracts were unsuccessful.

Experimental support for this hypothesis was provided by employing a variety of techniques to demonstrate the presence of a Krebs cycle complex, which Robinson and Srere (1985) have termed a metabolon.

(i) Studies with intact swollen mitochondria

In swollen mitochondria one would expect a reduction in the rate of oxidation of substrate since the mean free path between an intermediate and the next enzyme is increased. Extensive swelling of mitochondria accompanied by gross disorganisation of the matrix and at least a three-fold increase in mitochondrial volume resulted in the reduction of rates of respiration with NAD-linked Krebs TCA cycle enzymes. This reduction of rates of respiration was due not solely to the loss of NAD, since it did not restore respiration completely. Matlib and Srere (1976) suggested that this could be explained by the disruption of a putative Krebs cycle complex.

(ii) Studies with crosslinking reagents

It was postulated that if Krebs cycle enzymes interacted with the inner mitochondrial membrane, crosslinking with a

variety of reagents would stabilise these structures. Indeed, disorganisation of the membrane by either sonication or swelling reduced the degree of crosslinking. From this it was concluded that Krebs cycle enzymes were preferentially located within the matrix next to the inner membrane.

(iii) Studies with purified enzymes

Studies with purified enzymes have demonstrated specific interactions between sequential enzymes in metabolic pathways i.e. between fumarase and malate dehydrogenase (Beekman and Kanarek, 1981); citrate synthase and malate dehydrogenase (Halper and Srere, 1977), and PDC with citrate synthase (Sumegi *et al.*, 1980). Immobilised enzyme systems of malate dehydrogenase and citrate synthase (either immobilised as separate enzymes or as aggregates on a solid support e.g. Sepharose beads) were shown to operate at a faster rate than the equivalent amounts of free enzymes (Koch-Schmidt *et al.*, 1977).

Criticisms of these observations were primarily concerned with those interactions which had not been detected at physiological ionic strength. Srere (1980, 1981, 1982) has argued that we have no accurate assessment of ionic strength within the cell and since protein concentrations within the mitochondrial matrix are approximately 100 mg/ml, these interactions are possible under physiological conditions. Srere (1984) also argued that these interactions were specific for enzymes of sequential metabolic reactions.

To date, investigation of these protein:protein interactions has relied solely on the ability to assay for enzymic activity and the limitations of this approach will be discussed in Section 3.4.

Although commonly regarded as a soluble multienzyme system, there have been occasional reports which suggest that PDC may interact with the inner mitochondrial membrane (Stanley and Perham, 1980; Matlib and O'Brien, 1975). Stanley and Perham (1980) found that by including detergents in the initial buffers employed to disrupt mitochondria a three-fold increase in yield was achieved and that all of the assayable material was found in the soluble fraction. In earlier procedures (Linn et al., 1972; Cooper et al., 1974) no detergents were used; however, it was necessary to subject mitochondrial membranes to freeze/thaw treatment to induce the release of the enzyme. These observations are consistent with PDC being associated with the inner mitochondrial membrane.

Availability of subunit-specific antisera prompted us to investigate the nature of this association in a refined and sensitive fashion by immunological means. The major advantages of this approach are that it can detect (a) if specific components are involved in the binding to the inner membrane and (b) allow detection of antigens after washing the membrane under stringent conditions which cause loss of PDC activity.

3.2 METHODS

3.2.1 Preparation of antibodies to native PDC

Production of high-titre monospecific antiserum against ox heart PDC required highly-purified preparations of antigen. Samples of PDC (20-30 μg) were electrophoresed in 10% (w/v) SDS/polyacrylamide gels and their purity was estimated by densitometric scanning of the Coomassie-Blue stained bands. Samples judged to contain between 96-98% pure PDC, were used to raise antibodies to the whole complex.

PDC (1 mg), 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. This sample was injected subcutaneously at multiple sites on the neck and back of a New Zealand White rabbit. Similar amounts were administered at 2-3 week intervals thereafter, in Freund's incomplete adjuvant. Ten to fourteen days after the fourth injection blood was obtained from a marginal ear vein. Additional booster injections were administered at approximately one month intervals with 0.5 mg of protein with Freund's incomplete adjuvant.

Serum was collected, aliquoted in 1.0 ml fractions and frozen at -20°C until required.

3.2.2 Preparation of antibodies to PDC subunits

For the production of subunit-specific antisera, samples of PDC (approximately 2.5 mg) were resolved on preparative slab gels (Section 2.2.3; dimensions as for

analytical gels but gel thickness increased to 3 mm). After staining with Coomassie Blue, protein bands were excised from the gels with a scalpel, diced and rinsed twice with water to remove solvents. The gel sections were frozen using liquid nitrogen before being ground finely using a mortar and pestle. The frozen powder was weighed and stored at -20°C until use. This technique yielded between 100-300 µg protein per gram of gel (Mihard and Blobel, 1980).

Before injecting into rabbits, 1 g of gel was homogenised in 0.5 ml of 0.9% (w/v) NaCl with a tight fitting glass-teflon homogeniser. The gel slurry was mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously into a rabbit. The procedure for immunisation was as described in Section 3.2.1.

I am extremely grateful to Olga de Marcucci, who prepared and characterised these antisera.

3.2.3 Assay of mitochondrial fractions for enzyme activity

Bovine heart mitochondria were prepared as described in Section 2.2.9b and unless required immediately were frozen at -20°C. The mitochondria were sonicated (4 x 15 sec. ? high amplitude setting 3) and centrifuged at 10 000 x g for 10 min to remove any intact mitochondria. The resultant supernatant fraction was then centrifuged at 100 000 x g for 1 h to yield two fractions (a) a membrane fraction which was resuspended in 50 mM potassium phosphate buffer, pH 7.4 and (b) a supernatant fraction. These fractions

were then assayed for PDC, fumarase and citrate synthase activity (Section 2.2.2), as previously described. When assaying for PDC activity in the membrane fraction competing activities were inhibited by the inclusion of 5 µg of either oligomycin or antimycin A in the assay mixture. The activity was calculated as a percentage of the total recorded in the sonicated mitochondrial fraction.

3.2.4 Treatment of bovine heart submitochondrial particles

Bovine heart submitochondrial particles were prepared (Section 2.2.9c) and either used immediately or frozen at -20°C until required. Equal aliquots of the membrane fractions were washed once in 20 mM potassium phosphate buffer, pH 7.4 and collected by centrifugation at 100 000 x g for 1 h. The pellets were resuspended in the appropriate solution, incubated on ice for 15 min, and again collected by centrifugation at 27 000 x g for 20 min. This procedure was repeated (x 1) before the pellets were washed in distilled H_2O. When membranes were incubated with 0.1M glycine buffer, pH 9.0 containing 1M NaCl, the incubation was for 1 h at room temperature. This is never shown!

The extract resulting from the first wash was, in each case, concentrated using either aquacide or precipitated in 4 volumes of ice-cold acetone and resuspended in 20 mM phosphate buffer, pH 7.4. In both samples, protein determination was by the method of Lowry. Samples were resolved on 10% (w/v) SDS polyacrylamide gels before being transferred to nitrocellulose and probed with a variety of antisera.

3.2.5 Labelling of PK-15 cells with [^{3}H] Palmitic Acid

(a) Preparation of [^{3}H] palmitate for labelling

1.5 mCi of [9, 10(n) - ^{3}H] palmitic acid was removed to a sterile conical tube. The tube was placed within a water bath which was heated to 60°C. The evaporation of toluene from the sample was facilitated by blowing N_2 over the surface of the liquid. When the evaporation was complete, the sample was dissolved in 60 µl of ethanol. 5 ml of filtered medium (NGM + 5% (v/v) FCS) was added to this and the solution was incubated at 37°C for 90 min before addition to the cells.

(b) Incubation of cells with [^{3}H] palmitate

Cells were grown in 10 cm diameter petri dishes, as previously described (Section 2.2.7). On reaching semi-confluency, normal growth medium was removed and replaced with either 1 ml of NGM containing 250 µCi of [^{3}H] palmitate or 4 ml of medium supplemented with 1 µCi of radiolabel. The cells were grown for 16 h in this medium before cell lysates were prepared as previously described (Section 2.2.8b).

The radiolabelled cell lysates were used for immunoprecipitation of [^{3}H] labelled polypeptides. Replica plates of cells were employed for a control incubation with [^{35}S] methionine, with the resultant lysate being used for the immunoprecipitation of radiolabelled polypeptides.

3.3 RESULTS

Preliminary studies showed that the bulk of PDC activity remains associated with the inner mitochondrial membrane under conditions which cause the release of other soluble enzymes into the supernatant fraction (Fig. 3.1A). Further analysis of this result shows that for the Krebs cycle enzymes, fumarase and citrate synthase, more than 90% of the activity of the enzymes partition into the soluble fraction in comparison to the 25% recorded for PDC.

Initially these experiments were performed on frozen mitochondria; however, the release of proteins from membranes by employing freeze/thaw treatment has been well documented. Using freshly-isolated mitochondria and subjecting these to sonication demonstrates that the activity profile for the two Krebs cycle enzymes is essentially as before, with < 10% of the total activity found in the sedimenting fraction (Fig. 3.1B). Comparable analysis of PDC indicates that approximately 10% of the activity is soluble while the remainder is associated with mitochondrial membranes. Thus, the freeze/thaw treatment employed in the initial study (Fig. 3.1A) where approximately 25% of the PDC was solubilised appears to have facilitated, to some extent, the release of enzymic activity from the inner membrane. However, immunoblot analysis of the supernatant fraction allowed the detection of a small residual amount of succinate dehydrogenase, suggesting that any activity recovered in the soluble fraction, either PDC or SDH, may result from non-sedimenting membrane particles.

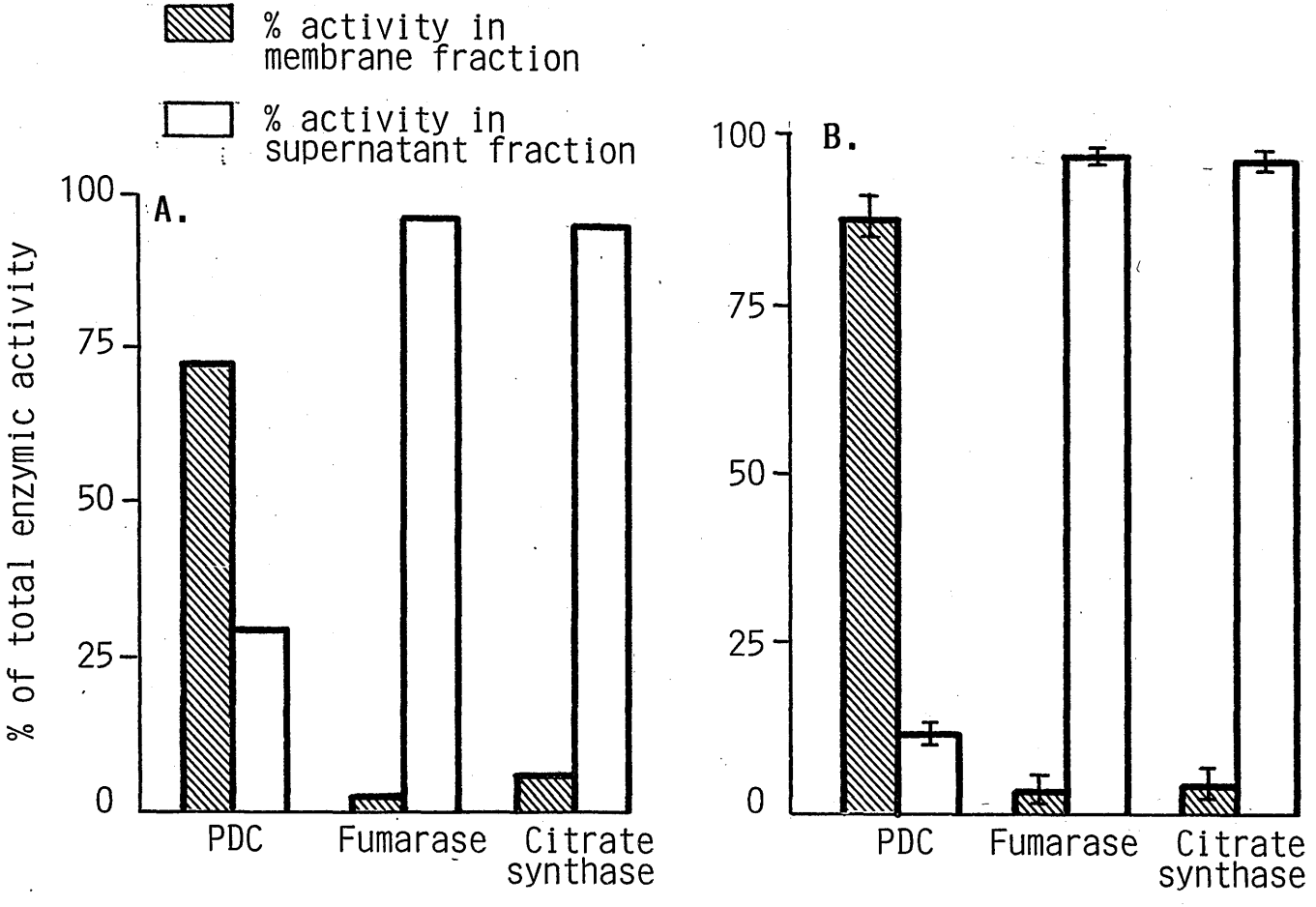
Fig. 3.1: Preferential association of PDC with the mitochondrial inner membrane

Mitochondria were sonicated (4 x 15 secs) with a recovery time of 15 secs between bursts. Intact mitochondria were removed by low speed centrifugation. The resultant extract was centrifuged at 100 000 x g for 1 h to yield a membraneous fraction (SMP) and a supernatant fraction. These fractions were assayed for PDC, fumarase and citrate synthase, as previously described. Activity was calculated as a percentage of the total activity found in sonicated mitochondrial fractions.

(A) SMP prepared from frozen mitochondria.

(B) SMP prepared from freshly prepared mitochondria.

Fig. 3.1 Preferential association of PDC with the mitochondrial inner membrane:



The availability of high-titre, monospecific polyclonal antisera against PDC and its constituent enzymes has allowed us to investigate this preferential association of PDC with the inner mitochondrial membrane under conditions which lead to a rapid inactivation of the enzyme.

This point is illustrated in Figure 3.2, where mitochondrial membranes were washed repeatedly in the presence and absence of 0.15M NaCl. It is apparent that PDC remains membrane associated whilst enzyme activity could only be recorded in those washed in the absence of salt. The presence of 0.15M NaCl is not known to inactivate the soluble enzyme, thus this result suggests that the higher ionic strength buffer may be removing a necessary co-factor from the membranes e.g. TPP. In an attempt to stabilise the enzyme activity, bovine heart mitochondria were sonicated and washed in a phosphate buffer containing 0.1 mM EDTA, 0.5 mM DTT, 0.1 mM TPP, 0.5 mM $MgCl_2$ and 0.15 μM leupeptin. The result of this experiment is demonstrated in Figure 3.3. As before, PDC could be detected, immunologically, in each of the fractions, although analysis of the resultant extract showed that less protein had been released than on the previous occasion. Control enzymes, which as demonstrated in Figure 3.1, are readily released into the supernatant fluid, could be detected in the membraneous pellet. Immunoblot analysis (Fig. 3.3B) shows citrate synthase to be membrane-associated even after four washes in a low ionic strength buffer. These results

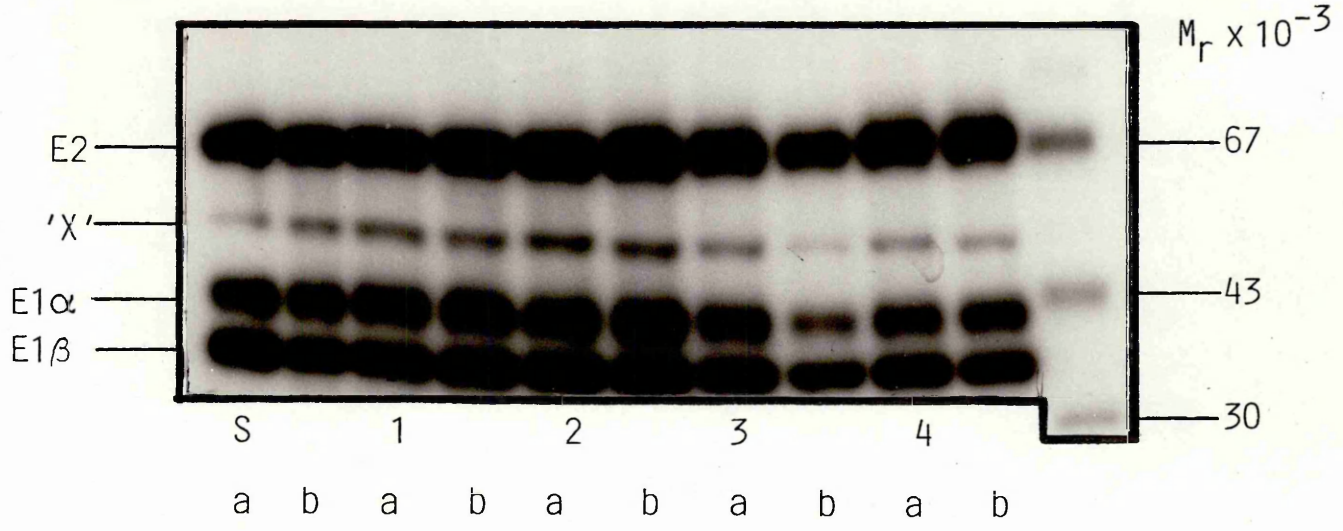
Fig. 3.2: Immune Blot Analysis using Anti-PDC-Serum of Bovine Heart Submitochondrial Particles Repeatedly Washed in Buffers +/- 0.15M NaCl

Bovine heart submitochondrial particles were prepared as previously described (2.2.9c) and washed repeatedly in 50 mM potassium phosphate buffer, pH 7.4 (a) or phosphate buffer containing 0.15M NaCl (b). Aliquots were withdrawn following each wash and analysed by immunoblotting using anti-PDC-serum. Each fraction was also assayed for PDC activity (data not shown).

1,2,3,4 corresponds to the number of washes.

45 µg of membrane protein loaded/track.

S = sonicated mitochondria.


M_r x 10^{-3}
E2
'X'
E1α
E1β
67
43
30
S
1
2
3
4
a b a b a b a b a b

suggest that either the mitochondria remained intact or that following sonic disruption have sealed right-side-out and subsequently, soluble activities (fumarase and citrate synthase) were trapped within closed membrane vesicles. The formation of right-side out vesicles induced by the presence of Mg^{2+} has been reported previously for red blood cells (Steck and Kant, 1974).

Specific attachment of PDC to the inner mitochondrial membrane was further investigated by washing submitochondrial particles under a variety of conditions, some of which were known to dissociate the soluble complex to its constituent enzymes, and other standard procedures for removing peripherally-bound membrane proteins (Fig. 3.4).

Immunoblot analysis of the resultant membranes shows that PDC is stable after washing in low ionic strength buffer (Fig. 3.4A, lane 4). However, increasing the salt concentration, from 0.15M NaCl to 3M NaCl, causes the gradual release of E1β (Fig. 3.5C, lanes 5-9) into the soluble fraction with E2 and X remaining bound to the inner membrane fraction. The parallel release of E1α and E3, under similar conditions, had been reported previously (de Marcucci, 1985). Attempts to differentiate between the attachment of E2 and X to the inner membrane using increasing concentrations of urea proved unsuccessful due to the chaotropic nature of this reagent.

Dissociation of the complex into its constituent enzymes was achieved by washing submitochondrial particles in 0.25M $MgCl_2$ or 0.1M glycine buffer pH 9.5. Subsequent immunoblot analysi illustrates that the membranes are depleted of E1α and E1β (Fig. 3.4A, lanes 3 and 6) with a corresponding enrichment of E2 and component X. It is not clear whether this specific association of protein X

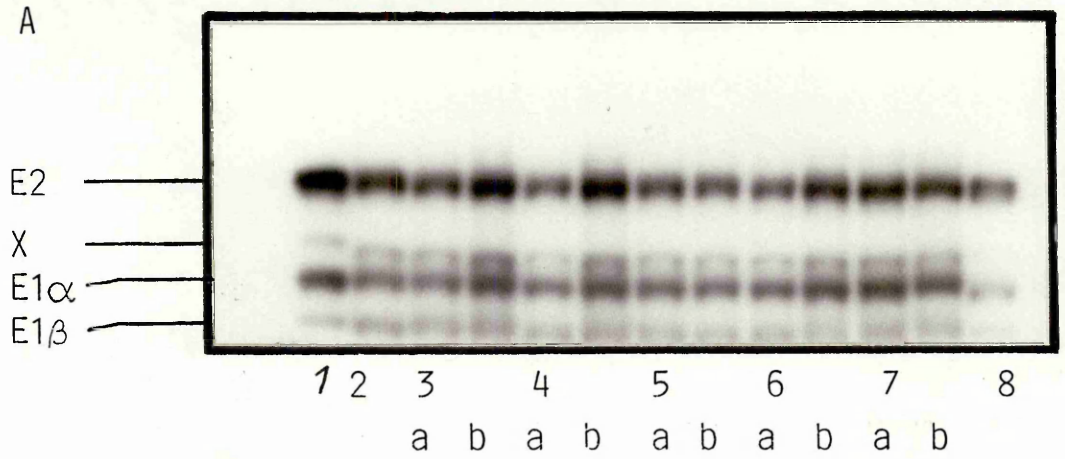
Fig. 3.3: Factors Affecting the Orientation of Vesicle Formation

Bovine heart mitochondria were sonicated in 20 mM potassium phosphate buffer, pH 6.5, containing 0.1 mM EDTA, 0.5 mM DTT, 0.1 mM TPP, 0.5 mM $MgCl_2$ and 0.15 µM leupeptin (a) or as (a) but supplemented with 0.15M NaCl (b). SMP resulting from the sonicate were washed (x 3) either in buffer (a) or (b), with aliquots being retained following each incubation. These aliquots were assayed for enzyme activity. Protein concentration of the membrane fraction was estimated by the method of Lowry. Proteins were resolved by SDS-PAGE before being analysed by immunoblotting prior to autoradiography.

(A) anti-PDC-serum

(B) anti-citrate synthase-serum.

Lane (1) purified antigen, 0.25 µg; (2) intact mitochondria, 40 µg; (3)a,b sonicated mitochondria 40 µg (4) a,b membranes following first wash; 40 µg (5) a,b membranes following second wash; 40 µg; (6) a,b membranes following third wash; 40 µg; (7) a,b membranes following fourth wash; 40 µg; (8) purified antigen 0.25 µg (9) I^{125} low M_r standard proteins.


A
E2
X
E1α
E1β
1 2 3 4 5 6 7 8
a b a b a b a b a b

B

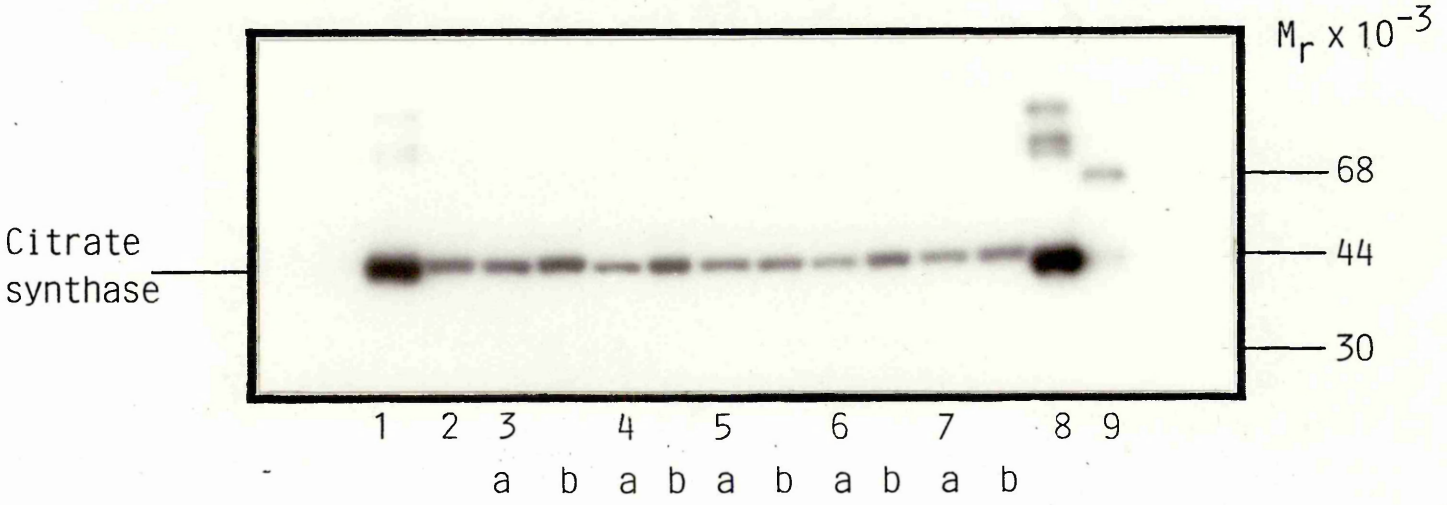

$M_r \times 10^{-3}$
68
44
30
Citrate synthase
1 2 3 4 5 6 7 8 9
a b a b a b a b a b

Fig. 3.4: Immune Blot Analysis Using anti-PDC-serum of the Effect of Washing Bovine Heart Submitochondrial Particles.

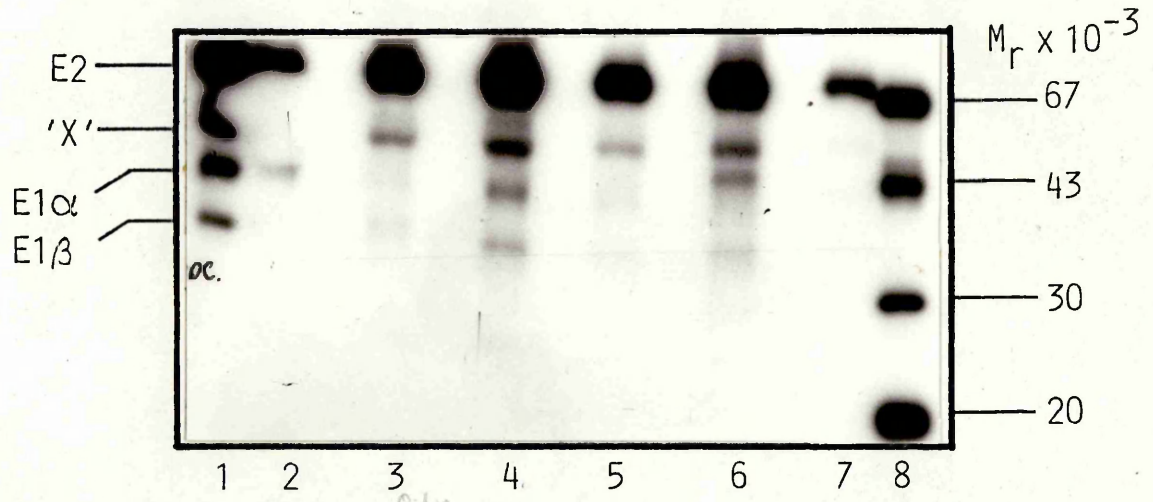
Bovine heart SMPs were washed under a variety of conditions which were known to either remove peripherally bound membrane proteins or to dissociate native PDC. The soluble fraction was separated from the membrane fraction by centrifuging at 27 000 x g for 15 min. The samples were resolved by SDS-PAGE (10% w/v) and analysed by immunoblotting. The various incubations were as follows (lanes 2-7):

Lane (1) Purified PDC (1 µg); (2) 20 mM potassium phosphate buffer, pH 7.4; (3) 0.1M glycine buffer, pH 9.5; (4) 0.5M NaCl; (5) 1M NaCl; (6) 0.25M $MgCl_2$; (7) 6M urea; (8) I^{125}-labelled low M_r standard proteins 45 µg of protein loaded per track (lanes 2-7).

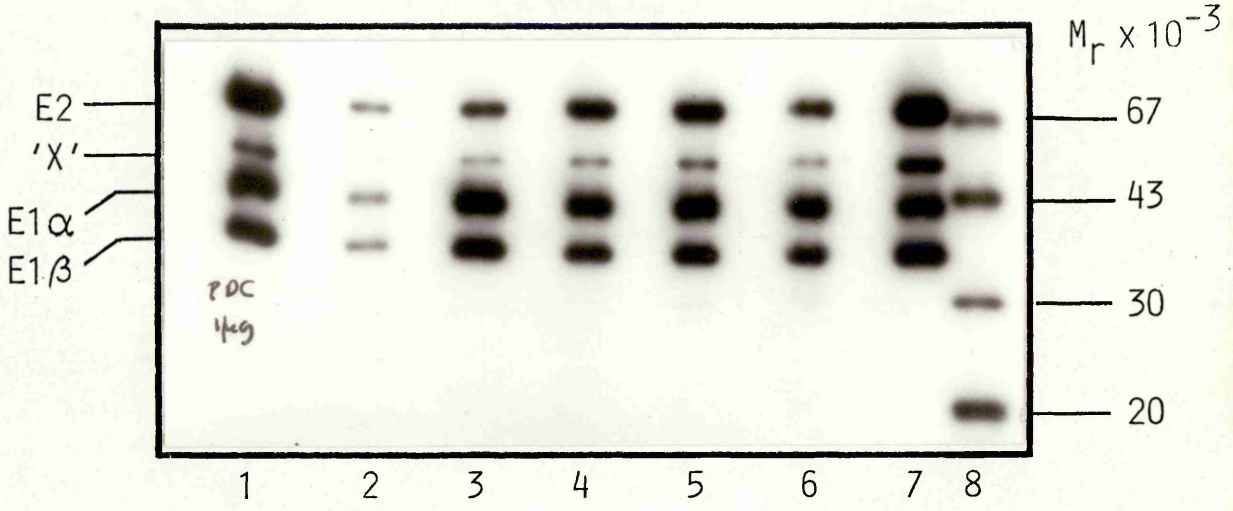
(A) mitochondrial membrane fraction

(B) mitochondrial extract.

A


E2
'X'
E1α
E1β
oc.
$M_r \times 10^{-3}$
67
43
30
20
1 2 3 4 5 6 7 8
KPi
0.1m gly pH 9.5
0.5M NaCl
1M NaCl
0.25M MgCl2
6M urea

B


E2
'X'
E1α
E1β
POC
1µg
$M_r \times 10^{-3}$
67
43
30
20
1 2 3 4 5 6 7 8

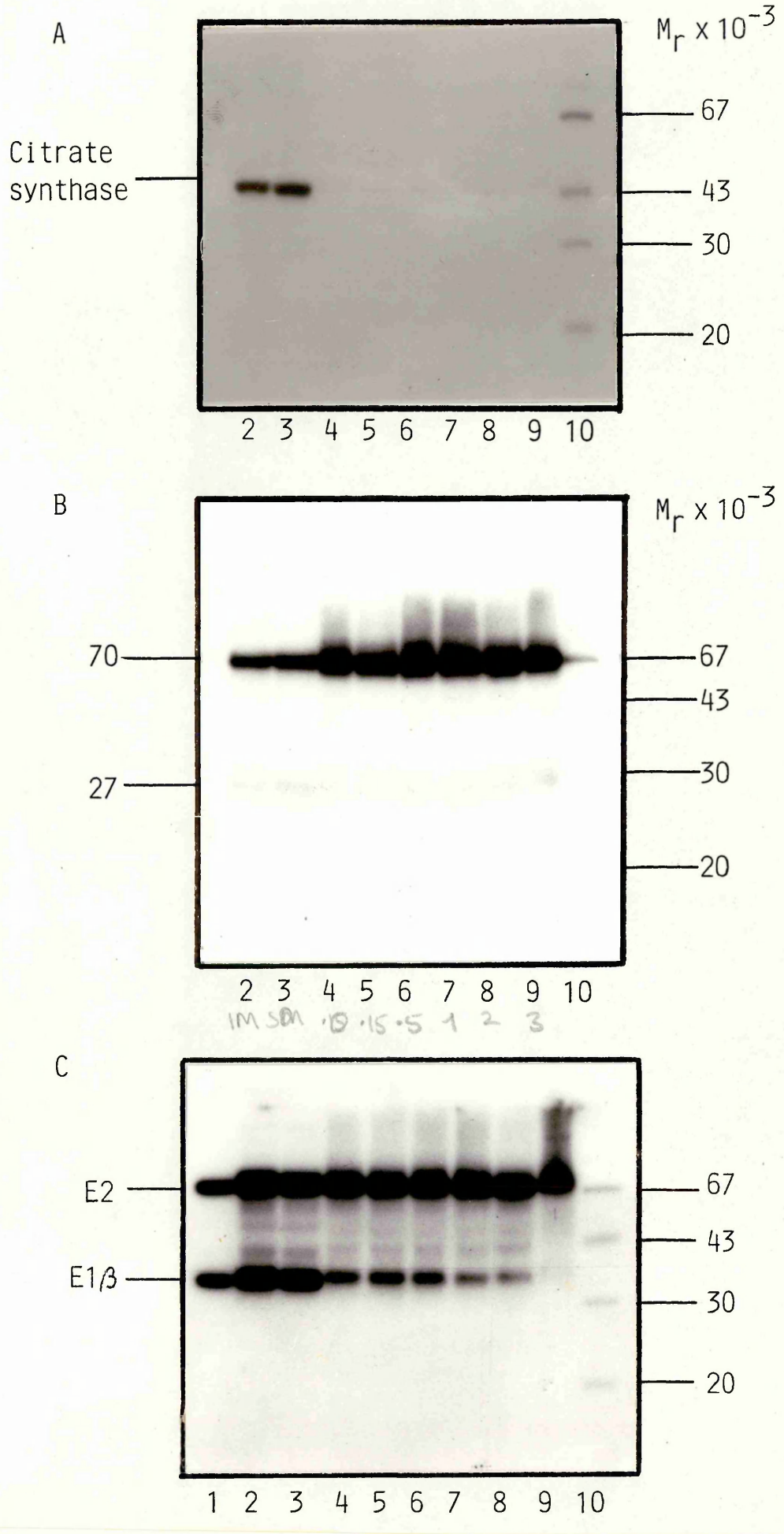
with the membrane fraction occurs by virtue of its strong association with E2 or specifically with the inner mitochondrial membrane.

This preferential interaction of E2 and/or protein X with the membrane fraction was further examined by determining the susceptibility of other mitochondrial enzymes to washing in the same range of buffers. This washing procedure successfully removes citrate synthase, even in the absence of salt (Fig. 3.5A, lane 3). In contrast, the integral membrane protein, succinate dehydrogenase, remains associated following prolonged washing in 3M NaCl (Fig. 3.5B, lane 9). Inspection of Figure 3.5C reveals that E1β is depleted from the membrane fraction by repeated washing, although not as readily as the control enzyme, citrate synthase. Further analysis of this figure shows that the core enzyme, E2 remains in stable association with the membrane even after stringent washing in 3M NaCl, a situation analogous to SDH. Only under the strongly denaturing conditions employed to remove tightly bound peripheral proteins e.g. 6M urea (Fig. 3.4, lane 7) or 0.1M Na_2CO_3, pH 11.5 (data not shown) is the bulk of PDC released from the membranes. However, the possibility exists that these stringent conditions are denaturing the binding sites for this complex rather than primarily removing the protein from the membrane, although the parallel release of SDH from the lipid bilayer under these conditions suggests that the integrity of the membrane is susceptible to these chaotropic reagents.

Fig. 3.5: Immune-blot Analysis of Salt Washed Bovine Heart Submitochondrial Particles Using Subunit Specific Antisera (E2 and E1β of PDC)

Submitochondrial particles were prepared and incubated with various concentrations of salt. The membrane fraction was collected by centrifugation (27 000 x g for 15 secs) and the wash procedures repeated before membranes were washed (x 2) in ice-cold distilled H_2O. Samples were resolved on 10% (w/v) SDS-polyacrylamide gels, transferred to nitrocellulose and probed for antigens using (C) anti-E2-serum, followed by anti-E1β-serum; (B) anti-SDH-serum; (A) anti-citrate synthase-serum. Lanes (1) Purified PDC, 0.5 µg; (2) intact mitochondria, 40 µg; (3) sonicated mitochondria 40 µg; (4) 0M salt wash, 80 µg; (5) 0.15M salt wash, 80 µg; (6) 0.5M salt wash, 80 µg; (7) 1M salt wash, 80 µg; (8) 2M salt wash, 80 µg; (9) 3M salt wash, 80 µg; (10) I^{125}-labelled low M_r standard.

Degradation of the E2 subunit is evident by the presence of lower M_r bands which also react with anti-E2-serum.


A
$M_r \times 10^{-3}$
Citrate synthase
67
43
30
20
2 3 4 5 6 7 8 9 10
B
$M_r \times 10^{-3}$
70
27
67
43
30
20
2 3 4 5 6 7 8 9 10
C
E2
E1β
67
43
30
20
1 2 3 4 5 6 7 8 9 10

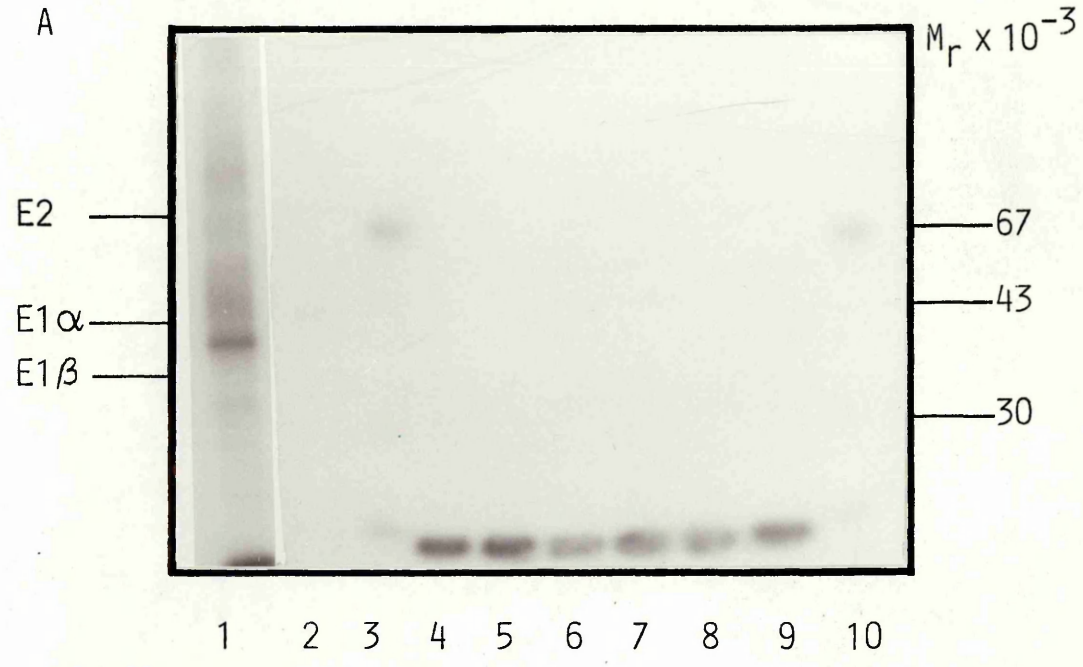
Having established the preferential association of PDC, and specifically the E2 and/or X subunits, with the inner mitochondrial membrane, the nature of this interaction was further investigated. It was possible that PDC was associated with the membrane via an interaction with either other proteins or perhaps by a covalent attachment to membrane lipids.

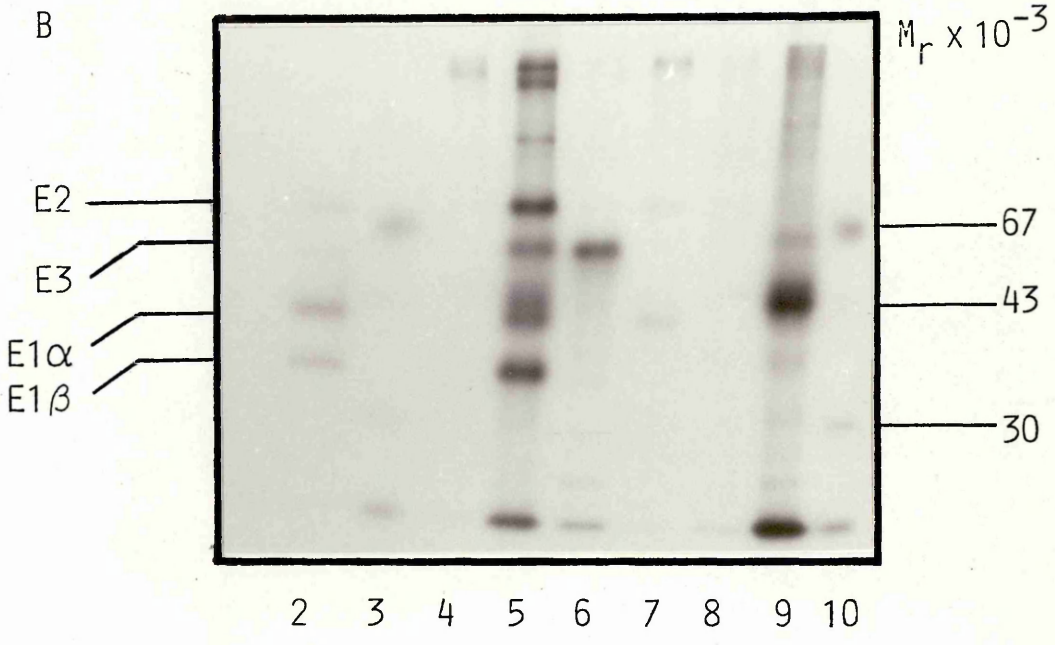
The latter possibility was investigated by incubating mammalian tissue culture cells overnight in the presence of [^{3}H] -palmitate (Section 3.2.5). Cell lysates were prepared as previously described (Section 2.2.7) and used subsequently for immunoprecipitation studies. The results obtained are illustrated in Figure 3.6. Analysis of the acetone-precipitated cell lysate demonstrates the association of radiolabel with a variety of cellular proteins (Fig. 3.6A, lane 1). Immunoprecipitation studies using this lysate in conjunction with a variety of antisera did not demonstrate a specific attachment of [^{3}H] -palmitate or [^{3}H]-myristate to any component of PDC (Fig. 3.6A, lanes 4-9). Control immunoprecipitations using identical cells with [^{35}S] - methionine successfully precipitated the chosen antigen by this procedure. Thus, in light of these results, the possible attachment of PDC with the inner membrane via a covalent bonding to lipids seems unlikely.

Other workers (Sumegi and Srere, 1984) have reported on the specific association of several NAD-coupled dehydrogenases with Complex I of the electron transport

Fig. 3.6: Labelling of PK 15 Cell With $[^3H]$ Palmitic Acid.

PK-15 cells were labelled for 16 h either in the presence of $[^{35}S]$ methionine or $[^3H]$ - palmitic acid, as described in Section 3.2.5. Cell lysates were prepared (2.2.7) and subsequently used for immunoprecipitation studies with a variety of antisera. The immunoprecipitation procedure is detailed in Section 2.2.11. Radiolabelled proteins were resolved on 10% (w/v) SDS-poly-acrylamide gels before being processed for fluorography. (A) $[^3H]$ palmitic acid labelled cell lysate (B) $[^{35}S]$ methionine labelled cell lysate used for immunoprecipitation.
Lane (1) $[^3H]$ palmitic acid-labelled cell lysate; lane (2) $[^{14}C]$ -labelled PDC; lanes 3,10 I^{125}-labelled low M_r standard proteins. Lanes(4-9) are immunoprecipitates with the following antisera: lane (4) non-immune rabbit serum; lane (5) anti-PDC-serum; lane (6) anti-E3-serum; lane (7), anti-E1α-serum; lane (8), anti-PTP-serum; lane (9) anti-citrate synthase-serum.


A
$M_r \times 10^{-3}$
E2
E1α
E1β
67
43
30
1 2 3 4 5 6 7 8 9 10


B
$M_r \times 10^{-3}$
E2
E3
E1α
E1β
67
43
30
2 3 4 5 6 7 8 9 10

chain. Complex I is known to consist of approximately 26 polypeptides (Heron et al., 1979), only a few of which have a known function. These workers (Sumegi and Srere, 1984) further postulated that perhaps some of the unassigned polypeptides of Complex I may be involved in binding these enzymes, in particular PDC, to the inner mitochondrial membrane, thus linking the enzymes involved in NADH production to its oxidation.

Further investigation of this point was achieved by resolving both Complex I and PDC on SDS-polyacrylamide gels (Fig. 3.7A) and scanning the resultant Coomassie Blue profiles. It was found that none of the five constituent polypeptides of PDC co-migrated with any of the major components of Complex I, eliminating the possibility that PDC is an integral part of Complex I. It has been reported that, on purifying PDC to apparent homogeneity by a variety of methods (Linn et al., 1972; Cooper et al., 1974; Stanley and Perham, 1980), that some Complex I activity is always recorded in the final preparation. Immunoblot analysis, with a variety of antisera, was used to ascertain the degree of cross-contamination of purified Complex I with PDC and other mitochondrial proteins. Figure 3.7B,C demonstrates that this preparation was not contaminated to any significant extent with either citrate synthase or fumarase. Probing Complex I with anti-SDH-serum also failed to detect the presence of the parent antigen (Fig. 3.7D).

In contrast, the clear detection of the constituent PDC polypeptides within Complex I argues for a specificity of interaction with this region of the respiratory chain. As PDC does not co-migrate with any component of Complex I, the immune detection of these bands suggests only that PDC polypeptides are a minor contaminant of this preparation. However, failure to detect SDH, which is both an integral membrane protein and a component of the electron transport chain, within this preparation suggests that there may be a normal interaction, in situ, between Complex I and PDC.

The results presented in this Chapter suggest that PDC binds to the mitochondrial inner membrane with a greater affinity than other matrix enzymes. The particular strength of interaction of E2 and/or component X with this membrane suggests that the complex is a tightly-bound peripheral protein and that binding is mediated via one or both of these subunits.

Fig. 3.7: Immune Blot Analysis of Complex I Using a Variety of Antisera

Purified Complex I was resolved on a 10% (w/v) SDS-polyacrylamide gel and either stained with Coomassie Blue (A) or transferred to nitrocellulose before being screened using a variety of antisera. (B) anti-fumarase-serum; (C) anti-citrate synthase serum; (D) anti-SDH-serum; (E) anti-PDC-serum.

(A): (1) Purified PDC, 2.5 µg; (2), (3), (4) 10,20, 40 µg respectively, of purified Complex I;
(5) Low M_r standard proteins

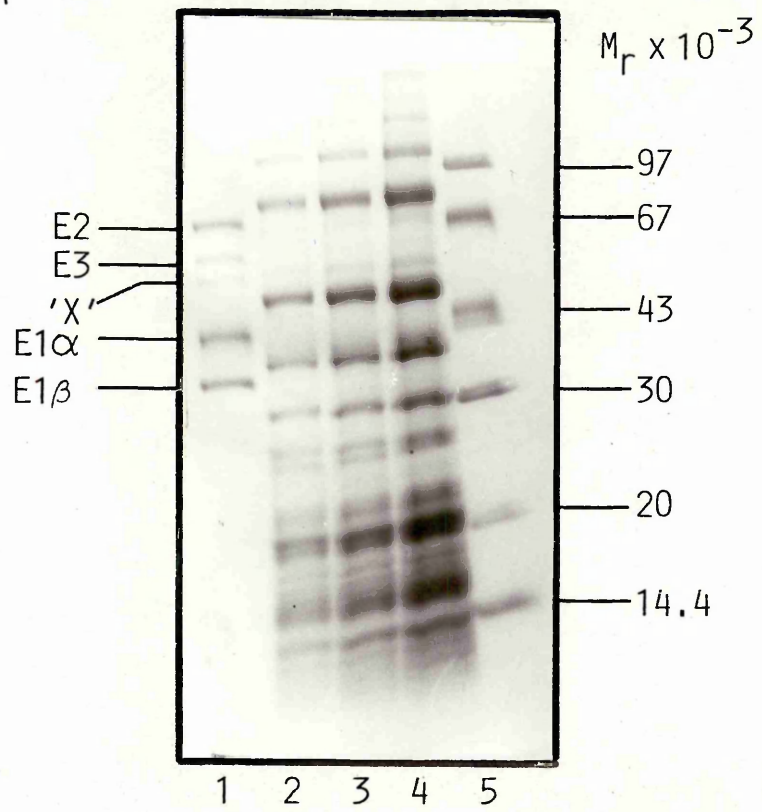
(B): (1) Purified fumarase, 0.2 µg;
(2) Purified Complex I, 20 µg;
(3) I^{125}-labelled low M_r standard proteins

(C): (1) Purified citrate synthase, 0.2 µg
(2) Purified Complex I, 20 µg
(3) I^{125}-labelled low M_r standard proteins

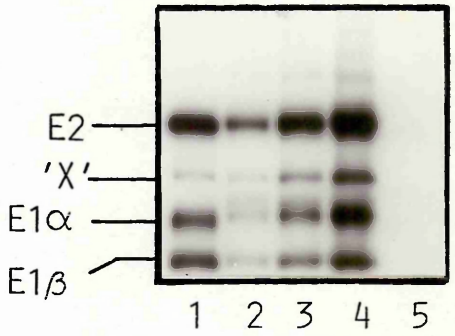
(D): (1) Sonicated mitochondria, 40 µg
(2) Purified Complex I, 20 µg
(3) I^{125}-labelled low M_r standard proteins

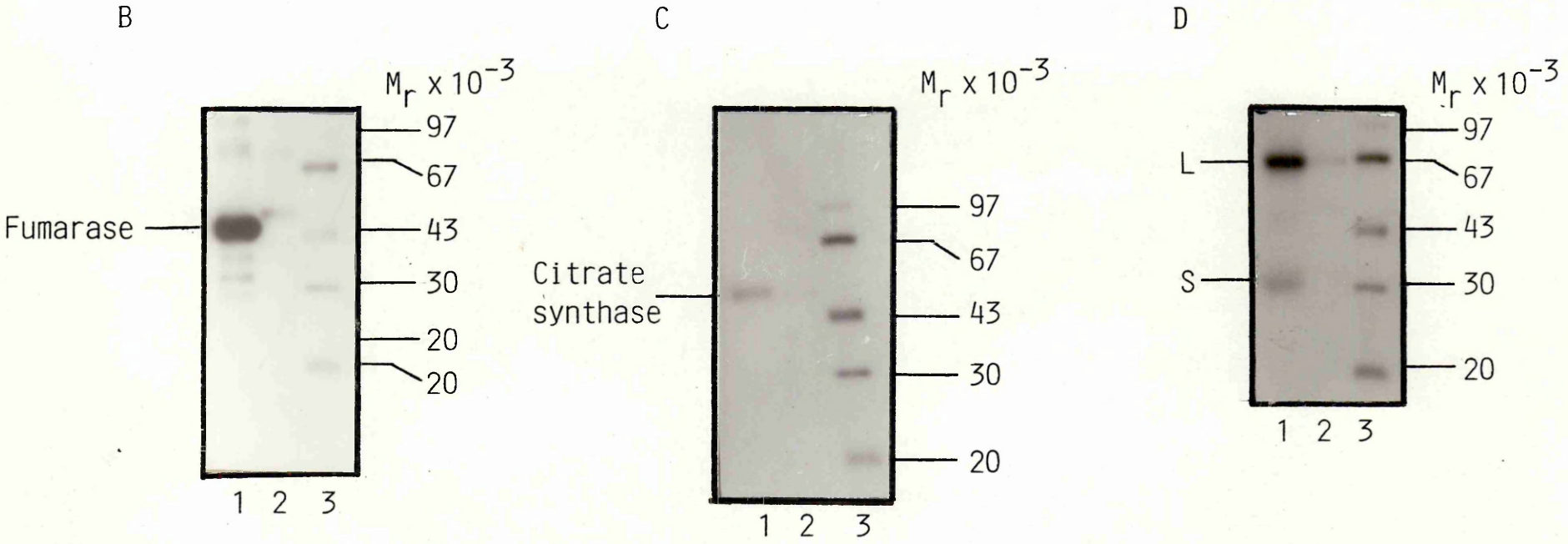
(E): (1) Purified PDC, 0.25 µg;
(2), (3), (4) as (A)
(5) I^{125}-labelled low M_r standard proteins.

A


$M_r \times 10^{-3}$
97
67
43
30
20
14.4
E2
E3
'X'
E1α
E1β
1 2 3 4 5

E


E2
'X'
E1α
E1β
1 2 3 4 5


B
$M_r \times 10^{-3}$
97
67
43
30
20
20
Fumarase
1 2 3
C
$M_r \times 10^{-3}$
97
67
43
30
20
Citrate synthase
1 2 3
D
$M_r \times 10^{-3}$
97
67
43
30
20
L
S
1 2 3

3.4 DISCUSSION

Evidence presented in this chapter suggests that PDC is tightly bound to the inner mitochondrial membrane of ox heart. This interaction between PDC and the inner mitochondrial membrane has been demonstrated to be significantly stronger than for two other matrix enzymes, fumarase and citrate synthase. Despite having similar characteristics to the membrane-binding of SDH, data suggest that PDC belongs to a class of tightly-bound membrane-associated proteins rather than that of integral membrane components.

Initially these investigations were performed on mitochondria isolated from rat liver. Several disadvantages of this system became apparent, not the least of which was the lower amount of enzyme present in comparison to that from bovine heart. The identification of a rat liver protease which specifically inactivates PDC (Wieland, 1975) presented an additional disadvantage. In contrast, we found no evidence for the rapid degradation of the ox heart enzyme in crude mitochondrial extracts, thus subsequent experiments were performed using this tissue.

By assaying sonicated mitochondria for enzyme activity, it was found that the bulk of PDC activity partitioned with the membrane, with less than 25% of the total assayable activity present in the soluble fraction. Whether this observed distribution is of functional significance and perhaps reflects structural differences between the two populations or is merely an artefact of the preparation remains to be established. However, detection of SDH in

the supernatant fraction suggests that this result may reflect the presence of non-sedimenting membrane particles rather than a distinct soluble population of PDC. It should be noted that > 5% of purified PDC was found to sediment under these conditions (data not shown).

Several laboratories have reported on the association of matrix enzymes with the inner mitochondrial membrane (D'Souza and Srere, 1984; Robinson and Srere, 1985). There have been conflicting reports on the internal mitochondrial location of some enzymes, in particular those of the Krebs cycle, (Wit-Peeters et al., 1971; Landriscima et al., 1970) although it is likely that these mainly reflect methodological differences.

Our data have established that E2 remains firmly attached to the matrix surface of the mitochondrial inner membrane (Fig. 3.4) and that the distribution of this subunit is radically different to that of E1 or E3, which are both readily released from the complex (de Marcucci, 1985). In contrast to this, Surmegi and Srere, (1984) have reported on the association of PDC with the inner mitochondrial membrane and observe that each of the constituent enzymes bind with equal affinity. This group relied primarily on the ability to detect PDC activity as the sole criterion for binding. Figure 3.3 demonstrates the presence of PDC on the inner mitochondrial membrane under conditions which inactivate the enzyme. Inactivated but membrane bound enzyme was not accounted for in these studies. These workers (Sumegi and Srere, 1984) suggest

that the inability to recover total enzyme activity (< 30% of total PDC was recovered) reflects that the reconstituted complex exhibits a lack of co-operativity not seen in the soluble, unbound enzyme. The significantly greater activity recorded for the partial reactions of E1, E2 and E3, assayed independently may be accounted for by either non-specific binding of each of the subunits to the membrane or more probably reflects an interaction of each of these constituents with residual E2 still associated with the membrane. Studies demonstrate that PDC remains membrane-associated following prolonged sonication and Figure 3.5 illustrates that PDC remains bound after repeated washing of the membrane in low ionic strength buffer. The bulk of PDC activity was only released after washing membranes in 6M urea (Fig. 3.4, lane 7). With reference to the studies of Sumegi and Srere (1984) it seems unlikely that they have prepared membranes completely depleted of PDC polypeptides and thus their results should be interpreted with caution.

Our studies have illustrated the limitations of assessing binding by assaying for enzyme activity and have demonstrated the presence of membrane-bound E2, via immunoblotting, under conditions which inactivate the enzyme. Figure 3.5 illustrates that E2 remains associated even at high ionic strength (3M NaCl) while E1β is readily released. This result confirms that PDC associates with the inner mitochondrial membrane, via an interaction with E2 with an affinity comparable to SDH more than

citrate synthase. Component X is also observed to be associated with the membrane fraction more tightly than either E1 or E3, which are both readily released. Whether

Thus an alternative to PDC binding to the membrane through E2 is that it binds through component X. Another possibility is that there is a direct involvement with both subunits.

We have investigated the possibility that PDC could interact with the mitochondrial inner membrane via a covalent attachment to membrane lipids, in particular with myristic acid. This method of membrane association has been reported for several proteins, e.g. SV40 large T antigen (Klockmann and Doppert, 1985); retinoid binding protein (Bazan et al., 1985) picornavirus capsid protein, VP4, (Chow et al., 1987) and VP2 protein of SV40, (Streuli and Griffin, 1987). Despite being able to incorporate radiolabel into a variety of cellular proteins (Fig. 3.6, lane 1), none of these was found to co-migrate with polypeptides of PDC, or to be immunoprecipitated with anti-PDC-serum or subunit-specific sera (Fig. 3.6A). Thus, in light of this, it seems unlikely that PDC is associated with the inner mitochondrial membrane via a covalent binding to myristic and/or palmitic acid.

Sumegi and Srere (1984) have suggested that this interaction may exist via Complex I of the electron transport chain. By examining the interaction of a variety of dehydrogenases, including OGDC, PDC and malate dehydrogenase, with the multimeric inner membrane complex, these

workers were able to demonstrate a specificity of interaction which could not be detected for either fumarase or citrate synthase. The application of these *in vitro* observations has important implications *in situ*, not the least of which is the linking of those enzymes involved in NADH production with those of oxidation.

The results presented (Fig.3.7) show the presence of PDC in Complex I, whereas another membrane protein, SDH, was not detected. This may support the idea of a specific association between PDC and Complex I, or may be the result of Complex I being contaminated with PDC and not with SDH.

It is still possible that two distinct populations of PDC exist in the mitochondria, one that is free in solution and a second which is associated with the inner membrane as a tightly-bound extrinsic protein although our data suggest that this association exists via the E2 and/or X components of the complex. The possible function of these two populations may be to feed acetyl CoA to different pools within the mitochondria (Von Glutz and Walter, 1975) e.g. that PDC which binds to citrate synthase (Sumegi *et al.*, 1980) will channel acetyl CoA to the Krebs cycle whilst that which interacts with the membrane will feed the acetyl groups to coenzyme Q or perhaps to enzymes involved in fatty acid synthesis.

The available evidence indicates that the mitochondrial matrix and inner membrane compartments are a semicontinuous compartment crowded with proteins that are interacting

with each other in each semiautonomous phase and with proteins in the adjacent phase. In this way it is possible to envisage that only portions of cycles are organised such as fumarase, malate dehydrogenase, citrate synthesis and PDC, which may be responsible for the formation of citrate, whilst the dehydrogenases of the Krebs cycle might be bound to Complex I. This proposed variety of organisational variants may be dependent on cell type, on the metabolic requirement of that particular cell and perhaps even be responsible for the conflicting reports on protein organisation within compartments.

CHAPTER 4

PURIFICATION OF THE PHOSPHATE TRANSPORT PROTEIN AND CHARACTERISATION OF ANTISERUM

4.1 INTRODUCTION

The initial stages of this project were concerned primarily with purifying the phosphate transport protein from both rat liver and beef heart mitochondria. The purified protein was utilised to raise specific antiserum, which was subsequently employed for studies on the topography and biosynthesis of the carrier protein.

The phosphate transport protein has proven difficult to purify as demonstrated by the apparent heterogeneity of preparations. Prior to the publication by Gibb *et al.* (1986) other preparation schemes had at least one other protein band evident at the final stage (Kolbe and Wohlrab, 1985). This lack of a homogeneous preparation presents obvious difficulties when attempting to raise antibodies against the PTP. Indeed, any contaminating bands may prove to be more immunogenic than the chosen antigen.

The use of antibodies as a biochemical tool has been well documented, particularly over the last few years. The advantages offered by antibodies are manifold, not the least of which is that small amounts of protein can be detected by immunoblotting and thus antibodies are ideal probes for peptide mapping studies. They allow the detection of antigen in both other tissues and the same tissue of different species. This has important implications when studying the evolutionary aspects of proteins where antibodies could be used to detect conserved regions of protein sequence.

Only one other group (Rasmussen and Wohlrab, 1986) has reported on the use of antibodies to further characterise the PTP. In their hands, anti-PTP-serum elicits a low sensitivity of detection of the parent antigen in crude extracts when immunoblotted.

4.2 METHODS

4.2.1 Purification of the phosphate transport protein from rat liver and bovine heart mitochondria

This procedure is as described in Section 2.2.10. Fractions were retained for analysis at each stage in the purification. Proteins were precipitated by the addition of four volumes of ice-cold acetone and finally prepared for analysis by SDS-PAGE as described in Section 2.2.3.

4.2.2 Preparation of antiserum to purified rat liver phosphate transport protein

Densitometric scanning of Coomassie Blue-stained gels allowed the selection of preparations of greater than 95% purity for immunisation of rabbits with PTP.

Purified PTP was acetone precipitated and the resultant pellet (1 mg) was resuspended in 0.5 ml of PBS, mixed with an equal volume of Freund's complete adjuvant and injected subcutaneously at multiple sites on the neck and back of a New Zealand White rabbit. As a result of the low titre of antibody that was obtained on previous occasions, this procedure was repeated x 3 with complete adjuvant over a period of six weeks in an attempt to increase the immune response. Similar amounts were injected at 2-3 week

intervals thereafter with Freund's incomplete adjuvant. Ten days after the sixth injection, blood was obtained from a marginal ear vein. Additional booster injections were administered at one monthly intervals with 0.5 mg of protein. The serum was collected and stored in 0.5 ml aliquots at -20°C until required.

4.3 RESULTS

4.3.1 SDS-PAGE analysis of the isolated phosphate transport protein

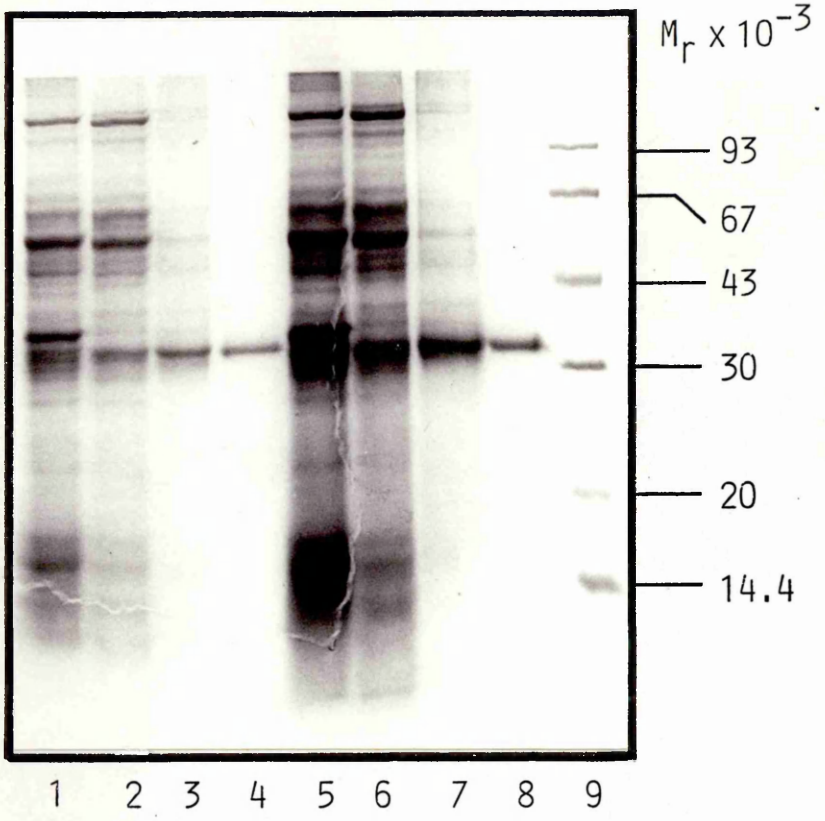
The purification procedure is detailed in Section 2.2.10. The Coomassie-Blue staining profiles of different stages of the purification of both rat liver and bovine heart mitochondrial protein is illustrated in Figure 4.1. Following analysis by SDS-PAGE a single apparently homogeneous band is evident after chromatography on Celite. The inclusion of a 0.5% (w/v) Triton X-100 pre-extraction by Gibb *et al.* (1986) successfully removes approximately 90% of mitochondrial proteins (Lane 1, Fig. 4.1a,b). This and the subsequent 8% (w/v) Triton X-100 extraction removes many of the contaminating proteins, enriching the sample in the PTP. By subjecting the 8% (w/v) Triton X-100 extract to batch chromatography on hydroxlapatite, it can be seen that most proteins remain bound to the column matrix whilst the PTP and a few other proteins of similar M_r value are present in the eluate. The supernatant from this stage in the purification was applied to a Celite column and resulted in the adsorption of all other contaminating bands.

Fig. 4.1: SDS-PAGE Analysis of Fractions from the Purification of the PTP

Fractions from the various stages of the PTP purification were subjected to polyacrylamide gel electrophoresis on a 10% (w/v) SDS-polyacrylamide slab gel. The proteins were detected by staining with Coomassie Blue. (A) purification from rat liver mitochondria; (B) purification from beef heart mitochondria.

Lane (1)	0.5% (w/v) Triton X-100 pellet	40 µg
(2)	8% (w/v) Triton X-100 extract	40 µg
(3)	HTP fraction	20 µg
(4)	Celite eluate	20 µg
(5)	0.5% (w/v) Triton X-100 pellet	40 µg
(6)	8% (w/v) Triton X-100 extract	40 µg
(7)	HTP eluate	20 µg
(8)	Celite eluate	20 µg
(9)	Low M_r standard proteins.	

(a)


$M_r \times 10^{-3}$
93
67
43
30
20
14.4
1 2 3 4 5 6 7 8 9

(b)

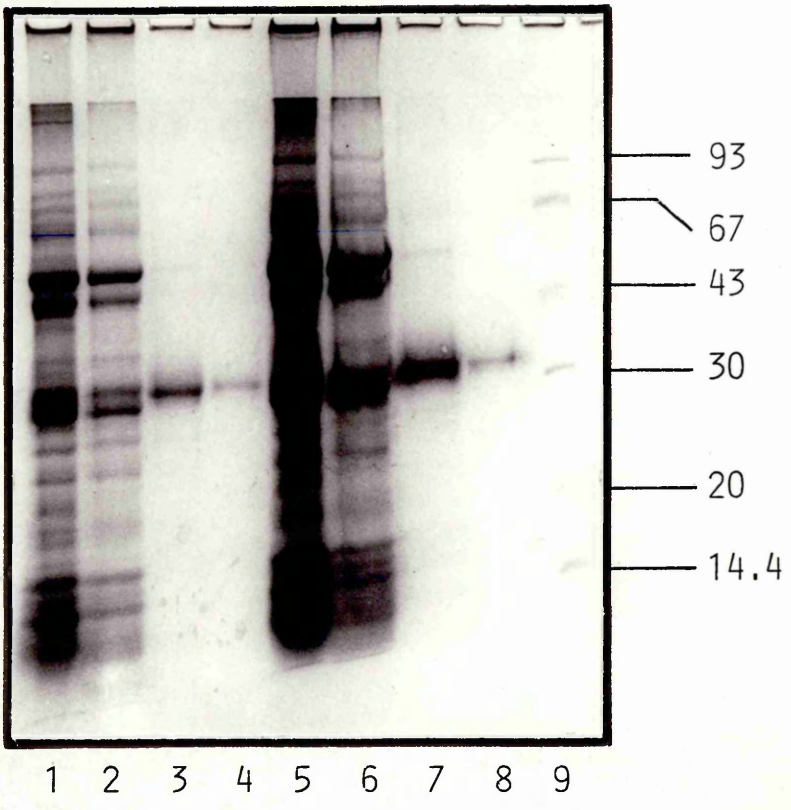
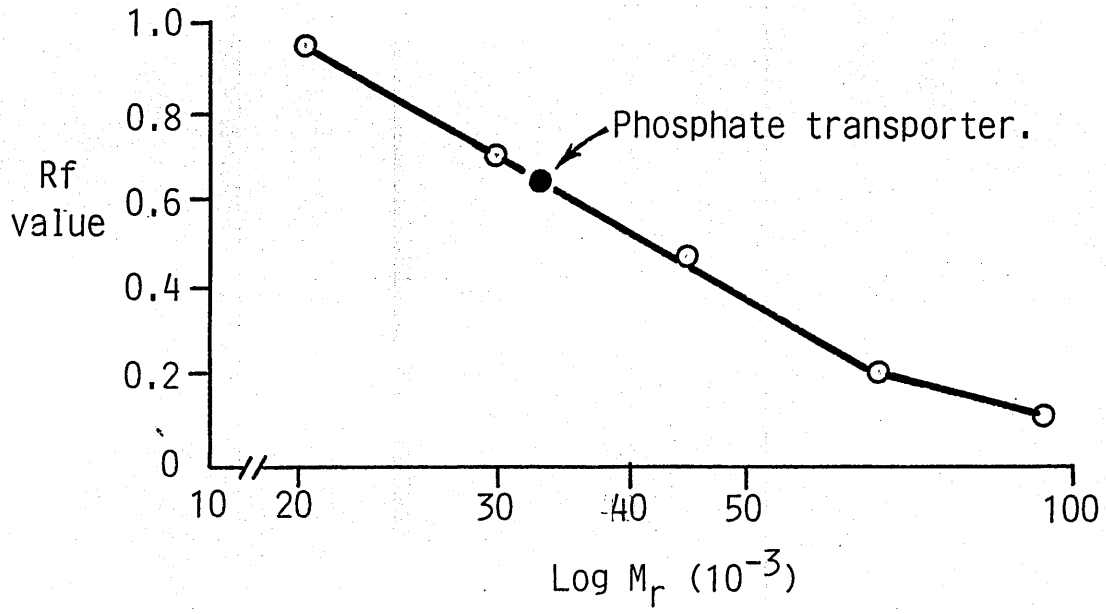

93
67
43
30
20
14.4
1 2 3 4 5 6 7 8 9

Fig. 4.2
Standard curve of electrophoretic mobility of marker proteins versus log subunit M_r value.



To estimate the M_r value of the phosphate transport protein on an SDS-polyacrylamide gel (10% W/V) its relative mobility (Rf) was compared to that of a set of standard proteins (section 2.2.3d).

PTP was purified from bovine heart mitochondria

Figure 4.2 shows the standard curve used to determine the M_r value of the purified protein. This was determined by calculating the relative mobility of the PTP in comparison to a set of standard marker proteins of known M_r value. The value obtained, i.e. of 34 000 M_r, is in agreement with that of other workers (Gibb et al., 1986; Kolbe et al., 1981; and Wohlrab, 1980).

Densitometric scanning of the final stage of this preparation following SDS-PAGE indicated the protein to be of greater than 95% purity. This was then considered as a homogeneous preparation since any other contaminants were less than 1% of the preparation and not visible following staining with Coomassie Blue.

4.3.2 Native M_r determination of the PTP using gel filtration

Approximately 200 µg of the final Celite eluate from the PTP purification scheme was applied to a Superose 12 column after being passed through a Millex filter. The column had been pre-equilibriated in 20 mM Tris-HCl, pH 7.4 containing 0.5% (v/v) Triton X-100 and calibrated using standard M_r proteins (Section 2.2.4) which had been prepared in the above buffer. Figure 4.4 illustrates the trace obtained following gel filtration of the purified PTP.

The M_r value of the PTP was estimated relative to the elution volume of standard M_r proteins. Fractions corresponding to the observed peak were pooled and identified as the PTP by immunoblot analysis using anti-PTP-serum (Fig. 4.5).

The lower M_r value band observed was thought to be a degradation product of the 34 000 M_r polypeptide since immunoblot analysis prior to gel filtration failed to detect any other bands.

It has been suggested previously (Kolbe et al., 1982) that, like its sister protein, the adenine nucleotide translocase, PTP would exist as a dimer in its functional state. This study on M_r determination of the native protein and also cross-linking data, using both dimethyl suberimidate and phenylene dimaleimide under conditions which crosslink control proteins (aldolase and PDC) have failed to provide any evidence to support this theory (Fig. 4.6). Indeed all of our available information suggests that this protein exists as a 34 000 M_r monomer. Whether this subunit exists as a component of a multimeric transport system or as an integral functional unit remains to be seen.

4.4 DISCUSSION

The purified rat liver and beef heart mitochondrial phosphate transport proteins were subjected to SDS-PAGE and their Coomassie Blue staining profiles compared. In each case one major polypeptide of M_r 34 000 was observed following chromatography on Celite. In this and other preparations this final stage in the purification successfully removes a 31 000 M_r band which is the major contaminant following chromatography on hydroxlapatite. Wohlrab (1980) demonstrated this latter result when purifying the carrier protein from

Fig.4.3 Densitometric scan of the Coomassie Blue profile of the Celite fraction of purified PTP from bovine heart.

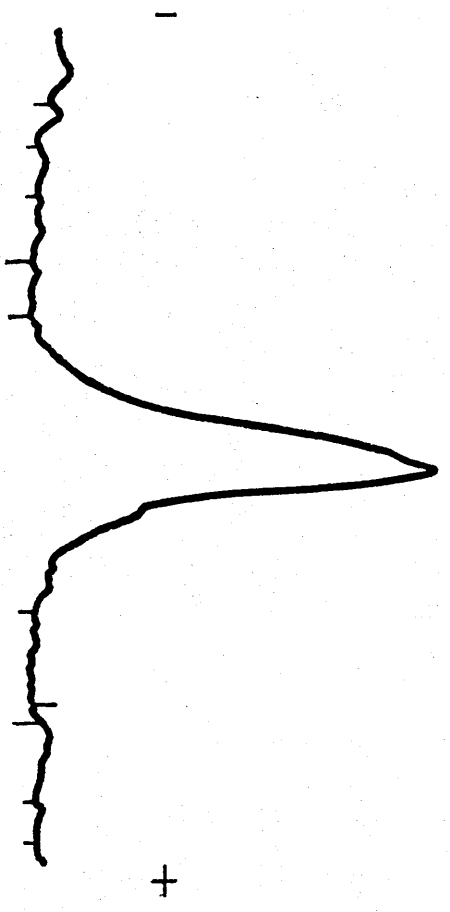


Fig. 4.4: Gel Filtration of Purified PTP Using the FPLC System and Superose 12 Column

PTP, purified as described in Section 2.2.10, was applied to a Superose 12 column. The flow rate was set at 0.5 ml/min and the column was pre-equilibriated in 20 mM Tris-HCl, pH 7.4 containing 0.5% (v/v) Triton X-100. 1.0 ml fractions were collected and those corresponding to the peak were pooled and precipitated in 4 volumes of ice-cold acetone. The resultant pellet was resuspended in 50 µl of Laemmli sample buffer containing 10 mM DTT in preparation for SDS-PAGE.

PTP was purified from bovine heart mitochondria.

The second peak represents the absorbance, at 280 nm, due to Triton X-100

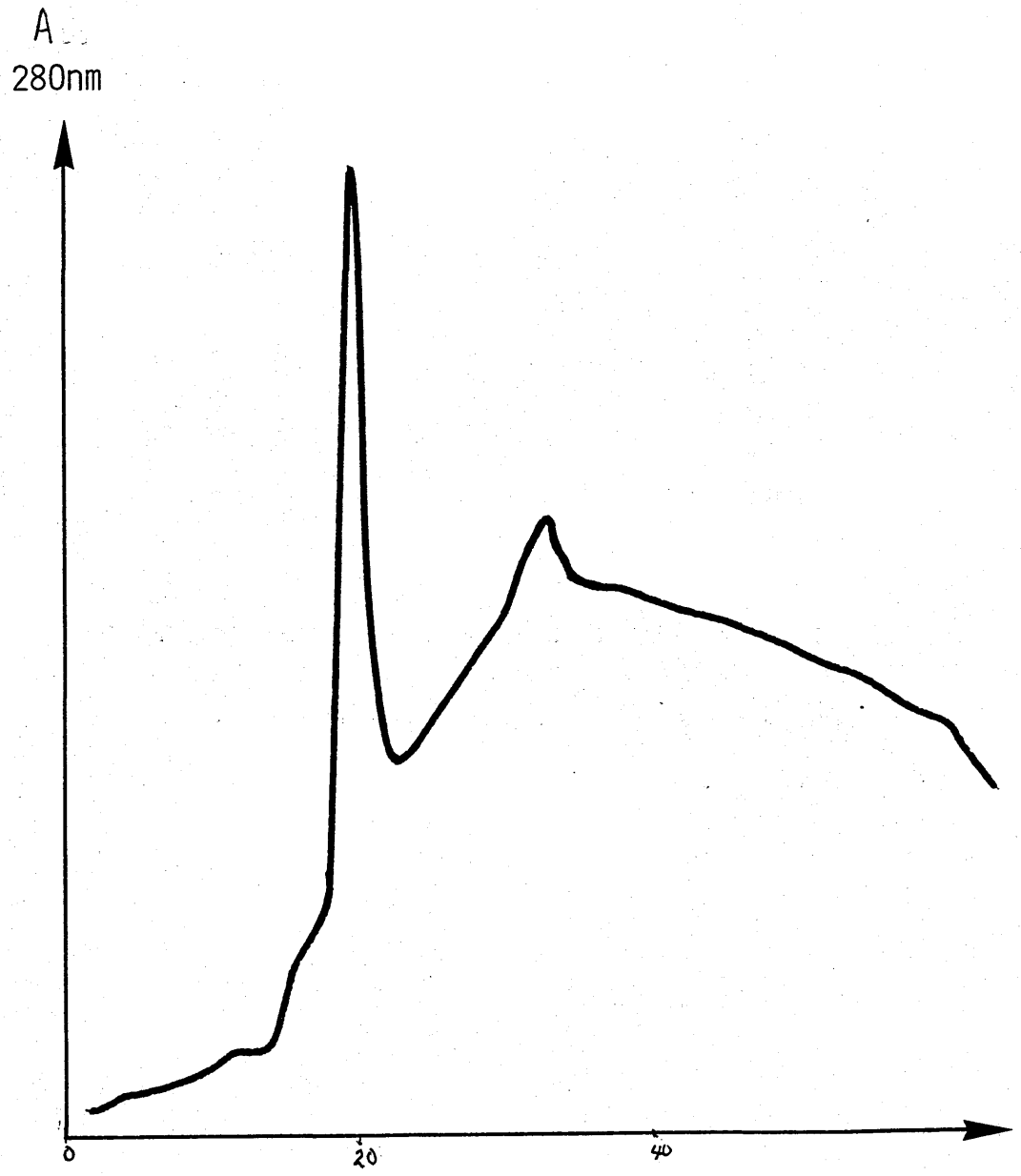

A
280nm
0
20
40
Volume (ml)

Table 4.1: Native M_r Determination of the PTP by Gel Filtration

Sample	$M_r \times 10^{-3}$	Elution vol. (mm)
BSA	67	84
Ovalbumin	44	90
α Chymotrypsinogen	21	140
Cytochrome c	12.5	152
BH PTP	34	96

Standard M_r proteins of known M_r value were applied to the Superose 12 column, as described in Section 4.3.2. The elution volume was measured as the mean value of at least three sample applications to the column.

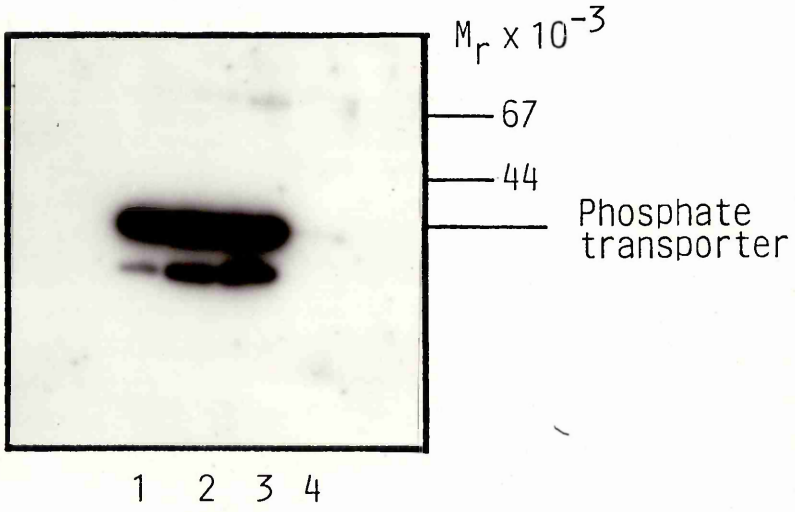
the same tissue and identified the contaminating protein as the adenine nucleotide translocase. In marked contrast to the findings of Kolbe et al., (1985) we find no evidence for the existence of any other band following chromatography on Celite. Alkylation of these bands, resolved by Kolbe et al., (1985), putatively designated α and β, caused them to co-migrate with an M_r value of 34 000 whilst peptide mapping failed to detect any differences. These results suggest that the apparent subunit composition may be artefactual and arise from intramolecular disulphide bond formation during electrophoresis and that PTP consists of one 34 000 M_r polypeptide.

Densitometric scanning of the Coomassie Blue profile (Fig. 4.3) allowed confirmation of the purity of the isolated carrier and estimated it to be greater than 95% pure. These preparations were used to mount an immune response. The protein was identified as the PTP as a result of its sensitivity to NEM (data not shown) and the M_r determination following purification of the protein by the method of Gibb et al. (1986). The antiserum was also shown to cross-react with the equivalent antigen from bovine heart mitochondria.

The high selectivity and sensitivity of the antiserum was monitored by immunoblotting against the purified protein and against whole cell and mitochondrial extracts. Transfer of proteins to nitrocellulose has several advantages: filters are easier to manage, the same replica can be used

Fig. 4.5: Immune Blot Analysis of the Fractions Corresponding to the Peak Obtained from Gel Filtration Studies

Fractions corresponding to the $A_{280\ nm}$ profile (Fig. 4.4) were pooled and precipitated in 4 volumes of ice-cold acetone. The resultant pellet was prepared for SDS-PAGE and analysed using the immune replica technique, probing with anti-PTP-serum. Lanes (1,2,3) 5 µl, 10 µl and 20 µl respectively of the resultant pellet. Lane 4, ^{125}I-labelled low M_r standard proteins.


$M_r \times 10^{-3}$
67
44
Phosphate transporter
1 2 3 4

for successive investigations, the proteins are readily accessible and therefore processing times and the amount of reagents used are reduced. One major disadvantage though, is that the elution of proteins from the gel is dependent on M_r value. However, the inclusion of 0.02% (w/v) SDS in the original transfer buffer of Towbin *et al.* (1979) facilitates the elution of higher M_r proteins.

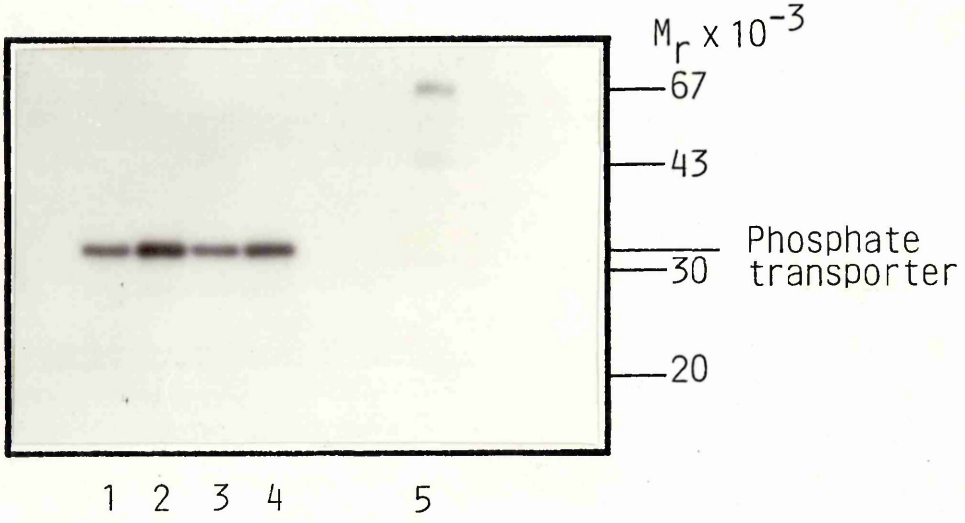
Following transfer of proteins to nitrocellulose, unbound sites on the paper must be quenched prior to the incubation with antiserum. Originally, this was achieved by supplementing the wash buffer with 3% (w/v) BSA, as described by Towbin *et al.* (1979). Improved backgrounds and specificity of interaction were achieved by substituting the non-ionic detergent Tween 20 as a blocking agent (Batteiger *et al.*, 1982). An additional advantage of this system is that the efficiency of electrophoretic transfer would be determined by staining for proteins directly on the blot. Comparison of the resultant autoradiogram with the stained blot from which it was derived allows accurate identification of the immunoreactive protein(s).

The highly sensitive technique of immunoblotting using samples prepared in Laemmli sample buffer plus 5% (v/v) mercaptoethanol revealed the presence of two non-protein bands (M_r 54 000 and 65 000). These artefactual bands have been reported previously (Tasheva and Dasser, 1983) and in contrast to this report, substituting DTT as the reducing agent removed this artefact.

Fig. 4.6: Crosslinking Studies on the PTP using Phenylene Dimaleimide (PDM)

Purified bovine heart PTP was incubated with a final concentration of 100 μm PDM. The reaction was allowed to proceed for 30 min. at room temperature before being stopped by precipitating the protein in 4 vol. of acetone. The resultant pellets were dried under air and boiled in Laemmli sample buffer containing 10 mM DTT, before being resolved by SDS-PAGE.

Lane 1, PTP incubated with 100 μM PDM, 5 μg; lane 2, as (1); 10 μg; lane 3, untreated PTP 5 μg; lane 4, untreated PTP, 10 μg; lane 5, ^{125}I-labelled low M_r standard proteins.


$M_r \times 10^{-3}$
67
43
Phosphate transporter
30
20
1 2 3 4 5

This method revealed that the antiserum directed against the rat liver phosphate transport protein cross-reacts very strongly with not only the parent antigen but also the carrier protein from bovine heart mitochondria (Fig. 4.7). It can also cross-react with the corresponding protein from porcine kidney, bovine kidney and rat liver cells (Fig. 4.8). It was not established whether the weaker cross-reaction observed against cells derived from the bovine kidney tissue reflected a genuine immunological difference or simply a lower abundance of the enzyme in this tissue since mitochondria from various tissues are known to differ significantly in their anion transporting capacities (Babcock *et al.*, 1983). On the basis of the cross-reactivity of the antiserum raised against the rat liver phosphate transport protein with the carrier from different tissues, it can be argued that the primary protein sequence must be highly conserved in certain regions and that this must result in the preservation of certain structural epitopes.

Despite attempts to ensure that only homogeneous protein preparations were used in the production of antiserum, Figures 4.7 and 4.8 demonstrate that this antiserum also cross-reacts with a 31 000 M_r polypeptide, tentatively identified as the adenine nucleotide translocase (ANT). It should be noted, however, that this cross-reaction is evident only in the hydroxlapatite fraction and is removed or only weakly visible following chromatography of this sample on Celite.

Fig. 4.7: Crossreaction of Anti-PTP-serum with Fractions from the Purification Procedure

Fractions were withdrawn at various stages of the purification of the phosphate transport protein and subjected to SDS-PAGE (10%(w/v)). The proteins were transferred to nitrocellulose and processed for detection of antigen using anti-PTP-serum. (Lanes 1-5; rat liver; lanes 6-8, bovine heart).

(1) intact mitochondria, 40 µg; (2) 0.5% (w/v) Triton X-100 pellet, 20 µg; (3) 8% (w/v) Triton X-100 extract, 20 µg; (4) HTP eluate, 10 µg; (5) Celite eluate, 10 µg; (6) 8% (w/v) Triton X-100 extract, 20 µg; (7) HTP eluate, 20 µg; (8) Celite eluate, 10 µg; (9) ^{125}I-labelled low M_r standard proteins.

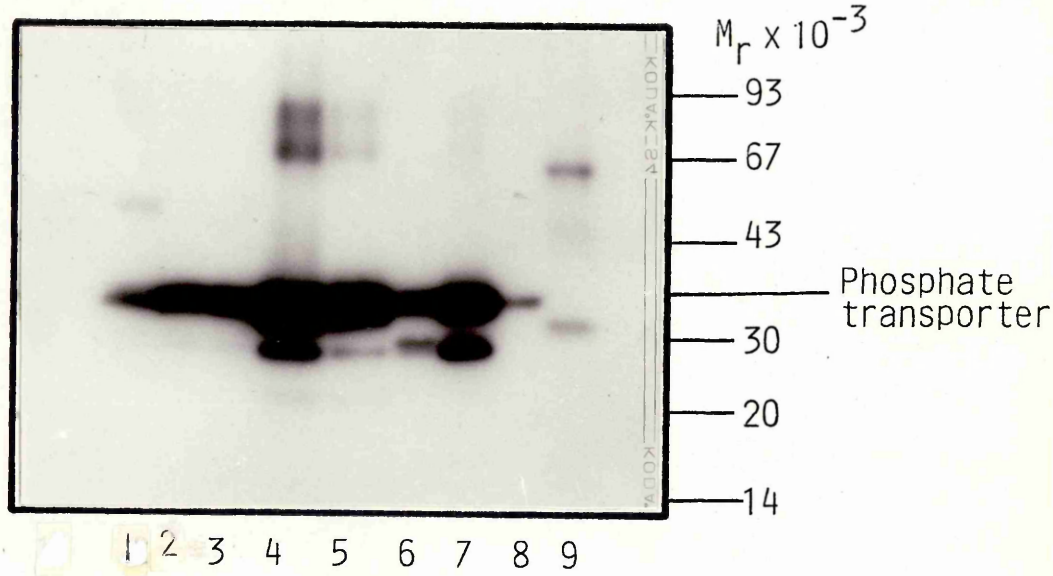

M_r x 10^{-3}
93
67
43
Phosphate transporter
30
20
14
1 2 3 4 5 6 7 8 9

Fig. 4.8: Immune Blot Analysis of Cultured Mammalian Cell Extracts Using Anti-PTP-serum.

Crude cell extracts and mitochondrial fractions from cultured mammalian cells were resolved in SDS-polyacrylamide gels (10% (w/v)) prior to immune blot analysis.

The autoradiographic profile of the following samples is illustrated:

Lane (1) BRL cell extract 80 µg

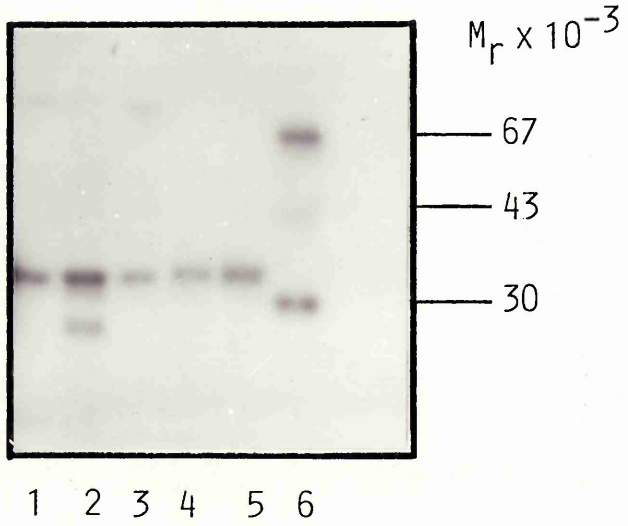
(2) BRL mitochondria 40 µg

(3) NBL cell extract 80 µg

(4) NBL mitochondria 40 µg

(5) PK 15 mitochondria 40 µg

(6) ^{125}I-labelled low M_r standard proteins.


$M_r \times 10^{-3}$
67
43
30
1 2 3 4 5 6

There are therefore two possible explanations for this cross-reactivity:

(i) minute amounts (< 2%), which are not detectable by Coomassie Blue staining, of the contaminating protein were present in the final Celite eluate used for injection of the rabbit. This subsequently resulted in raising a titre to not only the PTP but also to the protein identified as the ANT.

or (ii) the antiserum reacts with epitopes on the ANT which are in common with those on the PTP.

Evidence to date favours the second hypothesis. At the superficial level, the observation that the ANT copurifies with the PTP up to chromatography on Celite suggests that these proteins have similar properties. The poor affinity of both these proteins towards hydroxlapatite has been attributed to the extensive hydrophobic areas on the periphery of these integral membrane proteins (Klingenberg *et al.*, 1978) which probably still have lipid bound but are otherwise covered by detergent molecules. Both these proteins also possess phosphate binding sites. Kolbe and Wohlrab (1985) have established that homologies do exist in the primary structure of the ANT and the PTP which are similar to those preserved in evolution between the ANT from beef heart and in *N. crassa*. In a more recent publication (Runswick *et al.*, 1987) the amino acid sequence of the phosphate carrier has been compared with both the translocase and the uncoupling protein from brown fat mitochondria. This analysis demonstrates that all three

proteins contain a three-fold repeated sequence, approximately 100 amino acids in length and that strong homologies exist between these repeats. This result suggests that the three proteins have related 3 dimensional structures and mechanisms. It may also imply that the three proteins share a common evolutionary origin. The importance of three dimensional structure in determining the immunoreactivity of a protein has been noted previously (Westhof et al., 1984).

This would then suggest that the cross-reaction observed most probably reflects the common structural epitope(s) existing between the two proteins. This argument is strengthened by the observation that the cross-reaction is more apparent in the hydroxylapatite eluate which still contains the ANT fraction. Chromatography on Celite removes this contaminating polypeptide and no (or a very weak) cross-reaction with a 31 000 M_r is observed.

This chapter describes the purification of the PTP and raising and characterisation of antiserum directed against this protein. On the basis of the strong cross-reactivity observed with the parent antigen and the equivalent antigen from different sources biosynthetic and topographical analysis were initiated.

CHAPTER 5

BIOSYNTHETIC AND TOPOGRAPHICAL STUDIES ON THE PHOSPHATE TRANSPORT PROTEIN

5.1 INTRODUCTION

ATP is synthesised from ADP and inorganic phosphate by oxidative phosphorylation in mitochondria. The supply of substrates in the mitochondrial matrix is maintained by two proteins in the inner membrane, the adenine nucleotide translocase (ANT) and the phosphate transport protein.

Although the ANT and PTP are distinct proteins (Section 1.3), they possess several structural and mechanistic similarities. Both are proteins catalysing a transmembrane activity and each carrier possesses a phosphate binding site. Both proteins are solubilised by the non-ionic detergent, Triton X-100, and in each case the major purification step involves adsorption chromatography on hydroxylapatite (Wohlrab, 1980). The subunit monomer of the translocase has an M_r value of 30 000, with two monomers in dimeric form constituting the functional carrier unit (Hackenberg and Klingenberg, 1980). The subunit M_r value of PTP is 34 000, and although a dimeric functional unit has been suggested (Kolbe *et al.*, 1982), no compelling experimental evidence exists for this.

One aspect of this study is concerned with mitochondrial biogenesis and in particular with the biosynthesis of the nuclear encoded PTP. Only two other transmembrane activities have been studied with respect to mitochondrial biogenesis, one is the PTP's sister protein, the ANT and the other is the uncoupler protein from brown fat mitochondria. Early

studies on the biogenesis of the ADP/ATP translocator, provided the first demonstration that mitochondrial precursor pools were present in the cytoplasm (Hallermeyer et al., 1977). Subsequent analysis revealed that the soluble precursor form of the translocator is not synthesised with a transient N-terminal sequence which is characteristic of most other mitochondrially imported proteins (Hay et al., 1984). The precursor was shown to exist in a different conformation to that of the mature form (Zimmerman and Neupert, 1980) and it has since been established (Adrian et al., 1986) that the information required for the in vivo targeting of the Saccharomyces cerevisiae ANT is determined by residues within the first 115 amino acids.

This chapter is concerned with further characterisation of the PTP and attempts to use specific antibodies directed against this protein to study both the biosynthesis and final membrane organisation of this carrier protein.

When immunological detection of mitochondrial polypeptides is performed using an in vitro translation system primed with poly A^+ RNA, the major translation products are the precursor forms of the polypeptides. In vivo studies are hindered by the rapid import and processing of cytoplasmic pools of precursor molecules. By employing uncouplers of oxidative phosphorylation to dissipate the transmembrane gradient required for the import of proteins, these precursor molecules can accumulate in the cytoplasm, given that these molecules are relatively stable.

High titre monospecific antiserahave been a key tool in studying mitochondrial biogenesis. The criteria for initiating such studies are that the antibodies must exhibit a cross-reactivity with the equivalent polypeptide from different sources and be capable of recognising the precursor form of the antigen. This latter condition does not always hold in that the antibody to apocytochrome C fails to recognise the holocytochrome and vice-versa (Hennig and Neupert, 1981). In addition, it was apparent that pre-E2 was not recognised to any significant extent by antibody to native OGDC (Hunter, 1985).

Preliminary studies carried out in yeast cells grown in the presence of uncouplers of oxidative phosphorylation (Gibb, 1985) suggested that, like the ANT, the yeast PTP is synthesised with the same M_r value as the mature protein. Thus as our antiserum (Chapter 4) fulfilled the above criteria, an <u>in vivo</u> approach was chosen to extend these initial observations and to study the biosynthesis of PTP in mammalian tissue culture cells.

The second aspect of this chapter is concerned with the final organisation of this polypeptide in the inner mitochondrial membrane.

To date, very little information is available on the orientation of the PTP within this membrane. Topographical studies, and as a consequence of these, an understanding of the transmembrane activity of ANT, have been facilitated by the existence of two inhibitors which are known to bind to the translocase in an asymmetric fashion (carboxyatractyl-oside at the cytoplasmic face and bongkrekate at the matrix

surface). Also the complete amino acid sequence is known (Aquila et al., 1982) and reveals that the monomeric protein contains 4 cysteines in 297 amino acids. Masking or chemical modification of sulphydryl groups causes inhibition of the translocase activity.

In contrast to the ANT, very little information is available on the membrane organisation of the PTP. This, as discussed in 1.3, is partly due to the lack of specific inhibitors and also the low concentration of the protein within the membrane (ANT constitutes approximately 10% of total protein of the mitochondrial inner membrane).

Studies have been undertaken to further characterise the reactive sulphydryl groups essential for the activity of both proteins (Houstek and Pedersen, 1985) and to extend these observations to the catalytic mechanism. Maleimides have proven particularly useful in this respect. As their reaction with sulphydryl groups of proteins is not readily reversed, proteins containing these groups can be radioactively labelled and monitored by gel electrophoresis. Unfortunately, the most commonly used radioactive maleimide, NEM, is highly permeable (Fonyo, 1976). Analogues of low permeability, like carboxylated maleimides (Griffiths et al., 1981) or N-acetyl-4-sulphamoylphenylmaleimides (Vignais et al., 1975) are either not available commercially in radioactive form, or they are not completely impermeable.

Utilising the highly charged fluorescent probe, eosin-5-maleimide, which circumvents some of these problems, Houstek and Pedersen (1985) have suggested that the reactive

sulphydryl groups of the PTP are available only at the cytoplasmic surface of the inner mitochondrial membrane. In contrast to the analogous study on the ANT, this group has been shown to be reactive with NEM, and therefore essential to the catalytic function. These workers were unable to detect any reactive sulphydryls at the matrix surface of the inner mitochondrial membrane.

Whilst this study provides a new insight into the relative locations of the sulphydryl groups of both the ANT and PTP, it does not allude to the topographical organisation of either protein within the membrane structure. Indeed, the extent of fluorescence may reflect a combination of a) the abundance and b) the reactivity of each protein within the membrane. Although PTP possesses some of the most reactive sulphydryl groups in the mitochondrial inner membrane, maleimides, as discussed in 1.3, have a broad specificity. Thus, interpreting fluorescent labelling patterns may prove rather difficult due to a combination of these factors.

In this chapter the orientation of PTP in the inner mitochondrial membrane towards the cytoplasm or the matrix was investigated by proteolytic digestion of mitoplasts and submitochondrial particles. With the availability of high quality antiserum directed against the rat liver enzyme, the opportunity existed to investigate the topography of this carrier protein using an approach that had not been used in this context i.e. analysis of the protease digested mitoplasts or SMP's by immunoblotting to detect

membrane-bound protease-resistant fragments.

5.2 METHODS

5.2.1 Protease treatment of bovine heart submitochondrial particles

The incubation of bovine submitochondrial particles (SMP) (16 mg/ml), prepared as described in Section 2.2.9b, with a variety of proteases was performed at 30°C in 50 mM potassium phosphate buffer, pH 7.4. The protease was added, to a final concentration of 2% (w/w), to start the reaction. Control incubations were carried out in the absence of proteases. Samples (1 mg) were withdrawn at various time points, added immediately to 7 µl of 15 mM PMSF or 0.8 µg/µl leupeptin (or specific protease inhibitors where appropriate). The sample was immediately placed on ice before being diluted seven-fold with ice-cold phosphate buffer. The membrane fraction was collected by centrifugation (26 000 x g for 20 min.) and resuspended in phosphate buffer containing PMSF and 0.15M NaCl. This wash procedure was repeated twice before the samples were washed with distilled water (x 2). The final pellet was resuspended in Laemmli sample buffer containing 10 mM DTT and boiled for 3 min. IAA was then added to a final concentration of 100 mM. Samples were subsequently analysed by SDS-PAGE and immunoblotting with the appropriate antiserum. Detection of the antigen was by autoradiography.

An identical protocol was followed for protease digestion of mitoplasts. In these instances the incubation and wash buffers were supplemented with 0.25M sucrose.

5.3 RESULTS

5.3.1 Immunoprecipitation of PTP from mammalian tissue culture cells

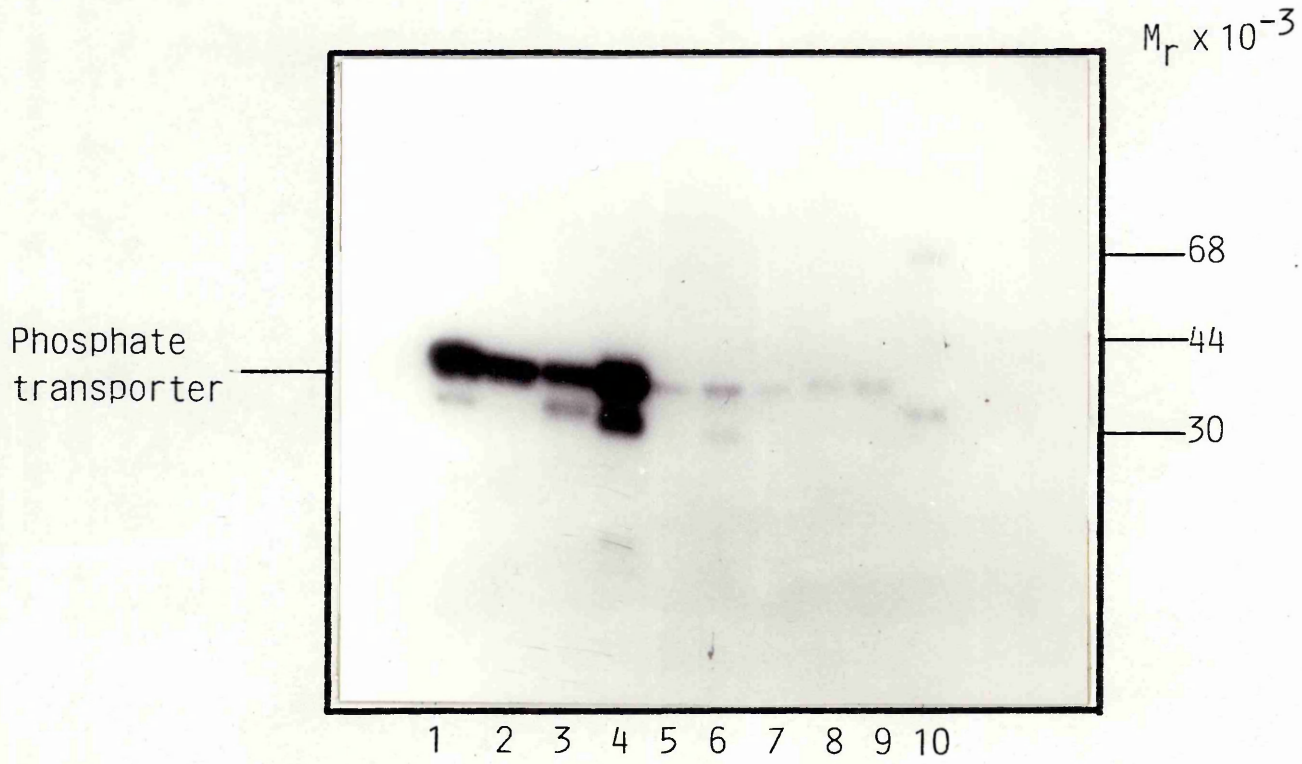
Figure 5.1 shows the cross-reactivity of the antiserum directed against the rat liver phosphate transport protein. As discussed in Chapter 4, this antiserum recognises the parent antigen in extracts of cultured cells with a high degree of specificity, a requirement for successful *in vivo* studies. It also cross-reacts, however, with a lower M_r polypeptide, tentatively identified as the adenine nucleotide translocase. As the ANT is not made as a larger precursor and being of lower molecular weight, it was judged not to interfere with the proposed study. Research on biosynthesis, translocation and membrane insertion of this carrier was initiated using the BRL cell line.

Cultured BRL cells were incubated with $[^{35}S]$ -methionine either overnight in the absence of uncouplers or for 4 h in their presence, to accumulate precursor molecules of nuclear encoded proteins. Radiolabelled cell lysates were prepared (Section 2.2.8b) and after incubating an aliquot of this lysate with specific antisera, the immunoprecipitated $[^{35}S]$ methionine-labelled polypeptides were analysed by SDS-PAGE and fluorography. After treatment of this lysate with anti-E1α-serum, from PDC, one major band of M_r value 42 000 was observed (Fig. 5.2, lane 2). Since the band was absent from the corresponding control incubation (lane 1) and was of the correct M_r value for E1α, the polypeptide confirmed the specificity of the antiserum. In the presence of 2 mM DNP

Fig. 5.1: Immunological Detection of PTP in Cultured Mammalian Cell Subfractions and from PTP Purification Fractions

Post-nuclear supernatant and mitochondrial fractions were prepared from cultured BRL, PK-15 and NBL-1 cells (Section 2.2.8) and electrophoresised on 10% (w/v) SDS-polyacrylamide slab gels, before being processed for detection of immunoreactive polypeptides using anti-PTP-serum.

Lane 1	Rat liver mitochondrial HTP eluate	(20 µg)
2	Rat liver mitochondrial Celite eluate	(20 µg)
3	Bovine heart mitochondrial 8% (w/v) Triton X-100 extract	(40 µg)
4	Bovine heart mitochondrial HTP eluate	(20 µg)
5	BRL cell lysate	(40 µg)
6	BRL mitochondria	(40 µg)
7	NBL-1 cell lysate	(40 µg)
8	NBL-1 mitochondria	(40 µg)
9	PK-15 cell lysate	(40 µg)
10	^{125}I-labelled low M_r standards	

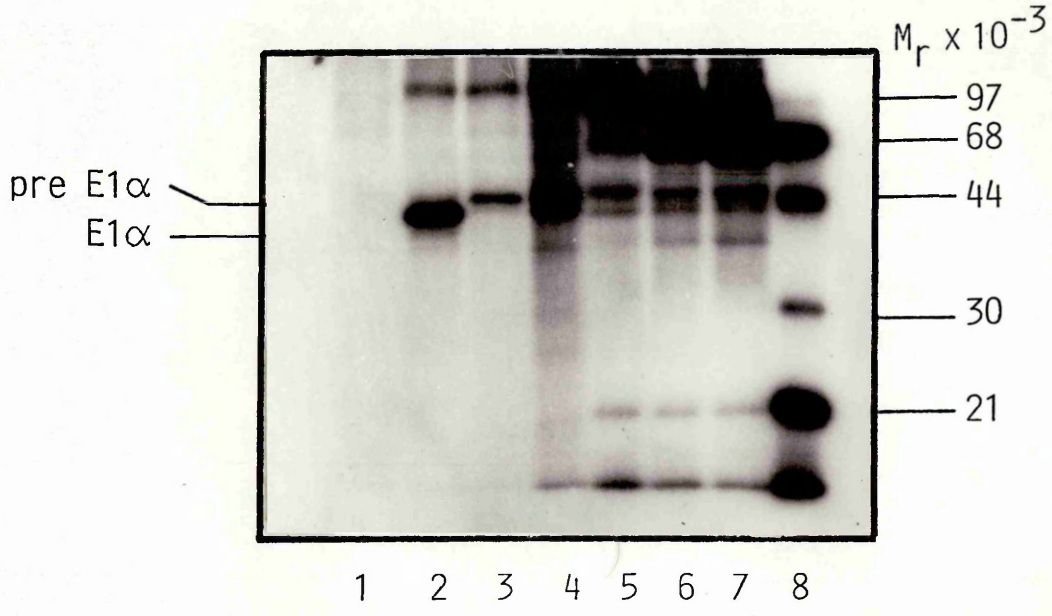

$M_r \times 10^{-3}$
68
44
30
Phosphate transporter
1 2 3 4 5 6 7 8 9 10

or 10 μM FCCP (lanes 3 and 4, respectively) newly synthesised (precursor) polypeptides were precipitated exhibiting M_r values 2-3000 greater than that of the mature subunit. Indeed, in the presence of 10 μM FCCP, processing of the precursor polypeptides to the mature counterpart is only partially inhibited as some conversion to the 42 000 M_r polypeptide can still be detected.

When the analogous incubations were performed with anti-PTP-serum, the major radiolabelled products (in the absence of uncouplers) exhibited an M_r value in excess of 97 000. These polypeptides were specifically immunoprecipitated by the anti-PTP-serum, as they were neither present in the control track (lane 1) or in the precipitation with anti-Elα-serum. The immunoprecipitates from cells radiolabelled in the presence of uncouplers, again, resulted in [^{35}S]-methionine-labelled high M_r polypeptides unique to this antiserum. The 34 000 M_r polypeptide demonstrated in crude cell extracts to cross-react with anti PTP-serum was not detected in the resultant fluorographs. Immune replica analysis of cell lysates prepared in the buffer system used for immunoprecipitation identified the PTP and discounted the possibility that the protein aggregated to a high M_r form under these conditions. To check that this discrepancy was not a characteristic of the BRL cell line, the corresponding experiments were performed in [^{35}S]-methionine labelled cell extracts from cultured pig kidney cells. The resultant fluorograph is shown in Figure 5.3.

Fig. 5.2: Immune Precipitation from BRL Cells Labelled with [^{35}S]-methionine in the Presence and Absence of Uncouplers

BRL cells were incubated either overnight with [^{35}S]-methionine (150 - 250 µCi/dish) in the absence of uncouplers or labelled for 4 h in the presence of uncouplers. After preparation of [^{35}S]-methionine labelled extracts (Section 2.2.8) indirect immunoprecipitation was performed using various antisera in conjunction with fixed S. aureus cells (Section 2.2.11c). The resultant immunoprecipitates were resolved on 10% (w/v) SDS-polyacrylamide gels and identification of radiolabelled bands was by fluorography. Lane 1, overnight label, with non-immune rabbit serum; lane 2, overnight label, anti-Elα-serum; lanes 3,4, 4 h label in the presence of 2 mM DNP or 10 µM FCCp respectively with anti-Elα-serum; lane 5, overnight label, anti-PTP-serum; lanes 6,7, 4 h label in the presence of 2 mM DNP or 10 µM FCCP respectively, anti-PTP-serum; Lane 8, ^{125}I-labelled low M_r standard proteins.


$M_r \times 10^{-3}$
97
68
44
30
21
pre E1α
E1α
1 2 3 4 5 6 7 8

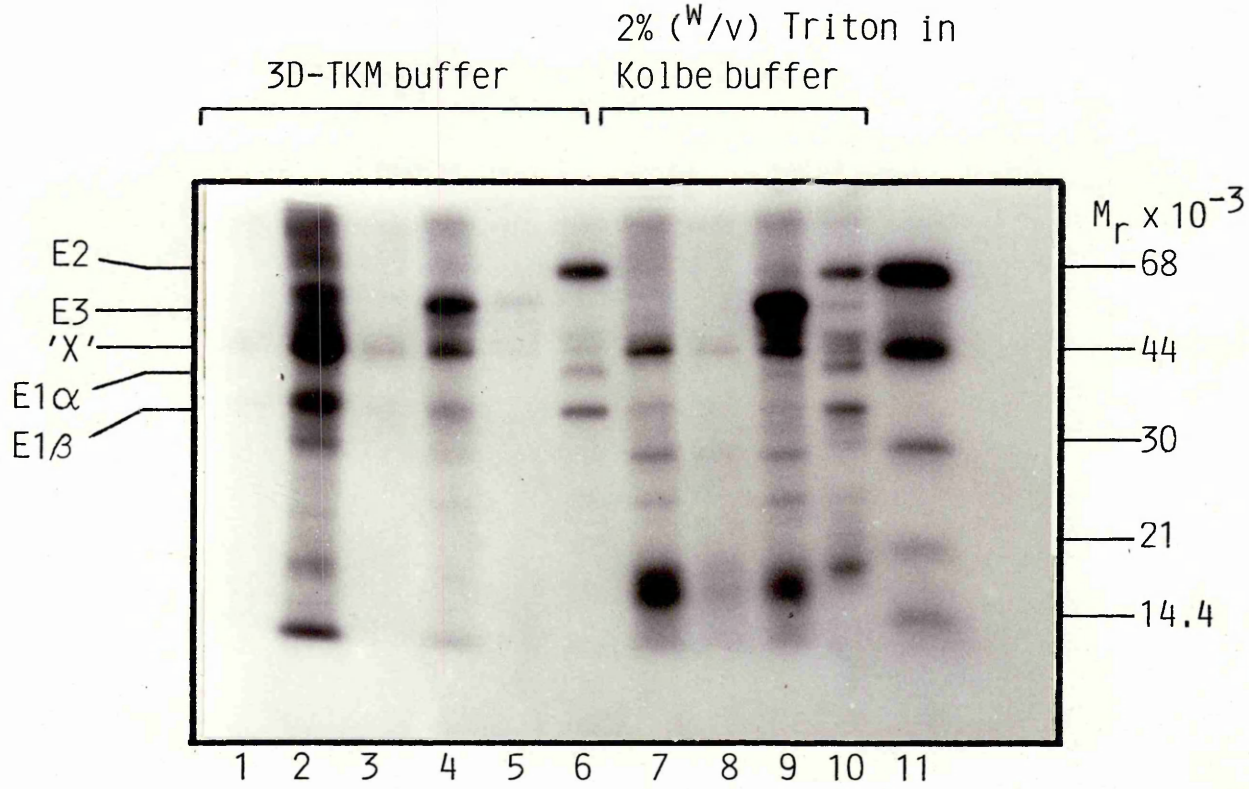
Lanes (1-6) show the immunoprecipitates from cell lysates prepared in 3D-TKM buffer (Section 2.2.8a). In this instance, using antiserum directed against PTP (lane 2), higher M_r radiolabelled bands can be detected which are neither present in the incubation with control serum (lane 1), nor with anti-E3-serum (lane 4) or anti-PDC-serum (lane 6). Each of these incubations successfully precipitates the parent antigen. An interesting result in the precipitation with anti-PDC-serum is that it fails to recognise the corresponding E3 subunit (lane 6). This has been shown to be indicative of the low immunogenecity of the E3 polypeptide, which is common to all three 2-oxo acid dehydrogenases (de Marcucci et al., 1985).

Lanes(7-10)of this figure result from incubations performed from cell lysates prepared in Kolbe Buffer (Section 2.2.10) containing 2% (w/v) Triton X-100 and 0.15M NaCl. This buffer was chosen as it is known to release PTP from rat liver mitochondria; the inclusion of 0.15M NaCl was shown to reduce the non-specific background binding that previously predominated (data not shown). This experiment was performed to exclude the possibility that the PTP has not been solubilised in the chosen detergent system.

Lane 9 shows the resulting immunoprecipitation with anti-E3-serum and demonstrates the expected radiolabelled polypeptide of 55 000 M_r. Lane 10 exhibits bands corresponding to the subunits of PDC. Both these antigens are solubilised, therefore, by the 2% (w/v) Triton X-100 Kolbe Buffer and

Fig. 5.3: Immune Precipitation from PK-15 cells Labelled with [^{35}S]-methionine in the Presence and Absence of Uncouplers

PK 15 cells were incubated either overnight with [^{35}S]-methionine (150 - 250 µCi/dish) in the absence of uncouplers or labelled for 4 h in the presence of uncouplers. After the preparation of [^{35}S]-methionine-labelled extracts (Section 2.2.11c), indirect immunoprecipitation using various antiserum in conjunction with formalinised S. aureus cells was perf The resultant immunoprecipitates were resolved on 10% (w/v) SDS-polyacrylamide gels prior to identification of radiolabelled bands by fluorography. In lanes (1-6) the immunoprecipitation was performed in 3D-TKM buffer whilst in lanes (7-10) Kolbe Buffer containing 2% (w/v) Triton X-100 and 0.15M NaCl was used. Lane 1, overnight label, non-immune rabbit serum; lane 2, overnight label, anti-PTP-serum; lane 3, 4 h label with 10 µM FCCP, anti-PTP-serum; lane 4, overnight label, anti-E3-serum; lane 5, 4 h with 10 µM FCCP, anti-E3-serum; lane 6, overnight label, anti-PDC-serum; lane 7, overnight label, anti-PTP-serum; lane 8, 4 h label with 10 µM FCCP, anti-PTP-serum; lane 9, overnight label, anti-E3-serum; lane 10, overnight label, anti-PDC-serum; lane 11, ^{125}I-labelled low M_r standard proteins.


2% (W/v) Triton in
Kolbe buffer
3D-TKM buffer
E2
E3
'X'
E1α
E1β
Mr x 10-3
68
44
30
21
14.4
1 2 3 4 5 6 7 8 9 10 11

it appears that the inclusion of 0.15M NaCl has increased the stringency of the wash procedure, resulting in a decreased background. Following the incubation with anti-PTP-serum, (lane 7), the only major polypeptide precipitated (approximately M_r 45 000) is also present in those resulting from incubating with anti-E3-serum and anti-PDC-serum. This band could not be competed out by the addition of non-radiolabelling PTP to the incubation and thus represents a non-specific contaminant.

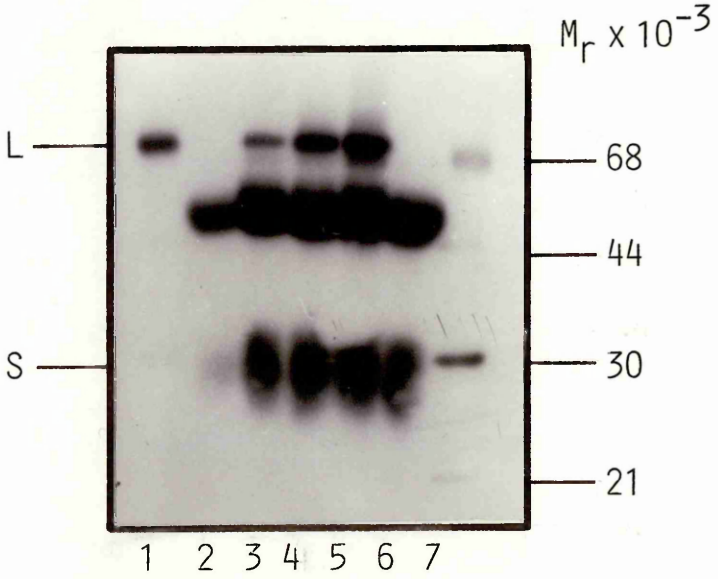
To examine the possibility that the anti-PTP-serum did not recognise the parent antigen during the immunoprecipitation procedure, a protocol was devised to immunoprecipitate the PTP from solubilised bovine heart mitochondria and to detect the polypeptides by immunoblot analysis using anti-PTP-serum.

Bovine heart SMPs were solubilised by heating in 1% (w/v) SDS and non-specific dissociations were decreased by a preadsorption with non-immune serum. Immunoprecipitates (Section 2.2.11d) were resolved on 10% (w/v) SDS-polyacrylamide gels before being transferred to nitrocellulose. Detection of the antigen was by decorating with ^{125}I-labelled protein A followed by autoradiography.

Figure 5.4 illustrates the results obtained with anti-SDH-serum. The large subunit of this enzyme (70 000 M_r) is known to be a component of the inner mitochondrial membrane and thus serves as a control for the solubilisation procedures. SDH can be detected in intact bovine heart mitochondria following immunoblotting, lane 1 establishes the specificity

Fig. 5.4: Immune Blot Analysis of an Immune Precipitation of SDH from Bovine Heart Sub-mitochondrial Particles

Bovine heart submitochondrial particles were solubilised in 1% (v/v) SDS and the immune precipitation procedure (Section 2.2.11d) was followed. The resultant immune-precipitates were resolved on 10% (w/v) SDS polyacrylamide gels before being processed for immunoblotting. Detection of antigen was by iodinated protein A followed by autoradiography. Lane 1, 40 µg of intact mitochondria; lane 2, non-immune rabbit serum used in immunoprecipitation; lane 3, 10 µl anti-SDH-serum; lane 4, 50 µl anti-SDH-serum; lane 5, 100 µl anti-SDH-serum; lane 6, 50 µl anti-L-serum, used in immune precipitation. Lane 7, ^{125}I-labelled low M_r standard protein. 0.8 mg of solubilised membrane used per incubation.


$M_r \times 10^{-3}$
L
S
68
44
30
21
1 2 3 4 5 6 7

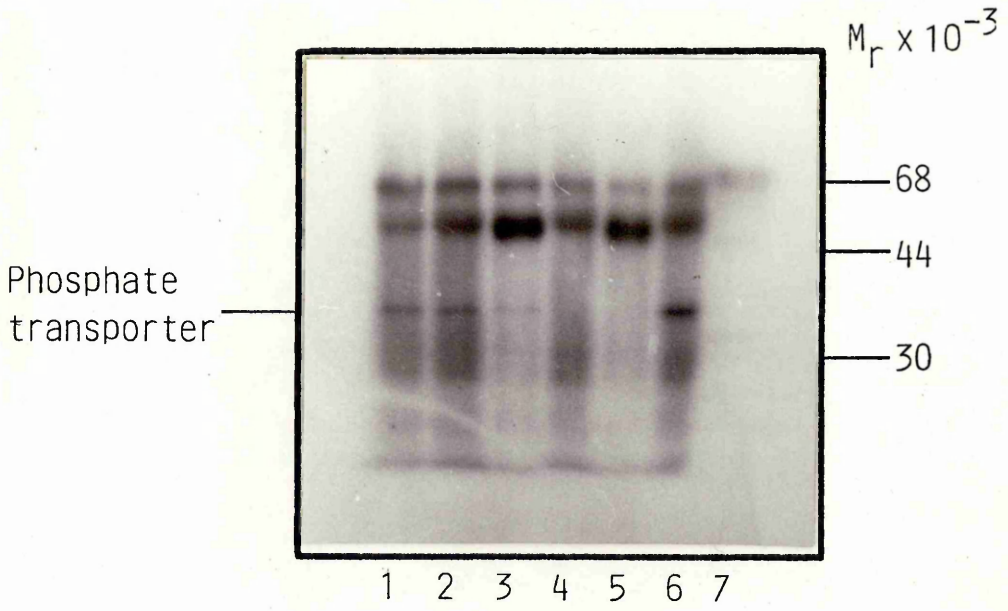
of the antiserum, reacting with both the L and S subunits. Lanes (3-5) show the presence of the 70 000 M_r polypeptide following precipitation with anti-SDH-serum. The incubation with non-immune rabbit serum (lane 2) further establishes the specificity of the antiserum and resolves the heavy (50 000 M_r) and light chain (25 000 M_r) of the immunoglobulin molecules. The lower M_r subunit of 27 000 M_r only weakly cross-reacts with the antiserum (lane 1) and if successfully precipitated would co-migrate with the observed IgG light chain.

The comparable experiment with anti-PTP-serum is shown in Figure 5.5. Lane 5 illustrates the result of the immunoprecipitation using non-immune rabbit serum. The heavy and light chains of the immunoglobulin molecule are observed and a 66 000 M_r polypeptide. This result is replicated in lane 5 by anti-PTP-IgG. The 34 000 M_r polypeptide can be identified in lanes 1, 2 and 3. By incubating 0.4, 0.8 and 1.2 mg of solubilised membrane with anti-PTP-serum, the parent antigen has been collected during immunoprecipitation and identified by immunoblotting with the antiserum. This result is further illustrated in lane 6, where purified PTP is added to the incubation.

Thus it appears that the PTP is indeed recognised by the corresponding antiserum and can be directly precipitated in conjunction with protein A.

Fig. 5.5: Immune Blot Analysis of an Immune Precipitation of the PTP from Bovine Heart Submitochondrial Particles

Bovine heart submitochondrial particles were solubilised in 1% (v/v) SDS and the immune precipitation procedure (Section 2.2.11d) was followed. The resultant immunoprecipitates were resolved on 10% (w/v) SDS-polyacrylamide gels before being transferred to nitrocellulose paper. The antigen was detected using ^{125}I-labelled protein A followed by autoradiography. Lanes 1,2, 3: 0.4 mg, 0.8 mg and 1.2 mg of solubilised membrane protein used in the immunoprecipitate with 10 µl of anti-PTP-serum. In lanes 4,5,6 0.8 mg of solubilised membrane protein was used with the following antisera. Lane 4, anti-PTP-IgG; lane 5, non-immune rabbit serum; lane 6, as 2 but supplemented with approximately 5 µg of purified PTP; lane 7, ^{125}I-labelled low M_r standard proteins.


$M_r \times 10^{-3}$
68
44
30
Phosphate transporter
1 2 3 4 5 6 7

5.3.2 Protease treatment of bovine heart submitochondrial particles

Submitochondrial particles generated by sonication of intact mitochondria are predominantly inverted with respect to their orientation in situ (Wehrle et al., 1978; Godinot and Gautheron, 1979), in that more than 90% of the vesicles formed are sealed 'inside out'. Therefore, by applying the protease treatment/immunoblot procedure, described in Section 5.2, to SMPs it was possible to investigate the presence of exposed regions of PTP located specifically at the matrix surface of the inner mitochondrial membrane.

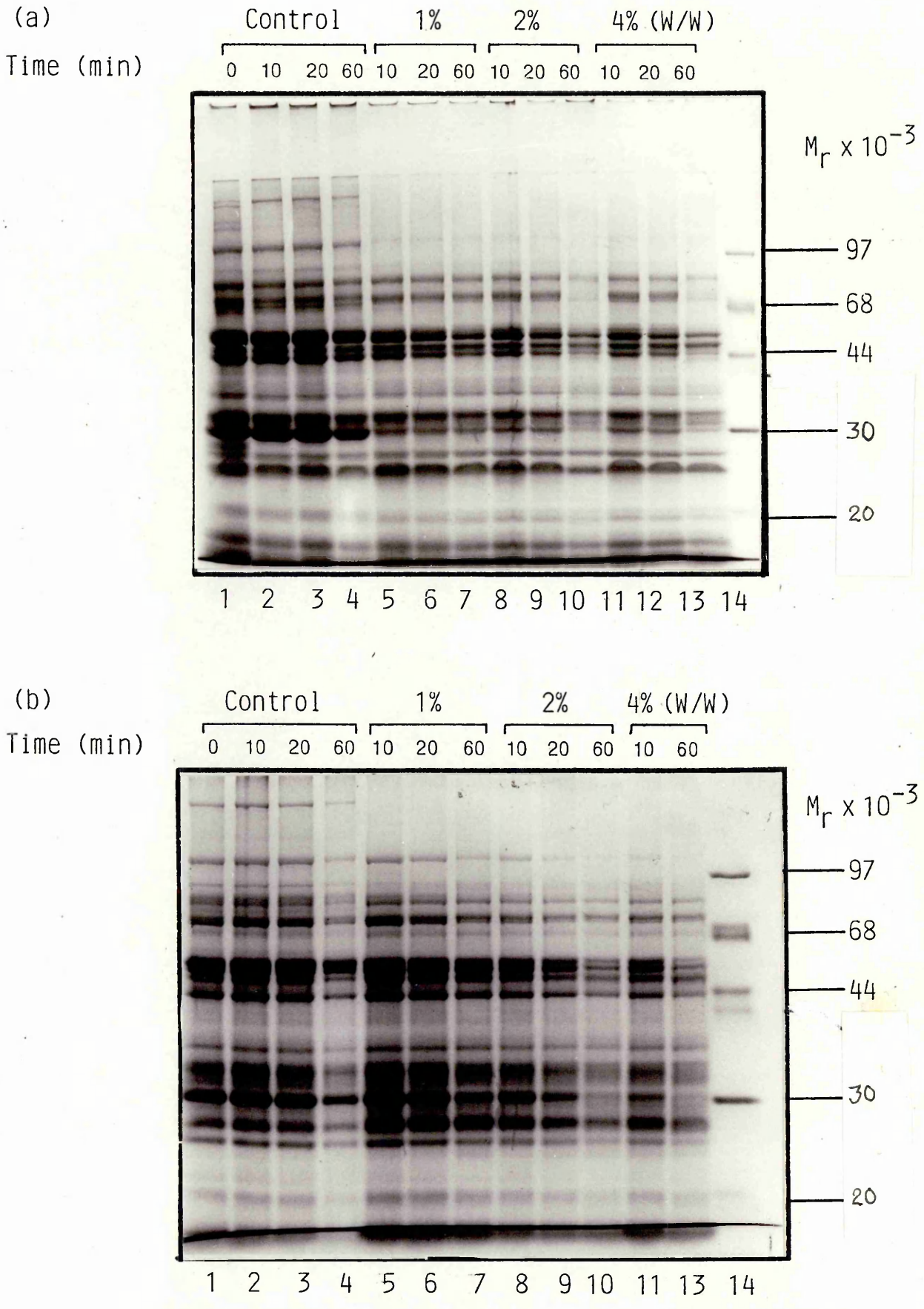
Various proteases, including pronase, protease K, papain, trypsin, chymotrypsin and elastase, were studied for their effect on cleavage of PTP in SMPs. The influence of the cumulative addition of chymotrypsin and trypsin on the polypeptide pattern of submitochondrial particles is shown in Figure 5.6. The membrane associated peptides were resolved by SDS-PAGE. With increased incubation period and protease concentration, a decrease in intensity of stained bands in the high M_r region, relative to control incubations (lanes 1-4), The two proteases formed different proteolytic breakdown products which remained unchanged at the higher protease concentrations (4%, w/w), indicative of the stability of the inner mitochondrial membrane.

For identification of proteolytic fragments derived from PTP, samples were subjected to immune-replica analysis. Individual polypeptides and their immunoreactive cleavage

Fig. 5.6: SDS-Polyacrylamide Gel of Protease Treated Bovine Heart Submitochondrial Particles

Bovine heart submitochondrial particles were digested for up to 60 min with increasing concentrations of (A) Trypsin or (B) Chymotrypsin. The resultant membrane associated peptides were resolved on 10% (w/v) SDS-polyacrylamide gels. Lanes (1-4) control incubation in the absence of protease; lanes (5-7) 1% (w/w) protease; lanes (8-10) 2% (w/w) protease; lanes (11-13) 4% (w/w) protease*. 45 µg of protein loaded per track. Lane 14, low M_r standard proteins.

*20 min time point not processed in chymotrypsin digest.


(a)
Control
1%
2%
4% (W/W)
Time (min)
0 10 20 60 10 20 60 10 20 60 10 20 60
$M_r \times 10^{-3}$
97
68
44
30
20
1 2 3 4 5 6 7 8 9 10 11 12 13 14
(b)
Control
1%
2%
4% (W/W)
Time (min)
0 10 20 60 10 20 60 10 20 60 10 60
$M_r \times 10^{-3}$
97
68
44
30
20
1 2 3 4 5 6 7 8 9 10 11 13 14

products could be identified by subsequent incubation with specific antisera and iodinated protein A. Figure 5.7 illustrates the immunoblot pattern derived from incubating bovine heart SMPs with pronase, protease K and trypsin for the indicated time intervals and probing with anti-PTP-serum. With the exception of the incubation with 2% (w/w) protease K for 2 h, the PTP remains resistant to digestion i.e. essentially as the control incubation, and no cleavage peptides can be observed. This stability of PTP to proteolysis was demonstrated also for elastase, papain, chymotrypsin and V-8 protease.

On the basis of this result two other explanations presented themselves: (i) that for some reason the proteases were rapidly inactivated during the experiment or (ii) the matrix surface of the inner mitochondrial membrane was not available to externally added proteases. To test both these hypotheses a parallel incubation with the selected proteases and the subsequent immunoblot analysis was carried out on succinate dehydrogenase (SDH).

Succinate dehydrogenase, an enzyme involved both in the Krebs cycle and the electron transport chain, is traditionally regarded as a marker for the inner mitochondrial membrane (Ernster and Kuylenstierna, 1970). The asymmetric distribution of this enzyme with respect to the matrix surface of the mitochondrial inner membrane has been documented using both antiserum directed against the holo-enzyme (Merli et al., 1979) and chemical reagents (Klingenberg and Buchholz, 1970; Girdlestone et al., 1981). Thus,

protease treatment of SMPs and analysis using anti-SDH-serum provides an excellent control system for these studies.

Figure 5.8 shows the result of the parallel incubation of SMPs with the range of proteases used in monitoring the accessibility of PTP, and subsequent immunoblot analysis. Incubation in the absence of protease (lanes 1,2) shows that neither the large nor small subunit of SDH is susceptible to degradation under the conditions employed i.e. 30°C for 120 min. Inclusion of either pronase, protease K or trypsin during the incubation leads to rapid degradation of one or both subunits.

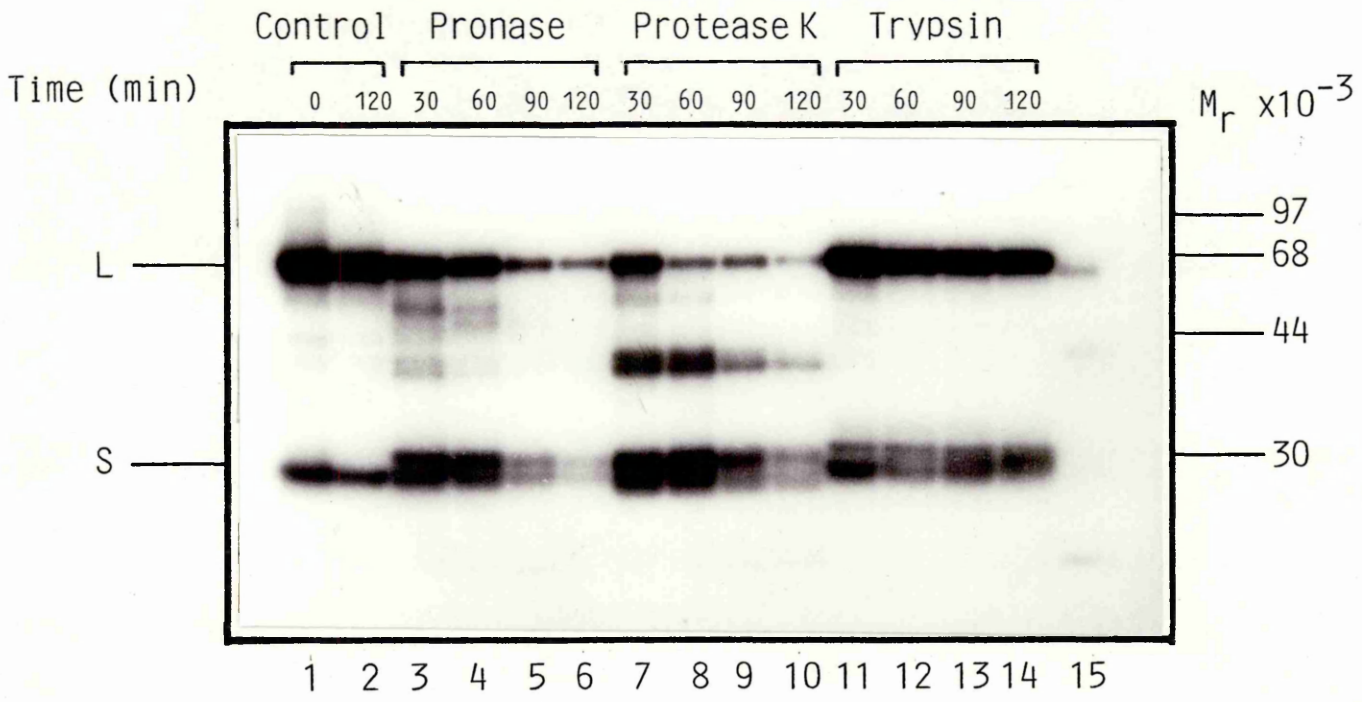
Pronase digestion (lanes 3-6) demonstrates that the large 70 000 M_r subunit of SDH is degraded to yield ultimately a 32 000 M_r membrane-bound fragment whilst the 27 000 M_r peptide remains resistant to digestion until the lipid bilayer is disrupted. Diminution of the band corresponding to the large subunit with the concomitant appearance of the immunoreactive 32 000 M_r polypeptide is evident following incubation with both protease K (lanes 7-10) and trypsin (lanes 11-14). The incubation with protease K indicated that the 27 000 M_r small subunit was degraded to a slightly smaller peptide (24 000 M_r) but only under conditions which totally degraded the 70 000 M_r subunit does the S subunit become susceptible to proteases. The presence of 0.15M NaCl in the wash buffer ensures that any weakly interacting peptides are removed and that those detected via immunoblot analysis are membrane-bound peptides.

Fig. 5.7: Immune Blot Analysis of the Effect of Protease on PTP in Bovine Heart Submitochondrial Particles

Bovine heart submitochondrial particles were incubated with 2% (w/w) protease for 120 min. Aliquots were withdrawn at 30, 60, 90 and 120 min, the protease added to a final concentration of 2% (w/w) following each time point. The reaction was stopped by the addition of protease inhibitors (2 mM PMSF, 0.8 µg/µl leupeptin) and by diluting samples in 7 volumes of ice-cold wash buffer. Following washing, the membrane fractions were boiled in Laemmli sample buffer containing 10 mM DTT, resolved on 10% (w/v) SDS-polyacrylamide gels prior to analysis by immunoblotting with anti-PTP-serum. 45 µg of protein was loaded in lanes (1-14) inclusive. Lane 15, ^{125}I-labelled low M_r standard proteins.

Fig. 5.8: Immune Blot Analysis of the Effect of Proteases on SDH in Bovine Heart Submitochondrial Particles

Bovine heart submitochondrial particles were incubated with 2% (w/w) protease for 120 min. Samples were removed and treated as detailed in Figure 5.7. Immune-blot analysis was performed using anti-SDH-serum. 45 μg of protein was loaded in lanes (1-14) inclusive; lane 15, ^{125}I-labelled low M_r standard proteins.


Control
Pronase
Protease K
Trypsin
Time (min)
0 120 30 60 90 120 30 60 90 120 30 60 90 120
M_r x10^{-3}
97
68
44
30
L
S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Subsequent investigation, using both anti-SDH-serum and subunit-specific antisera (Clarkson, 1987), of this proteolysis pattern has revealed that the 32 000 M_r peptide was conclusively derived from the 70 000 M_r subunit and was, indeed, embedded within the membrane bilayer i.e. it is an integral component of the inner mitochondrial membrane. Thus, this result provides an ideal marker enzyme for further protease studies on the inner mitochondrial membrane. It also establishes that the vesicles are in the correct orientation and that the proteases are active.

To eliminate the third possibility i.e. that PTP, in its membrane environment, is inherently resistant to proteolysis under the conditions described, the above experiment was repeated using SMPs that had been solubilised using SDS or Triton X-100.

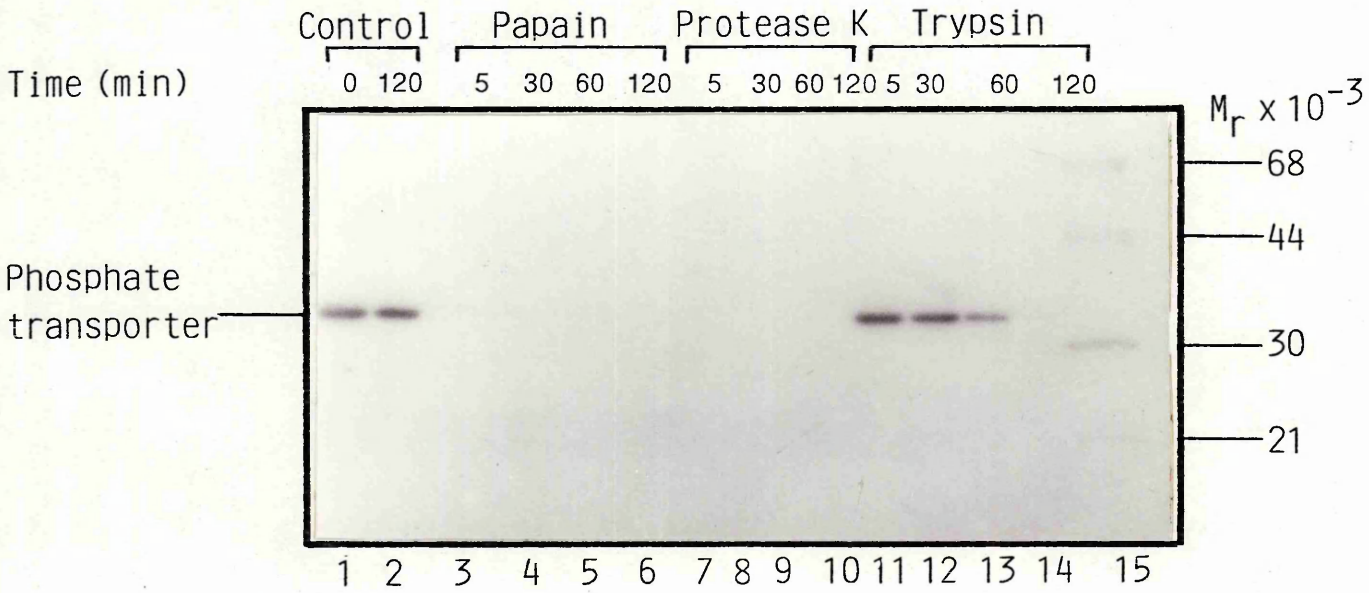
Figure 5.9B illustrates an immunoblot analysis of the control enzyme, SDH. Lanes (1-2) depict that again, SDH is stable under the chosen experimental conditions i.e. 30°C for 120 min. The inclusion of either 2% (w/w) papain or protease K to this incubation results in the enzyme being totally degraded. There is no evidence of the 32 000 M_r membrane bound peptide, previously detected following an incubation with these proteases in the absence of detergent. This result suggests that the 'protecting' lipid bilayer has been removed, although it does not exclude the possibility of more protease sensitive sites becoming available due to the presence of detergent. This is further illustrated in tryptic digests of SDS-solubilised membranes (lanes 11-14).

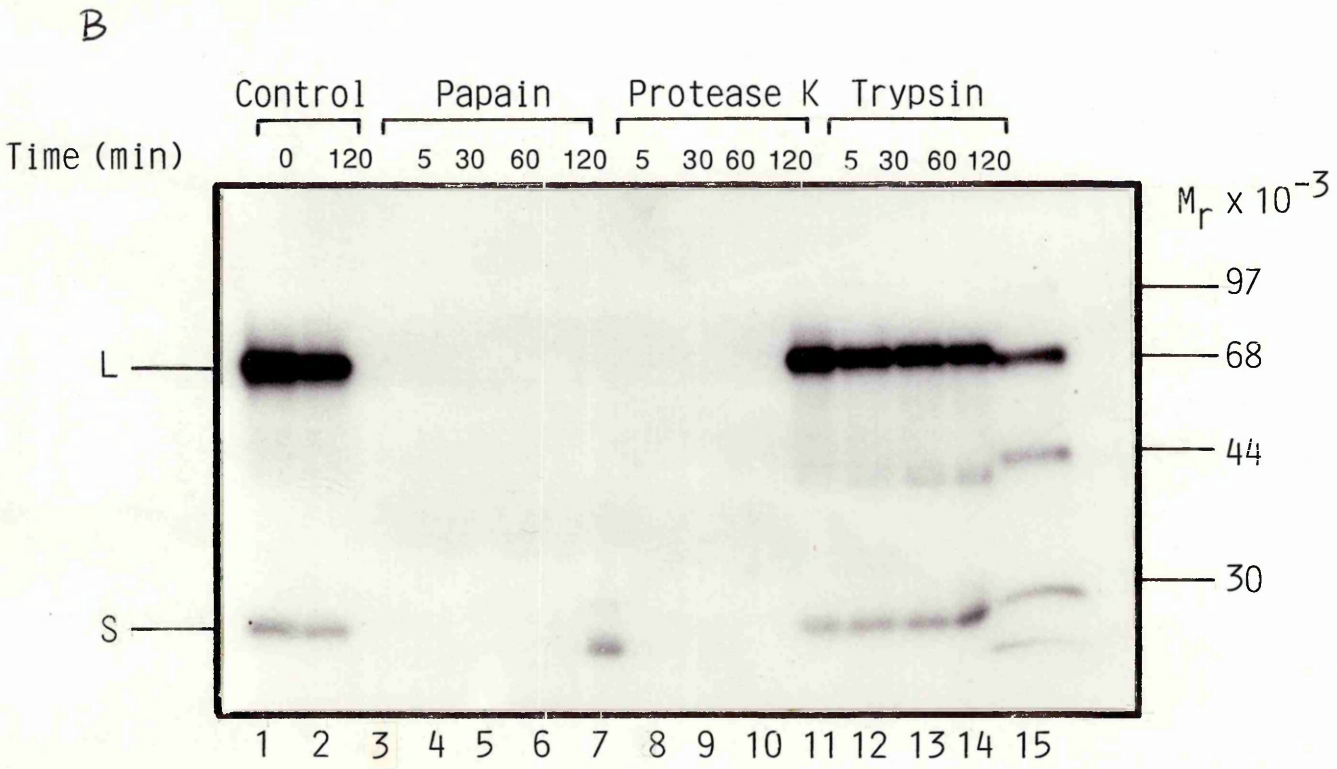
Fig. 5.9: Immune Blot Analysis of the Effect of Proteases on Bovine Heart SMP's Solubilised in 0.1% (w/v) SDS

Bovine heart submitochondrial particles were solubilised with a final concentration of 0.1% (v/v) SDS before being incubated with proteases (2% (w/w)). Aliquots were removed at 5, 30, 60 and 120 min and proteases added to the final concentration above following each time point. The reaction was stopped by the addition of protease inhibitors and by diluting into 7 volumes of ice-cold wash buffer. Analysis of membrane associated peptides was by SDS-PAGE followed by immune blotting with (A) anti-PTP-serum, or (B) anti-SDH-serum.

45 µg of protein was loaded to lanes (1-14) inclusive; lane 15, ^{125}I-labelled low M_r standard proteins.

A


Control
Papain
Protease K
Trypsin
Time (min)
0 120
5 30 60 120
5 30 60 120
5 30 60 120
M_r x 10^{-3}
68
44
30
21
Phosphate transporter
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15


B
Control
Papain
Protease K
Trypsin
Time (min)
0 120
5 30 60 120
5 30 60 120
5 30 60 120
M_r x 10^{-3}
97
68
44
30
L
S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

The immune profile obtained in these tracks is essentially as the control incubation and results from the rapid inactivation of the protease by SDS.

Analysis of PTP under these conditions (Fig. 5.9A) illustrates that in the absence of proteases the protein is stable during the incubation. Immunoblot analysis following digestion with papain (lanes 3-6) or protease K (lanes 7-10) failed to detect any immunoreactive peptides i.e. the protein was completely degraded. The parallel study with trypsin in the presence of 0.1% ($_w$/v) SDS demonstrates that the protease is inhibited by the detergent and that only following prolonged incubation (120 min) does PTP become degraded.

This result strongly suggests that the PTP is not inherently resistant to protease digestion by virtue of its native structure but that it is protected from proteolysis by the membrane bilayer. This analysis results from studies on inverted vesicles, and as Figure 5.7 and 5.9A demonstrate no shift in mobility to a lower M_r band, suggests that the PTP has no protease-sensitive domains exposed at the matrix surface of the mitochondrial inner membrane.

5.3.3 Protease treatment of intact bovine heart mitochondria

It is well documented that in preparations of freshly isolated mitochondria, the cytoplasmic surface of the inner mitochondrial membrane is accessible to labelling reagents such as lactoperoxidase, indicating that the outer membrane has been removed by the isolation procedure (Boxer, 1975). It was therefore possible to investigate the exposed regions of the PTP on the cytoplasmic surface of the mitochondria

by applying the protease treatment coupled to immunoblotting analysis, as detailed above.

Figure 5.10 shows the results of incubating freshly-isolated bovine heart mitochondria at 30°C for up to 120 min. in the absence of protease (lanes 1,2) and after the addition of 2% (w/w) trypsin (lanes 3-6), pronase (lanes 7-10) or protease K (lanes 11-14), as analysed by immunoblotting using anti-citrate-synthase serum. Inspection of this figure reveals that the parent antigen is stable under the conditions employed, both in the presence and absence of added protease. The incubation of intact mitochondria with each of the proteases had no effect on the intensity of the band corresponding to citrate synthase.

Citrate synthase is a component of the Krebs cycle and is a soluble enzyme located within the confines of the matrix inner membrane compartment. The native enzyme is susceptible to the added protease (data not shown) so this observation i.e. no diminution of the 50 000 M_r polypeptide to smaller immunoreactive peptides, is consistent with the mitochondria being isolated as intact organelles. These mitochondria can then be employed in studying the topography of PTP with respect to the cytoplasmic surface of the mitochondria.

Analysis of the corresponding investigation using anti-SDH-serum could not degrade the large 70 000 M_r subunit to the previously recognised 32 000 M_r peptide. The smaller 27 000 M_r subunit was also protected from the protease by the membrane structure. This observation suggests that neither subunit is available at the cytoplasmic surface of the inner membrane. This result has been verified by G. Clarkson in our laboratory.

Fig. 5.10: Immune Blot Analysis of the Effect of Proteases on Citrate Synthase in Intact Bovine Heart Mitochondria

Intact mitochondria were prepared (Section 2.2.9b) and incubated with a variety of proteases at a concentration of 2% (w/w), which was replenished after each time point. Aliquots were withdrawn at the indicated time points and the reaction was stopped by the addition of protease inhibitors (2 mM PMSF; 0.8 µg/µl leupeptin) prior to dilution with 7 vol. of ice-cold wash buffer. Subsequent analysis was by SDS-PAGE followed by immune-blotting using anti-citrate synthase serum. Lanes (1-14) contain 45 µg of mitochondrial protein. Lane 15, 0.2 µg of purified citrate synthase.

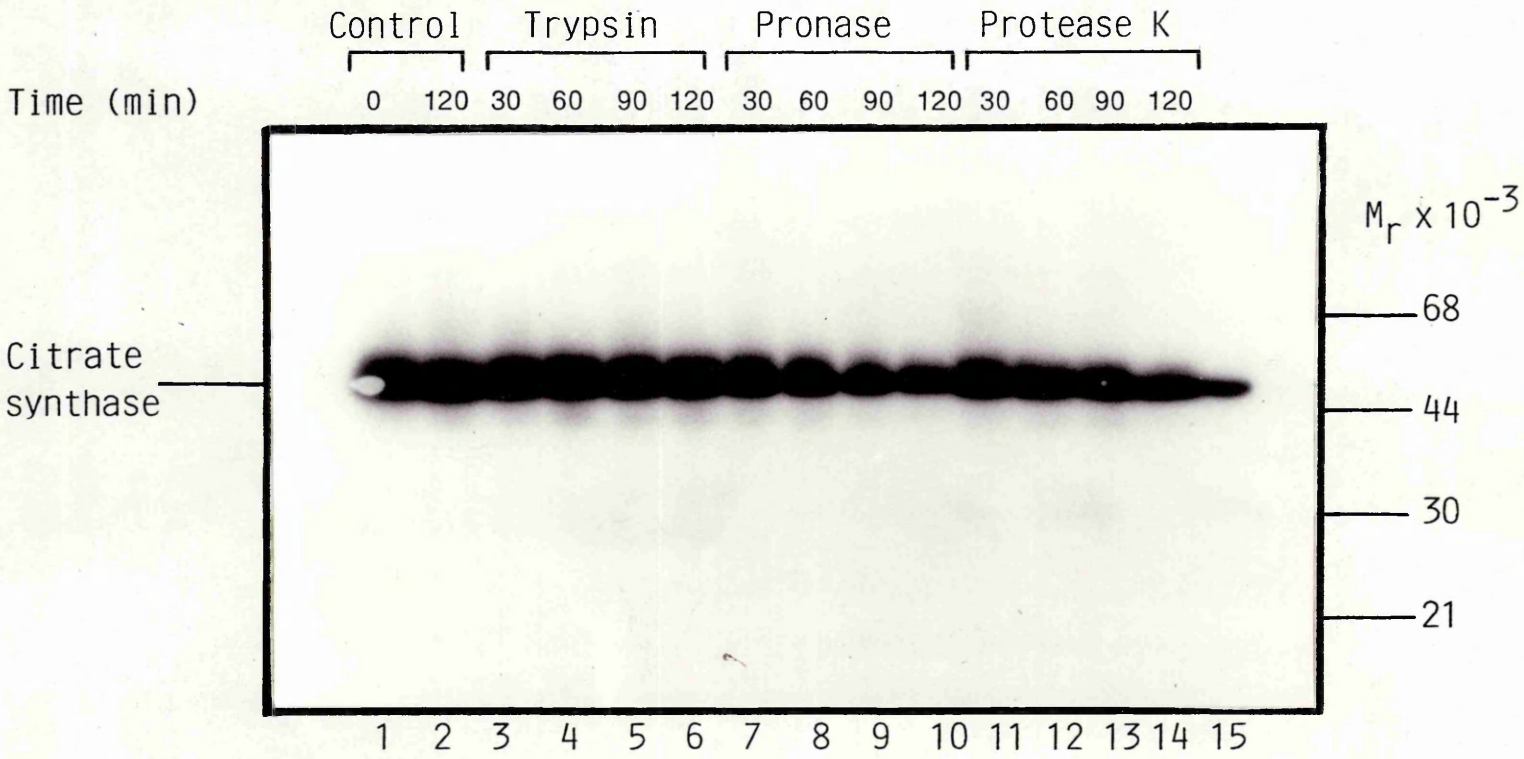

Control
Trypsin
Pronase
Protease K
Time (min)
0 120 30 60 90 120 30 60 90 120 30 60 90 120
Citrate synthase
$M_r \times 10^{-3}$
68
44
30
21
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Fig. 5.11: Immune Blot Analysis of the Effect of Proteases on SDH in Intact Bovine Heart Mitochondria

Intact bovine heart mitochondria were incubated at 30°C for up to 120 min in the absence of proteases (lanes 1,2) or with 2% (w/w) trypsin (lanes 3-6); pronase (lanes 7-10) or protease K lanes (11-14). Samples were removed at the given time point, protease inhibitors added and the samples diluted with 7 vol. of ice-cold wash buffer. After repeated washes samples were boiled in Laemmli sample buffer containing 10 mM DTT. Analysis was by SDS-PAGE followed by immune blotting with anti-SDH-serum. 45 µg of protein loaded/track.

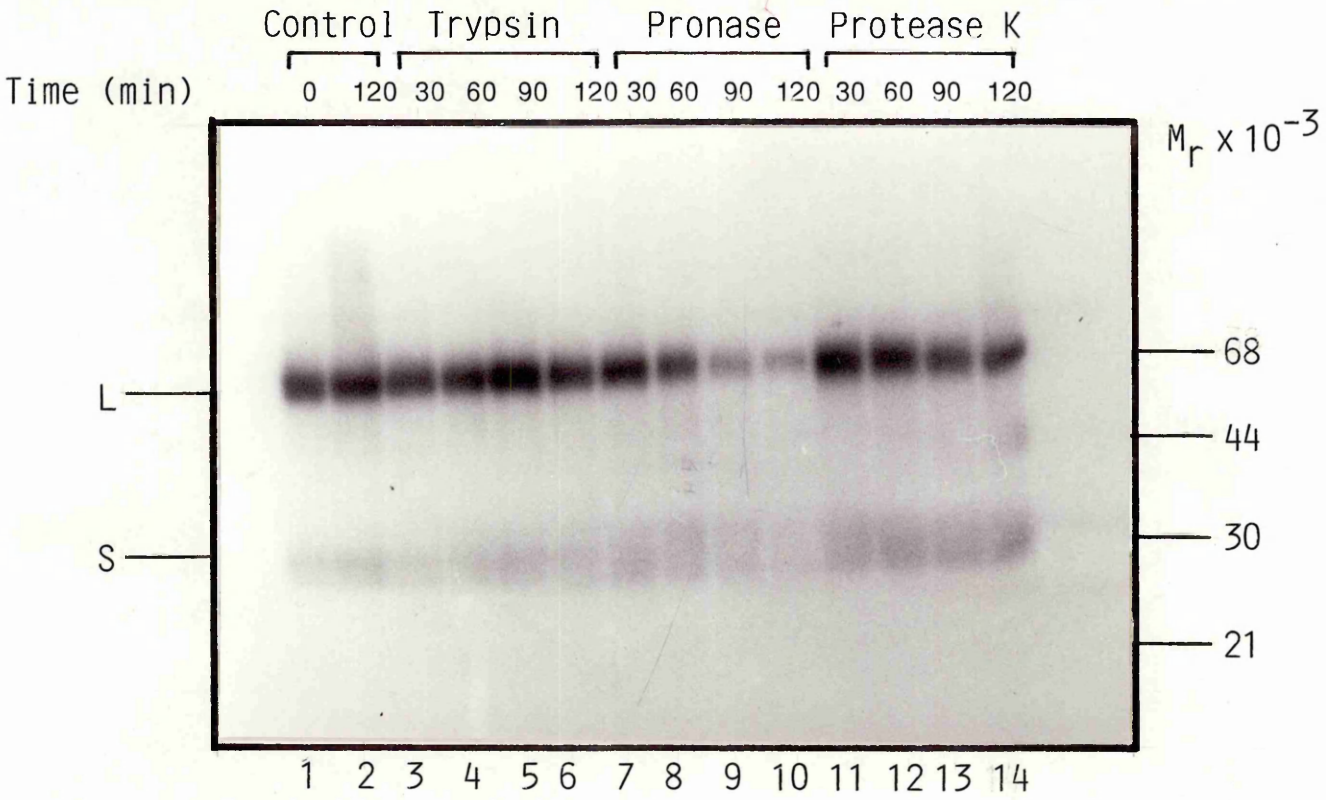
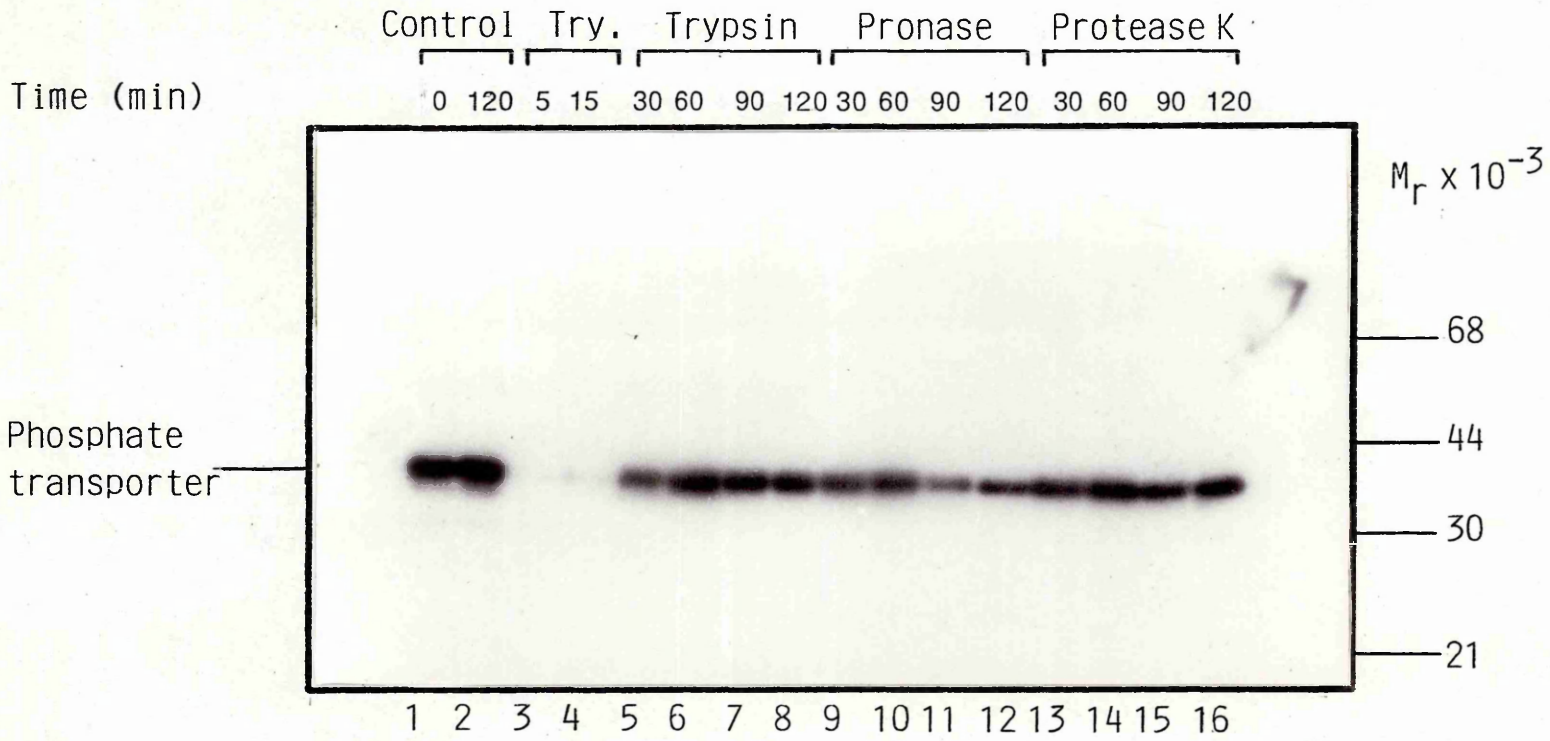

Control
Trypsin
Pronase
Protease K
Time (min)
0 120 30 60 90 120 30 60 90 120 30 60 90 120
$M_r \times 10^{-3}$
L
S
68
44
30
21
1 2 3 4 5 6 7 8 9 10 11 12 13 14

Fig. 5.12: Immune Blot Analysis of the Effect of Proteases on PTP in Intact Bovine Heart Mitochondria

Intact bovine heart mitochondria were incubated at 30°C for up to 120 min in the absence of proteases (lanes 1,2) or with the inclusion of 2% (w/w) trypsin (lanes 5-8), pronase (9-12) or protease K (lanes 13-16). Lanes 3,4 are intact mitochondria solubilised by the addition of 0.5% (v/v) Triton X-100 to the incubation buffer, prior to digestion with 2% (w/w) trypsin. Aliquots were withdrawn from the incubations and the reaction stopped by the addition of protease inhibitors (2 mM PMSF, 0.8 µg/µl leupeptin) and 7 volumes of ice-cold wash buffer. 45 µg of protein was loaded to each track.


Control
Try.
Trypsin
Pronase
Protease K
Time (min)
0 120 5 15 30 60 90 120 30 60 90 120 30 60 90 120
$M_r \times 10^{-3}$
68
44
30
21
Phosphate transporter
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16

Parallel research with anti-PTP-serum has also failed to demonstrate any immunoreactive peptides resulting from the incubation of intact mitochondria with the above proteases. Figure 5.12 illustrates that the intensity of the band corresponding to PTP is essentially as the control incubation (lanes 1,2). Subsequent solubilisation of the membrane with a final concentration of 0.5% (v/v) Triton X-100 demonstrates that the structure of PTP within the membrane does not render it resistant to proteolysis, but rather the lipid bilayer protects the protein from added proteases.

5.4 DISCUSSION

These studies have shown that it was difficult to immunoprecipitate PTP from radiolabelled cell lysates, despite demonstrating the antigen to be present in the chosen cell lines.

In each instance, under conditions whereby both soluble and membrane proteins can be precipitated by their respective antisera, we have been unable to identify a M_r of 34 000 polypeptide corresponding to the PTP.

Initially, it was thought that the strong cross-reaction elicited by the antibodies in immunoblots was by virtue of the antigen being present in a denatured fashion. Our antiserum was prepared from purified PTP which had been acetone-precipitated. To investigate the importance of antigen presentation, radiolabelled cell lysates were denatured under a range of standard conditions e.g. heat denaturation or acetone-precipitation prior to resuspending in 3D-TKM buffer. Each of these failed to precipitate the

parent antigen from the cell lysate. Substitution of the detergents used e.g. CHAPS for Triton X-100 in the 3D-TKM buffer system did not clarify the resultant immunoprecipitates.

During the immunoprecipitation procedure, the antibody-antigen complex is indirectly precipitated by its interaction with formalinised S. aureus cells. The PTP has marked hydrophobic properties which may cause the protein to adsorb to the bacterial cell wall, subsequent elution of this protein may prove difficult. This possibility was investigated by the substitution of S. aureus cells with Sepharose beads. This did not result in the elution of the 34 000 M_r antigen.

Thus under a variety of conditions, a range of buffer and detergent systems or source of cell lines it has proven difficult to immunoprecipitate a reproducible polypeptide pattern that can be identified with the PTP. In each of these situations the presence of control incubations has demonstrated this to be a specific problem of anti-PTP-serum in conjunction with the antigen.

The possibility exists, therefore, that the phosphate transport protein, is not being recognised by the antiserum during the immunoprecipitation procedure, although Figure 5.5 demonstrates that immunoblot analysis of an immuno-precipitation using anti-PTP-serum does, indeed precipitate the parent antigen. This was achieved by solubilising the membrane system in 1% (w/v) SDS before immunoprecipitation. Extension of this protocol to radiolabelled cell lysates did not resolve a polypeptide of 34 000 M_r with anti-PTP-serum, although the previously recognised high M_r bands were no longer visible.

The 3D-TKM buffer system (Section 2.2.8a) used in these immunoprecipitation studies was selected for its proven ability to promote highly specific precipitations of maximally dissociated polypeptides from cellular extracts. However, on occasions, incomplete dissociation of polypeptides has been observed even in the presence of this detergent system (Hunter, 1985).

The presence of higher M_r polypeptides in radiolabelled immunoprecipitates, in contrast to Figure 5.5 suggests that the antiserum may recognise the PTP but that by virtue of strong interactions, associated polypeptides may also be precipitated. It should be noted that boiling radiolabelled lysates in SDS prior to immunoprecipitation seemed to destroy these interactions. These polypeptides may be involved with phosphate transport in mitochondria and the existence of a phosphate transport system has been suggested previously (Kolbe *et al.*, 1982) though evidence in support of this has not been forthcoming. It is also possible that these bands may associate with the antigen by virtue of non-specific protein interactions, which arise as a result of the hydrophobicity of this protein.

Amino acid composition data (Gibb *et al.*, 1986) has shown that PTP has a very low methionine content (< 2% of total amino acids) and this may account for the difficulty in fluorographic detection of radiolabelled proteins. Alternative radiolabels are available, e.g. (4,5 - ^{3}H) leucine. Leucine is five-fold more abundant than methionine, though experience has shown that any benefit gained from an

increased incorporation of radiolabel resulting from the higher leucine content has been offset by the markedly lower specific activity of this isotope and the low sensitivity of detection of ^{3}H. The low abundance and stability of PTP within the inner membrane i.e. turnover of newly synthesised protein within the membrane may also be a major factor in the immunoprecipitation of this protein.

The function of PTP within the inner mitochondrial membrane is inextricably associated with certain structural features. For example the location of the reactive sulphydryl group available only at the cytoplasmic surface of the mitochondria (Houstek and Pedersen, 1985) has been located at the N-terminus of the protein, i.e. at cysteine residue 42 (Kolbe et al., 1985) within the primary structure. Other workers (Tommasino et al., 1987) have carried out preliminary studies which will further characterise the phosphate binding domain of this protein. It is clear, therefore, that a knowledge of the topography of this protein is a pre-requisite for understanding the catalytic function of this carrier.

In this chapter, the combined approach of protease digestion of proteins exposed at either face of the inner mitochondrial membrane and the detection of protease resistant fragments by specific antiserum was used. Bovine heart mitochondria were chosen for these experiments primarily because of the higher inner membrane content of this organelle in comparison to liver mitochondria. This system has the added advantage that the method commonly used to

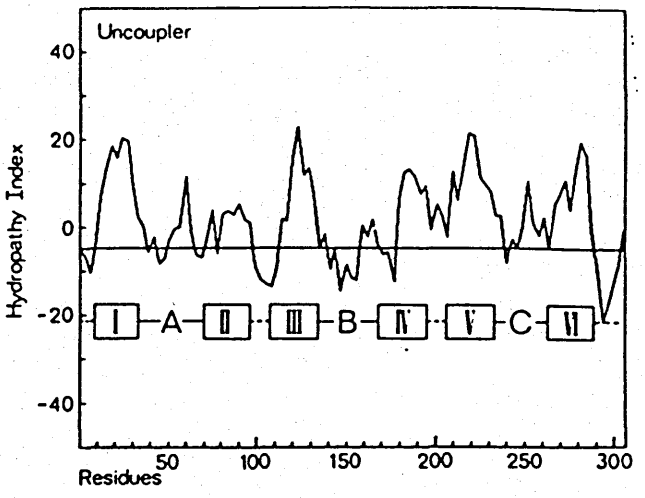
prepare these mitochondria (Smith et al., 1967) renders the cytoplasmic face of the inner membrane accessible to exogenously added enzyme (Boxer, 1975). Chapter 4 and Figure 5.1 also illustrate the strong cross-reaction elicited by the antiserum directed against the rat liver protein with the equivalent antigen in bovine heart.

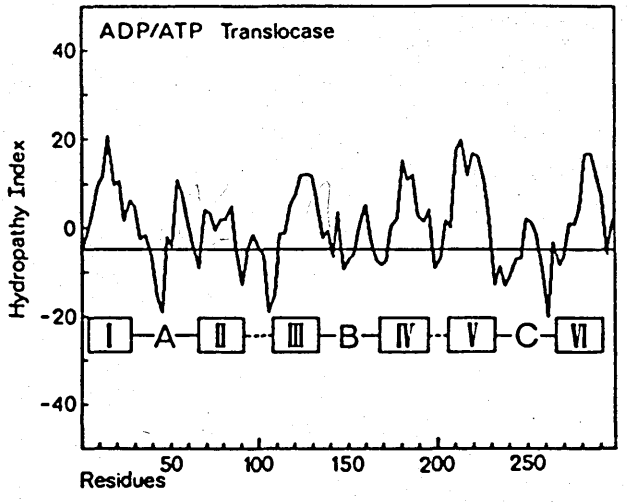
As discussed in this chapter, protease digestion of bovine heart mitochondria or submitochondrial particles has not demonstrated the presence of any protease sensitive domains exposed at either the cytoplasmic or matrix surface of the inner mitochondrial membrane. Incubation of sub-mitochondrial particles with protease K for 120 min. (Fig. 5.7) resulted in a decrease in the intensity of the PTP band with concomitant appearance of a 23 000 M_r peptide. Analysis of the parallel incubation (Fig. 5.8) with anti-SDH-serum demonstrates that the immunoreactive 32 000 M_r membrane-associated domain of the large subunit is also decreasing in intensity. This, then, suggests that the prolonged incubation with proteases, far from selectively degrading exposed regions, is destroying the integrity of the membrane structure.

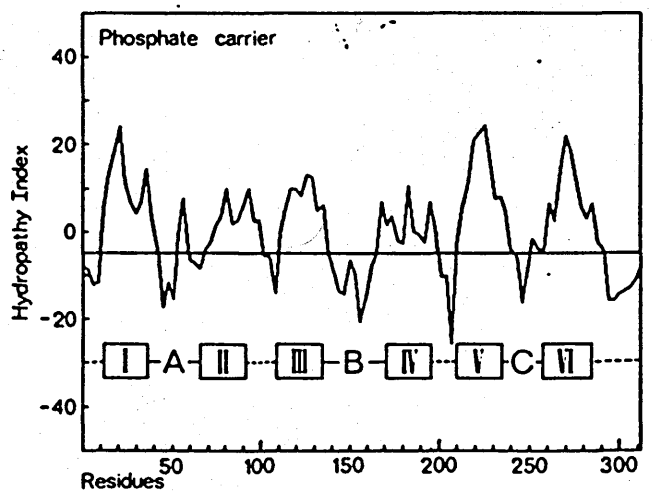
If the stability of the membrane structure is disrupted with the inclusion of detergents, SDS or Triton X-100 buffer, the previously resistant PTP becomes totally degraded, as does our control enzyme SDH (Fig. 5.9).

Fig. 5.13: Comparison of the hydrophobic profiles of the PTP with those of bovine ADP/ATP translocase and the hamster brown fat mitochondria uncoupler protein. The calculations were made with HYDROPLOT with a window of 11 amino acids. The horizontal bar represents an average hydrophobicity as defined by Kyte and Doolittle (1982).

(Adapted from Runswick et al., 1987).


Uncoupler
Hydropathy Index
40
20
0
-20
-40
I
A
II
III
B
IV
V
C
VI
50
100
150
200
250
300
Residues


ADP/ATP Translocase
Hydropathy Index
40
20
0
-20
-40
I
A
II
III
B
IV
V
C
VI
50
100
150
200
250
Residues


Phosphate carrier
Hydropathy Index
40
20
0
-20
-40
I
A
II
III
B
IV
V
C
VI
50
100
150
200
250
300
Residues

A knowledge of the primary structure of this protein would greatly facilitate the extension of this topographical information to a possible model structure of its arrangement within the lipid bilayer. Attempts, by ourselves in conjunction with Professor J. Fothergill at the University of Aberdeen, to sequence both the bovine heart and rat liver enzymes were unsuccessful. It is thought that the marked hydrophobicity and resultant solubility problems may be a major factor in this. Indeed, other workers (Kolbe *et al.*, 1984) have observed that PTP aggregates in the 'spinning cup' of the sequencer, and hence the N-terminus is inaccessible to the chemical reagents required for this analysis. To circumvent this problem these workers generated and isolated peptides from thermolysin and formic acid treated PTP (Section 1.3) and obtained sequence information on the first 47 amino acids of the N-terminus.

Runswick *et al.*, (1987) have constructed an oligonucleotide probe to this sequence which has subsequently been used to isolate and sequence a cognate cDNA clone from a bovine library. Analysis of the deduced amino acid sequence has demonstrated that the protein contains a N-terminal extension of 49 amino acids, exhibiting the properties of a mitochondrial signal peptide.

Comparison of the sequence with itself and other mitochondrial membrane proteins has established a strong homology with both the ANT and the brown fat mitochondrial uncoupling protein. This study demonstrates that the protein contains three internal repeats, previously noted for the

adenine nucleotide translocase (Saraste and Walker, 1982) and for the uncoupling protein (Aquila et al., 1985). Further analysis shows that all of the repeated elements (approximately 100 amino acids) are related to each other and that the most highly conserved residues are found in α-helices. The regions that connect these helices are poorly conserved and are found within the central part of the loops and at the N and C-terminal regions. These unconserved regions are thought to contribute to the specificity of the carrier. The cross-reactivity of the anti-PTP-serum with the 31 000 M_r polypeptide may result from common epitopes in the highly conserved α-helices, seen in both the ANT and the PTP. These may correspond to a phosphate binding domain or house a reactive cysteine residue.

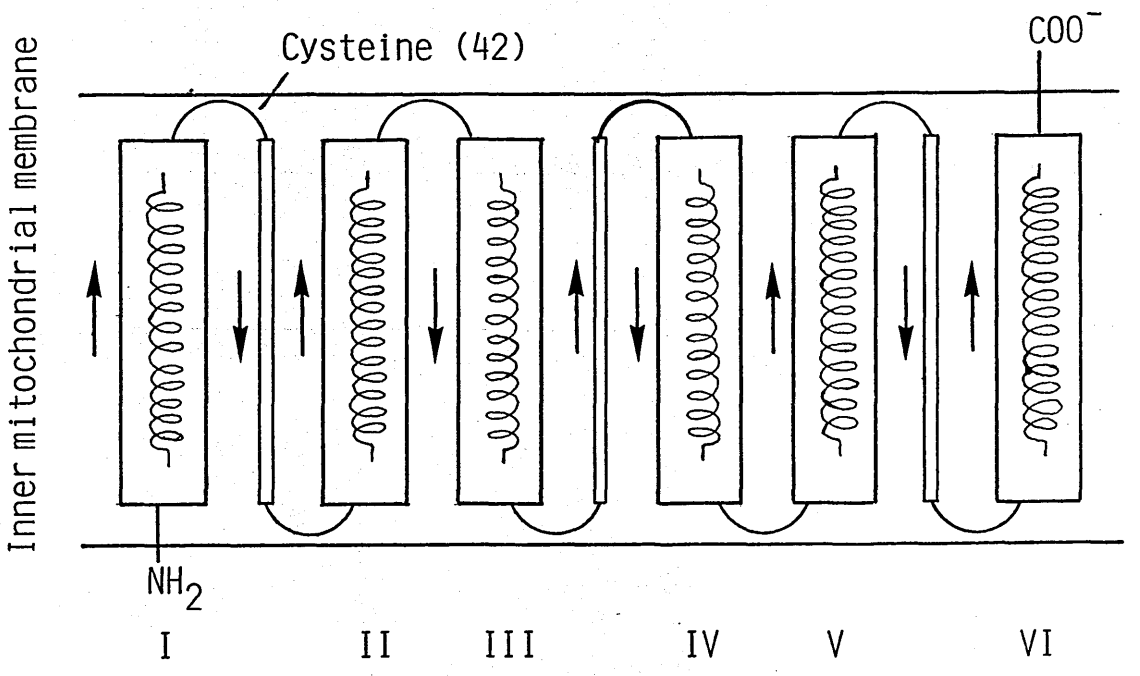
The significance of these membrane spanning α-helices and linking hydrophilic domains (Fig. 5.13) becomes apparent when taken into consideration with available topographical information. Data illustrated in this chapter suggests that PTP is an integral membrane component. This in conjunction with sequence analysis and biosynthetic data suggests that the hydrophilic regions may form a channel surrounded by the markedly hydrophobic α-helices (see Fig. 5.14), with neither domains being exposed at the membrane surface.

PTP has been shown to react with DCCD, which at low concentrations is a specific inhibitor of the H^+ATPase, due to covalent binding to a hydrophobic subunit of F_o. F_o is believed to contain a transmembrane channel through

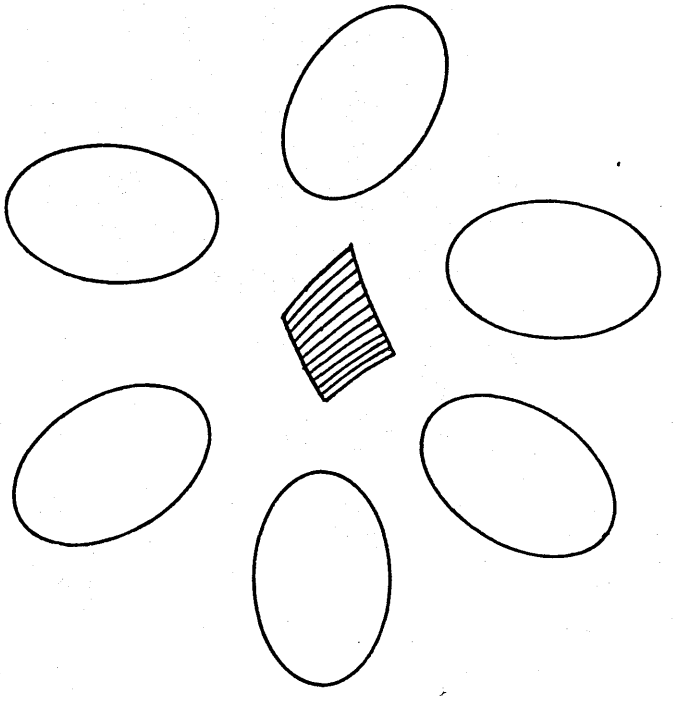
Fig. 5.14: Schematic Representation of the Arrangement of PTP within the Inner Mitochondrial Membrane

From the hydropathy profile (Fig. 5.13) it is predicted that the six membrane spanning regions will traverse the bilayer as α-helices. The intervening hydrophilic sequence will allow the formation of a pore or channel within which the DCCD-reactive lysine residues will be housed. The identification of the NEM reactive cysteine at residue 42 and the location of this group at the cytoplasmic surface of the membrane requires the N-terminus to be situated at the matrix surface. We find no evidence for the exposure of the molecule at either surface of the membrane nor for existence of a dimeric unit and thus propose that six α-helices arrange around an ion transporting channel (B), which is totally embedded within the lipid bilayer.

(A) Cytoplasm



(B) Matrix



which the protons flow to F_1. How the movement of protons through F_o; F_1 is coupled to the generation of ATP is currently the subject of intense debate and experimentation, as is the molecular relationship between the PTP and ATP synthesis. The F_o complex and PTP have, thus, certain structural features in common, not the least of which is the transport of H^+ through what is presumably a membrane channel. It is therefore feasible to postulate the molecular arrangement illustrated in Figure 5.14, where the tertiary structure forms a transmembrane channel, analogous to that of the F_o complex. This channel may explain the reactivity of PTP to DCCD (Houstek et al., 1981). This clustering of helices round an aqueous 'pore' has been proposed for other transport proteins which span the membrane several times (Eisenberg, 1984; Finer-Moore et al., 1984; Kopito and Lodish, 1985).

This model also allows for other 'associated' proteins, anticipated from biosynthetic data, to interact with the PTP either as peripheral proteins or as membrane bound components. The proposed structure of PTP also suggests that the activity of the transporter could be retained within a monomeric unit. This is in agreement with both native M_r determination and crosslinking studies which propose the 34 000 M_r protein to be a functional carrier.

This chapter has attempted to relate the structure of PTP to its biological function. Far less is known about the structures of membrane related proteins than of soluble proteins. In large part this is because X-ray crystallography and other biophysical tools are not so easily applied

to membrane proteins. The obvious difficulties associated with the PTP have been somewhat circumvented in this study by the use of antibodies, which have allowed detection and analysis of the protein in its native environment.

In the near absence of information of the three dimensional structure of membrane-related proteins, an abundance of information on their amino acid sequence has been gained from rapid DNA-sequencing methods. This has allowed investigators to infer various aspects of three-dimensional structures from sequence information. Thus, the coupling of the data of Runswick et al., (1987) to our own information has allowed us to propose a plausible model for the three dimensional structure of PTP within the inner mitochondrial membrane. The mapping of particular binding domains to amino acid residues within the PTP would clearly be of fundamental importance in understanding the functioning of the carrier.

CHAPTER SIX

DISCUSSION

6.1 BINDING OF PDC TO THE INNER MITOCHONDRIAL MEMBRANE

In recent years there has been considerable interest in the possible *in vivo* organisation of apparently soluble, metabolically sequential enzymes. In particular, much evidence, utilising a variety of techniques, has accumulated in favour of enzymes of the mitochondrial matrix being not only sequentially linked to those of the same pathway but also that they interact, in a specific manner, with the inner mitochondrial membrane (for review, Srere, 1985). It is now clear that specific, reversible protein:protein interactions may be the rule rather than the exception. Indeed, the association of enzymes to structural elements of the cell has been described and regulatory significance has been postulated for the binding or release of these enzymes.

Among the first experimental reports that there may be no freely soluble enzymes were those of Kempner and Muller (1968) using *Euglena* and those of Zalokar using *Neurospora* in which it was described that all enzymes in these cells sedimented as high M_r complexes. The range of such high M_r complexes is large and sequential site interactions are varied. These include multidomain proteins such as the arom complex in *Neurospora*, tightly but non-covalently bound multienzyme complexes such as the 2-oxoacid dehydrogenases from most organisms studied so far and the weak protein:protein interactions proposed for the Krebs cycle enzymes of rat liver. The optimal situation is thought to occur when the

active sites are covalently linked on the same polypeptide chain, thus allowing for the effective channeling of intermediates which would otherwise be diluted as part of a larger soluble pool.

The ability to isolate mitochondria free of other cellular components has allowed an extensive investigation of the organisation of proteins within this organelle. Fractionation and characterisation of protein components within the compartments created by the two membranes has permitted Srere and coworkers (Srere, 1985 and references therein) to propose that most enzymes of the mitochondrial matrix are organised as high M_r assemblies involving enzymes of the same pathway and that these aggregates bind with varying affinity to the matrix surface of the inner mitochondrial membrane. Some of these interactions have been shown to be affected by physiological concentrations of their substrates, so it is therefore possible to postulate regulatory behaviour based on the formation and dissolution of these complexes. Whilst much criticism has been levelled at the experimental approach of these studies, the kinetic and functional advantages of such interactions are evident e.g. they allow intermediates to be maintained at high concentrations on the surface of complexes and in the instance of membrane-associated enzymes the channeling of products to the next stage of metabolism would increase the specificity of interaction and the reaction rate.

The membrane association of proteins is still a subject of intense debate; Landriscina *et al.* (1970) proposed that

all proteins of the mitochondrial matrix interact with the inner membrane and vary only in their affinity for that surface. This hypothesis further suggests that rather than existing as two separate phases within the same organelle, the matrix and the inner membrane are a semi-continuous system. Thus, assigning a location to enzymes within such a system could be misleading when the organelle is subject to powerful disruptive techniques such as sonication. It should be noted that cytochrome c, which binds to the inner mitochondrial membrane with a Kd in the micromolar range is readily released following sonication (Erecinska et al., 1975).

With the availability of antisera directed against PDC and its constituent subunits we were able to investigate, in a more precise manner than previous workers (Sumegi & Srere, 1984), the association of this multienzyme complex with the matrix surface of the inner mitochondrial membrane. This enzyme system offers many advantages to such a study, not the least of which is that the complex and its association with the inner mitochondrial membrane is stable to prolonged sonication. It has been demonstrated, that PDC, although commonly regarded as a soluble enzyme, exhibits the characteristics of a tightly-bound, peripherally-located complex. The interaction is stable to repeated washing in buffers containing 0.15 - 3M NaCl. Dissociation of the membrane-bound enzymes results in the release of E1 and E3 while the E2-X core assembly remains bound to the inner membrane. The functional advantages of this association have been discussed in Chapter 3.

It is known that the outside surface of proteins help to locate the enzyme in the cell and to specify its interaction with other related enzymes e.g. the multisubunit array of the core of PDC provides the binding site for the other constituent enzymes i.e. E1 and E3. Workers investigating the conservation of amino acids within cellular proteins have indicated that much more of a protein is conserved than its active site (McConkey, 1982). PDC has an M_r value several million in excess of the analogous BCDC and OGDC. It is feasible to postulate that the increased dimensions of this complex relative to the other enzymes of this family, and in particular the size of the core enzyme, may serve to provide additional binding sites on the enzyme surface. These additional sites may serve to anchor the complex to the membrane surface i.e. via the E2 component or perhaps to locate component X on the surface of the complex. Payens (1983) has suggested that random, non-specific binding of the substrate to the additional sites on the enzyme surface may serve to trap substrates and enhance diffusion to the active site. This may have important implications for the transport of pyruvate across the inner mitochondrial membrane.

Work in our laboratory has provided evidence that component X is capable of becoming acetylated. It is possible that this 50 000 M_r polypeptide may provide an additional channel for the movement of acetyl groups to the membrane-associated biosynthetic processes e.g. ubiquinone formation or perhaps in the transport of acetyl groups to the cytoplasm.

In this context, it has been reported that citrate, in brain tissue, represents a poor source of acetyl groups required for the synthesis of acetylcholine. It is therefore possible that component X could fulfil this function and mediate the transfer of acetyl groups to the cytoplasm either as acetate or perhaps pyruvate.

From the wealth of information now available on protein:protein and protein:membrane interactions it is apparent that these levels of organisation are more widespread than previously believed. In no biological entity, be it a subcellular organelle, bacterial cell or eukaryote does it appear that structure depends on random juxtaposition. Organised arrays of biological components permit effective use of elements by the reduction in the number of molecules needed to achieve the same chemical reactions with seemingly opposite objectives being achieved in the same biospace.

The structural complexity of cells and organelles has been made clearly visible by electron microscopy. Despite the apparent need for usefulness of and physical existence of compartments and microenvironments within cells, a major problem has existed in terms of obtaining experimental evidence showing metabolic functioning of compartments *in vivo*.

6.2 FUTURE WORK ON PDC

Future work on the binding of PDC to the inner mitochondrial membrane will involve attempts to identify the putative membrane receptor(s). Employing substrate-dependent cross-

linking via the lipoyl-thiol group we would hope to locate crosslinked products containing E2 and/or X and specific membraneous polypeptides by immunoblotting with the appropriate subunit specific antisera. It may be possible to obtain subunit specific antisera to components of NADH dehydrogenase, in an attempt to make a formal identification of the binding site.

The association of the analogous 2-oxoglutarate and branched chain 2-oxoacid dehydrogenase with the membrane would also be investigated. Parallel studies on these and PDC may allude to a possible function of component X, if these complexes were found not to associate with the matrix surface of the inner membrane.

We would also hope to address the following points:

(i) does the phosphorylation state of PDC alter its affinity for the mitochondrial membrane?

(ii) to assay for the incorporation of acetyl groups from $[2\text{-}^{14}C]$ -pyruvate into E2 and protein X in the membrane-bound form in the absence of CoASH. This will allow us to ascertain whether $[^{14}C]$ -acetyl groups can be transferred into other membrane bound polypeptides or to the lipid components.

As these studies have already been initiated on the soluble enzyme it will also be of interest to compare the properties of the membrane-bound enzyme to that of the solubilised complex. This phenomenon of allotopy has already been reported for the ATP synthetase complex.

6.3 TOPOGRAPHICAL AND BIOSYNTHETIC STUDIES ON PTP

It is now clear that the limited coding potential of the mitochondrial genome necessitates that > 90% of mitochondrial polypeptides are nuclearly-encoded and synthesised on cytoplasmic ribosomes, normally as larger M_r precursors containing an N-terminal extension (Hay et al., 1981). Subsequent uptake to the organelle is a post-translational event dependent on the presence of an electro-chemical potential (μ_{H^+}) (Gasser et al., 1982; Schleyer et al., 1982). Proteolytic cleavage of the precursor molecule is achieved by a specific matrix protease during or shortly after uptake (Mori et al., 1980; McAda and Douglas, 1982; Böhni et al., 1983; Schmidt and Neupert, 1984). The final stage in the import process is the assembly of the mature protein to functional units within the mitochondrion. In contrast to the other areas of mitochondrial biogenesis this stage is poorly understood.

In recent years, rapid progress has been made in delineating the complex sequence of molecular events involved in mitochondrial protein import, primarily in yeast and fungal synthesis. With the cloning and sequencing of genes for several nuclear encoded mitochondrial proteins, the essential features of presequences have been analysed by the production of chimeric proteins and many gene fusion/deletion techniques. For example, the 25 amino acid extension sequence of yeast cytochrome c oxidase subunit IV can induce specific uptake and processing of mouse dihydrofolate

reductase, which is normally a cytoplasmic enzyme (Hurt et al., 1984). While it appears that a minimum of 12 residues may be necessary to direct uptake to the mitochondrion, there is considerable variability in the length of transit sequences reported to date (M_r 1500 - 70 000). Evidence suggests that in addition to directing polypeptides to the surface of the mitochondrion, these N-terminal sequences are involved in:
(i) regulating uptake to specific mitochondrial subcompartments, (ii) maintaining hydrophobic membrane proteins in a soluble state during transit and (iii) preventing premature assembly of multimeric aggregates in the cytoplasm.

In contrast to other areas of mitochondrial biogenesis very little information is available on the import of transmembrane activities to this organelle. Indeed, only the ANT from N. crassa and the brown fat mitochondria uncoupling protei have been studied in any detail, each being synthesised as precursors with the same M_r value as the mature protein (Arends and Sebald, 1984; Baker and Leaver, 1985; Adrian et al., 1986; Bouilland et al., 1986).

We studied the biosynthesis of PTP by direct immuno-precipitation studies on [^{35}S] -methionine-labelled detergent extracts of cultured mammalian cells which had been incubated for 4 h in the presence and absence of uncouplers e.g. FCCP or 2,4-DNP. This routine procedure has been utilised for the immune-precipitation and identification of several mitochondrial precursor polypeptides in permanent cell lines with a high rate of aerobic activity. The extension of

these studies to the PTP required the production of high quality, monospecific antiserum against this protein.

We and others have experienced considerable difficulty in raising monospecific antiserum to this integral membrane protein. It is believed that many of these problems have arisen due to the highly conserved nature of structural epitopes within membrane proteins. Sequence analysis of many membrane proteins has predicted that an α-helical structure is the most efficient way to reduce energetically unfavourable reactions between proteins and lipids in membranes. Since the mitochondrial inner membrane contains many transport proteins, these α-helical structures are thought to predominate and indeed this has been supported by spectroscopic evidence. It seems likely, therefore, that the poor specificity elicited by our previous antisera directed against this protein, has arisen primarily due to a cross reaction with epitopes common to other membrane proteins.

Despite obtaining antiserum which allows us to detect between 5-20 ng of PTP in SDS-extracts of whole cells with absolute specificity by immunoblot analysis, we have been unable to utilise this strong cross reaction to studying the biosynthesis of the PTP. The identification of a precursor molecule to the PTP has remained elusive when applying the in vivo approach successfully employed for many other mitochondrial proteins. This, again, appears to be a problem associated with membrane proteins in general.

It seems likely that many of the structural epitopes are maintained in a native state during the immunoprecipitation procedure and the specificity of interaction obtained with the SDS-denatured protein in immunoblotting could be obtained due to interaction of the antiserum with other proteins.

Many of the difficulties experienced in studying membrane proteins have been offset by the recent advances in gene sequencing. Runswick *et al.* (1987) have successfully isolated a cDNA clone from a bovine heart library and utilising rapid DNA sequencing techniques have deduced the amino acid sequence corresponding to the bovine heart PTP. They have shown that the protein contains an N-terminal extension which provides the PTP with a 5 000 M_r presequence. This result is in contrast to the information available on the comparable proteins i.e. the brown fat uncoupling protein and the ANT, where the precursors are synthesised with the same M_r value as the mature protein. Moreover, it has been demonstrated that in yeast, the PTP precursor is synthesised as the mature protein.

The additional advantage of DNA sequencing studies in deducing the amino acid sequences is that they circumvent many of the problems encountered by us and other workers (Kolbe and Wohlrab, 1985) utilising conventional protein sequencing techniques e.g. Edman degradation. This deduced primary structure has allowed Runswick *et al.* (1987) to propose, from structure prediction data bases, that the

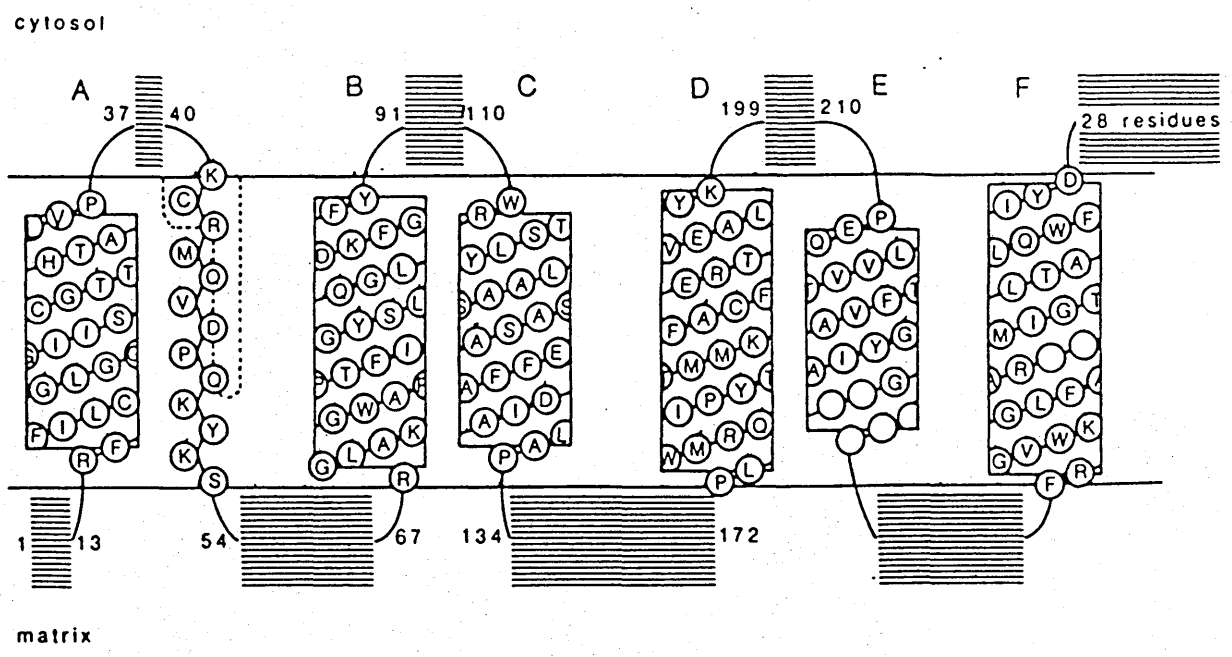


Fig. 6.1: Proposed model for the arrangement of PTP in the inner mitochondrial membrane (Aquila et al., 1987).

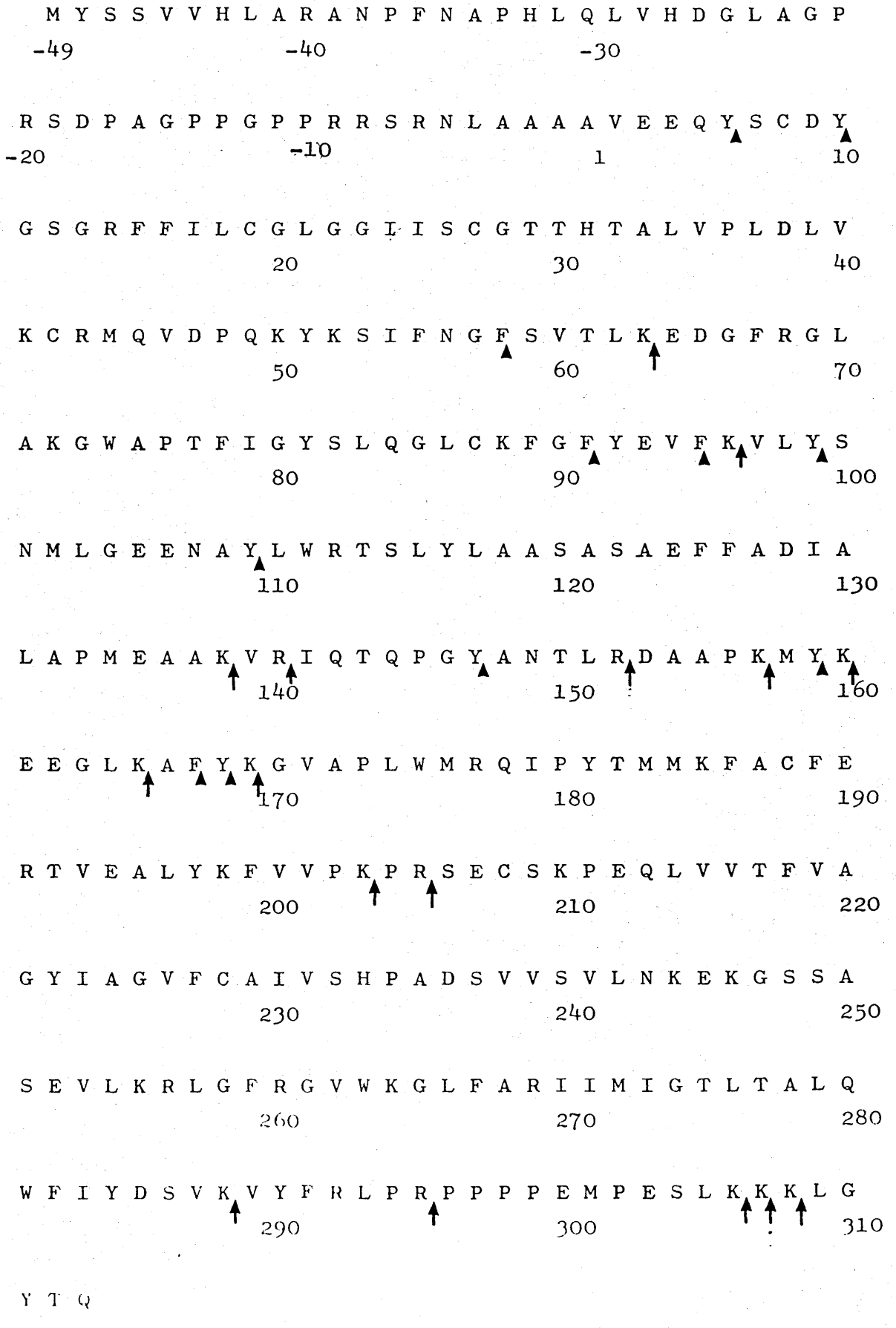
This suggested arrangement of the phosphate transport protein within the mitochondrial inner membrane. The six membrane spanning regions are labelled A-F from the amino terminus. A possible β strand near a pore (dashed line) is shown between helices A and B. The intervening hydrophilic segments are indicated by hatched boxes as their structure is unclear.

PTP contains six membrane spanning α-helices, a model also suggested by Aquila et al.,(1987) who have recently obtained sequence information on the majority of the protein. Comparison of this sequence information with that available for the ANT and the uncoupling protein has prompted Aquila et al.,(1987) to propose a common model for all three carrier proteins (Fig. 6.1). Aligning the sequence and topographical information contained within this model with the gene sequence presented by Runswick et al.,(1987) shows certain inconsistencies with data obtained from topographical studies (Chapter 5).

Extensive analysis of the susceptibility of the PTP, at both the matrix and cytoplasmic surface of the mitochondrial inner membrane to externally added proteases has allowed the testing of the predicted orientation of this membrane protein. As Figure 6.2 demonstrates, there are several chymotrypsin and trypsin sensitive sites available at both the matrix and cytoplasmic surface of the mitochondrial inner membrane. If the model predicted by Aquila et al.,(1987) is correct, a shift in mobility corresponding to the digestion of protease sensitive domains would be readily detected by immunoblot analysis following SDS-PAGE. As discussed in Chapter 5, PTP was found to be insensitive to proteolytic cleavage at either surface of the mitochondrial inner membrane. Thus, if the hydrophilic domains predicted by Aquila et al.,(1987) exist at the matrix or cytoplasmic surface they must be protected from degradation by other proteins or be situated very close to the bilayer.

Fig. 6.2: Deduced amino acid sequence of the bovine heart mitochondrial phosphate transport protein.

The amino acid sequence was deduced from the DNA sequence of a cDNA encoding the precursor of the bovine heart mitochondrial phosphate transport protein (Runswick et al., 1987). The import sequence of the protein runs from amino acids -49 to -1 and the mature protein from 1-313. Regions of the protein which are exposed at either surface of the mitochondrial inner membrane are illustrated in Figure 6.1. The protease sensitive sites within the exposed regions are indicated by (↑) for trypsin and (▲) for chymotrypsin.


M Y S S V V H L A R A N P F N A P H L Q L V H D G L A G P
-49
-40
-30
R S D P A G P P G P P R R S R N L A A A A V E E Q Y S C D Y
-20
-10
1
10
G S G R F F I L C G L G G I I S C G T T H T A L V P L D L V
20
30
40
K C R M Q V D P Q K Y K S I F N G F S V T L K E D G F R G L
50
60
70
A K G W A P T F I G Y S L Q G L C K F G F Y E V F K V L Y S
80
90
100
N M L G E E N A Y L W R T S L Y L A A S A S A E F F A D I A
110
120
130
L A P M E A A K V R I Q T Q P G Y A N T L R D A A P K M Y K
140
150
160
E E G L K A F Y K G V A P L W M R Q I P Y T M M K F A C F E
170
180
190
R T V E A L Y K F V V P K P R S E C S K P E Q L V V T F V A
200
210
220
G Y I A G V F C A I V S H P A D S V V S V L N K E K G S S A
230
240
250
S E V L K R L G F R G V W K G L F A R I I M I G T L T A L Q
260
270
280
W F I Y D S V K V Y F R L P R P P P P E M P E S L K K K L G
290
300
310
Y T Q

In contrast to their model, we propose that these hydrophilic domains are arranged within the structure of the protein such that they form a hydrophilic channel which will allow the movement of phosphate and H^+ across the inner mitochondrial membrane. This hypothesis is supported by the DCCD-reactivity of the phosphate carrier. The predicted N-terminal extension identified by Runswick *et al.*, (1987) which is in contrast to the precursors of both the ANT and uncoupling protein, may serve to maintain the precursor molecule in a conformation which will prevent its aggregation to the dimeric unit proposed for its companion protein, the ANT.

6.4 FUTURE WORK ON THE PTP

Future work on the PTP will concentrate primarily on isolating the gene encoding this mitochondrial protein. For this purpose we would hope to utilise the cross-reactivity of the anti-rat liver PTP - IgG to identify and develop an isolation procedure for the yeast carrier.

The major aim would be to clone and sequence the gene for the yeast PTP by immunological screening of a λgt 11-housed genomic library derived from *S. cerevisiae*. Previous work in our laboratory has demonstrated that the yeast phosphate carrier is synthesised with the same M_r value as the mature protein. It would therefore be of interest to establish whether this is a general feature of this protein or unique to the yeast protein.

The availability of the cloned gene would allow us to (a) investigate the structure and mechanism of PTP and (b) provide the opportunity for *in vitro* and *in vivo* studies in the control of its expression and mode of integration into the mitochondrial inner membrane.

The yeast system lends itself favourably to the technique of gene transplacement, this will allow us to produce a PTP^- strain and a range of strains in which alterations will be incorporated into the PTP. The phenotype of these strains will be assessed by their ability to grow on non-fermentable carbon sources e.g. as a criterion for the functional assembly of the PTP and hence the presence of mitochondria which are active in ATP production.

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