CHARACTERIZATION AND BIOSYNTHESIS OF MITOCHONDRIAL PROTEINS

by

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TO MY PARENTS

Research is the seeking of knowledge
and the beginning of wisdom but the
greatest asset is common sense.

LIST OF CONTENTS

LIST OF FIGURES

3.3.5	SDS-polyacrylamide gradient gel analysis 14-20% (w/v) of purified phosphate transport protein from rat liver and beef heart mitochondria.
3.3.6	Postulated mechanism of mitochondrial swelling in solutions of isotonic ammonium salts.
3.3.7 (a) & (b)	Inhibition of mitochondrial swelling in isotonic $(NH_4)_2HPO_4$ medium in the presence of N-ethylmaleimide.
3.3.8 (a) (b) & (c)	N-[^{3}H]-ethylmaleimide labelling studies of the phosphate transport protein from rat liver mitochondria.
3.3.9 (a) & (b)	Partial inhibition of phosphate uptake in rat liver mitoplasts pre-incubated with a specific antiserum to the phosphate transport protein as measured by the NH_4^+-ion swelling technique.
4.3.1 (A) & (B)	ELISA estimations of the antibody titres of phosphate transport protein and fumarase antisera respectively.
4.3.2	Immune-replica analysis of purified phosphate transport protein versus antiserum directed against the protein.
4.3.3	Immune-replica analysis of mammalian tissue-culture cell extracts versus phosphate transport protein antiserum.
4.3.4	Effect of polyclonal, rabbit anti-fumarase serum on purified enzymic activity.

4.3.5	Immune-replica analysis of purified pig heart fumarase versus anti-fumarase serum.
4.3.6	Immune-replica analysis of cultured mammalian cell extracts versus anti-fumarase serum.
4.3.7	Estimation of fumarase and citrate synthetase activities in digitonin treated PK-15 cells.
4.3.8	Immune-replica analysis of beef heart mitochondrial subfractions versus anti-fumarase serum.
5.3.1	The effect of pre-incubation time in (-) methionine (-) serum medium on the incorporation of [^{35}S]-methionine into BRL cell protein.
5.3.2	Time-course to measure the extent of incorporation of [^{35}S]-methionine into BRL cellular protein in (-) methionine and low methionine media.
5.3.3 (A) & (B)	A study of the effects of various inhibitory compounds on the synthesis of mitochondrially-encoded proteins.
5.3.4	Time-course of the effect of 10μM FCCP on the incorporation of [^{35}S]-methionine into total BRL cell proteins.
5.3.5	Immune-precipitation of fumarase from PK-15 and NBL-1 cells labelled overnight with [^{35}S]-methionine.
5.3.6	Biosynthesis of fumarase in PK-15 cells labelled with [^{35}S]-methionine in the presence of an inhibitor of mitochondrial import.

5.3.7 Biosynthesis of fumarase in BRL cells labelled with [^{35}S]-methionine in the presence of an inhibitor of mitochondrial import.

5.3.8 Effect of the uncoupler CCCP on the growth of the rho$^-$ mutant strain of S.cerevisiae.

5.3.9 (A) Immune-replica analysis of the yeast rho$^-$ mutant strain treated with increasing concentrations of CCCP (Fumarase antiserum).

(B) Quantitation of precursor accumulation in yeast cells after growth in the presence of CCCP.

5.3.10 (A) Immune-replica analysis of the yeast rho$^-$ mutant strain treated with increasing concentrations of CCCP (phosphate transport protein antiserum)

(B) Immune-replica analysis of a yeast mitochondrial extract with phosphate transport protein antiserum.

5.4.1 Generation of two mRNA species encoding the mitochondrial and cytosolic forms of fumarase.

LIST OF TABLES

ABBREVIATIONS

The abbreviations used in this thesis are as given in 'Instructions to Authors' published by the Biochemical Journal (1985) with the following exceptions:

BSA	- bovine serum albumin
CCCP	- carbonyl cyanide m-chlorophenylhydrazone
con A	- concanavalin A
DMSO	- dimethyl sulphoxide
DOC	- sodium deoxycholate
DTNB	- 5,5' dithio(bis)2-nitrobenzoic acid
DTT	- dithiothreitol
FCCP	- carbonyl cyanide 4-(trifluoromethoxy)phenyl-hydrazone
Bio-gel HTP	- hydroxylapatite
Leupeptin	- acetyl-L-leucyl-L-leucyl-L-argininal
Mops	- 4-morpholinopropanesulfonic acid
NEAA	- non-essential amino acids
OPD	- o-phenylene diamine
OAA	- oxaloacetic acid
PBS	- phosphate buffered saline
PMSF	- phenylmethylsulfony/fluoride
PPO	- 2,5-diphenyloxazole
SDS	- sodium dodecyl sulphate
TEMED	- N,N,N',N'-tetramethylethylenediamine
TMPD	- tetramethylphenylenediamine
Tween 20	- polyethylene sorbital mono-laureate
WGA	- wheat germ agglutinin

SUMMARY

Mitochondria have their own very limited genetic system which codes for a few proteins of the inner membrane respiratory chain. As such, the bulk of mitochondrial proteins are nuclear encoded, synthesised in the cytoplasm on free ribosomes (in general as larger M_r precursors) and subsequently imported into the correct compartment within the organelle. We were particularly interested in studying two mitochondrial proteins with respect to this problem of biosynthesis and import into the organelle.

The first of these proteins, the phosphate transport protein is an integral, inner transmembraneous protein of mitochondria which has a major role to play in energy metabolism in the cell. It had only been partially characterized when we started to investigate it and as such this represented an interesting area of research. Purification of the phosphate transport protein from rat liver mitochondria was carried out by extraction in an 8% (v/v) Triton X-100 buffer and then adsorption chromatography on hydroxylapatite followed by Celite. SDS-polyacrylamide gel electrophoresis 10% (w/v) demonstrated that the protein band was apparently homogeneous when stained with Coomassie Blue and had an M_r of 34,000. However, when this gel was overlayed with ^{125}I-labelled concanavalin A to detect glycoproteins and exposed to autoradiography, numerous low and high M_r glycoprotein contaminants could be visualized. To overcome this problem, mitochondria were pre-extracted with a 0.5% (v/v) Triton X-100 buffer prior to purification. This initial extraction solubilised about 80% of the mitochondrial protein and effected quantitative removal of the contaminants.

SDS-polyacrylamide gradient gel electrophoresis 14-20% (w/v) of the hydroxylapatite and Celite eluates revealed one major band of M_r 34,000

when stained with Coomassie Blue. Densitometric scanning of the gel demonstrated that the protein was greater than 95% pure. This result was in marked contrast to the recent findings of several groups who have purified the phosphate transport protein from bovine and pig heart mitochondria, respectively. The hydroxylapatite eluates, when resolved on a similar gradient gel, demonstrated four to five Coomassie Blue staining bands.

In order to identify the protein, the sulphydryl group sensitivity of the phosphate transport protein was employed. Labelling studies with N-[^{3}H]-ethylmaleimide showed that only the 34,000 M_r band was labelled in both the hydroxylapatite and Celite fractions, when purified from rat liver mitochondria. This result was consistent with the known sulphydryl group sensitivity of the phosphate transport protein. Further confirmation has been provided by preliminary experiments with an antiserum directed against the 34,000 M_r protein. Partial inhibition of mitoplast swelling in isotonic ammonium phosphate buffer was achieved when mitoplasts were incubated with the specific antiserum.

To study the biosynthesis and import of this protein into mitochondria, in general, cultured mammalian cell lines were employed. The rabbit, polyclonal antiserum raised against the 34,000 M_r band proved to be unsuccessful when testing its specificity by immune-blotting and later with the in vivo mitochondrial import studies. Numerous polypeptides were observed when crude tissue-culture extracts were challenged with the anti-phosphate transport protein serum and analysed by the immune-replica technique. However, a preliminary experiment in the yeast rho^{-} strain (ρ^{-}), which can accumulate large quantities of mitochondrial precursors in the presence of uncoupler, indicated that the protein was probably not made as a larger M_r precursor. The antiserum raised against the rat liver mitochondrial phosphate transport protein strongly cross-reacted with a yeast protein

of 29,000 M_r under the above conditions. In addition the identity of this cross-reacting yeast protein has been partially provided by the immune-replica technique, where the antiserum specifically recognised a protein of 29,000 M_r in a yeast mitochondrial extract.

The second protein of particular interest to us was the enzyme fumarase. This enzyme has been shown to be located in the cell cytosol as well as in the mitochondrial matrix. In the mitochondrial matrix, fumarase has a functional role to play in the operation of the 'citric acid cycle' and in the cell cytosol has an indirect role in the 'urea cycle', the tyrosine-oxidizing system and in purine biosynthesis. It has been reported in the literature that both forms of the enzyme are of the same M_r (subunit M_r of 49,000). In studying the biosynthesis of this enzyme, an additional problem to be overcome, is to determine the mechanism by which this bimodal distribution occurs.

The pig heart enzyme, which had a purity of greater than 95%, was purchased from Sigma. A rabbit, polyclonal antiserum was raised against the protein, so that we could monitor the biosynthesis of the enzyme in vivo employing cultured mammalian cells. Initially, the specificity of the polyclonal antiserum was tested in one of two ways:

(1) Enzymic activity was measured in the presence of the antiserum and resulted in a greater than 80% inhibition.

(2) Immune-blotting of the antiserum against various crude extracts, in which the antigen-antibody complexes formed were detected with ^{125}I-labelled protein A, showed that it was monospecific. The crude extracts included beef heart submitochondrial fractions.

To determine which of the following mammalian tissue-culture cell lines to employ for the in vivo biosynthetic studies, immune-blotting of

the antiserum against various crude cell extracts was carried out. The cell lines used were Buffalo rat liver (BRL), Pig kidney (PK-15) and Bovine kidney (NBL-1), respectively. All the crude cell extracts prepared from these cell lines cross-reacted specifically with the antiserum, although the PK-15 and NBL-1 cell extracts reacted most strongly. Additionally, in the case of BRL cells, the mitochondrial (M_r 45,000) and cytosolic fumarases (M_r 47,000) appeared to differ in M_r value.

Initial studies to determine the mechanism of synthesis of fumarase were carried out by labelling PK-15 and NBL-1 cells overnight in the presence of L-[^{35}S]-methionine. Immune-precipitation and subsequent detection by fluorography showed the presence of a band of M_r 47,000, corresponding to mature fumarase. Preliminary precursor experiments in which PK-15 cells were labelled in the presence of the uncoupler FCCP (10μM), demonstrated the existence of two polypeptide bands which had M_r values of 47,000 and 51,000 respectively. The 47,000 M_r band is assumed to represent cytosolically-located fumarase whereas the 51,000 M_r polypeptide is the cytosolic precursor form of the mitochondrially-located fumarase, under the conditions employed. When a similar experiment was performed in BRL cells, a single specific polypeptide of M_r 47,000 could be immune-precipitated from the cell extract. This polypeptide was assumed to represent both cytoplasmically-located fumarase as well as the precursor form of the mitochondrial enzyme. In the absence of uncoupler two proteins with M_r values of 47,000 and 45,000 respectively, were recognised in a specific manner by the antiserum, confirming the immune-replica data. Analogous examination of yeast (ρ) immune-blots, to those employed with the phosphate transport protein, suggest the accumulation of a larger M_r precursor to fumarase when cells were grown for 1-2 generations in increasing uncoupler concentration.

Finally, the data obtained has enabled hypothetical pathways to be

put forward to explain the mechanism by which this enzyme becomes distributed between two cellular compartments.

CHAPTER ONE

INTRODUCTION

1.1 THE MITOCHONDRIAL GENOME

1.1.1 Introduction

Mitochondria contain their own genetic system which is responsible for the synthesis of about 5% of the proteins found in the organelle. Additionally, the mitochondrial genome encodes all the ribosomal and transfer RNAs of its separate translation system. The mitochondrial genome is autonomously replicated and is expressed within the confines of the organelle. Each mitochondrion in a cell contains several copies of the closed circular DNA genome. The M_r value of the DNA varies from species to species within a narrow range: between 10×10^6 in animal tissue and 70×10^6 in higher plants.

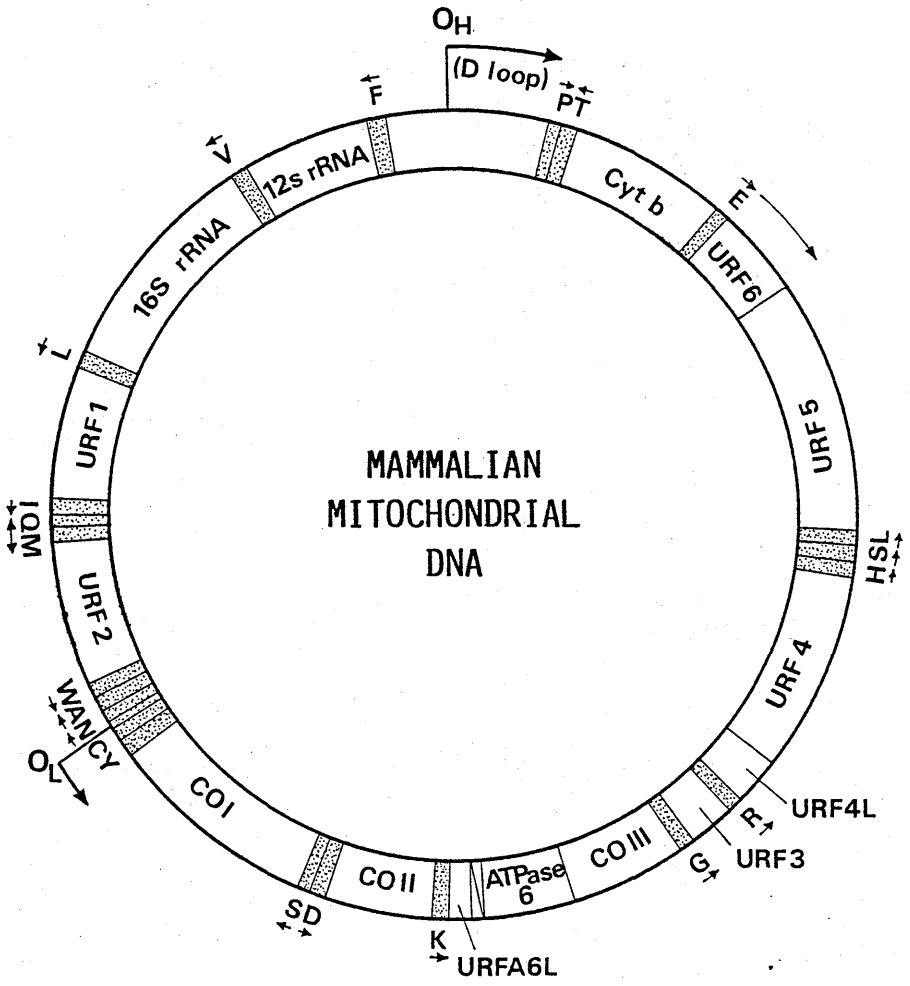
1.1.2 Genomic Organisation of Mitochondrial DNA

The complete sequence and organisation of three mammalian mitochondrial genomes have recently been elucidated (Bibb *et al.*, 1981, Anderson *et al.*, 1981; Anderson *et al.*, 1982). They all show extreme economy in organisation, in that there are none or very few non-coding bases between adjacent genes (Fig. 1.1.1). It can be seen that the mammalian mitochondrial genome encodes two ribosomal RNA genes (12S and 16S), 22 transfer RNA genes (tRNA) and 13 protein-coding genes. Encoded on the heavy strand of the genome (H-strand) are the two ribosomal RNA genes, 14 tRNA genes and 12 of the 13 protein-coding genes while the remaining eight tRNA genes and one protein-coding gene are expressed by the light strand (L-strand).

Protein products of known function have been assigned to five of the 13 reading frames. They were identified from the amino acid sequence of purified protein and by homology with DNA sequence of

Fig. 1.1.1

Genomic map of Mammalian Mitochondrial DNA



The dotted 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 counter clockwise 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: cytochrome b, URF: unassigned reading frame, O_H and O_L: the origins of H and L strand replication, respectively. (Reproduced from Clayton, 1984).

known genes in other mitochondrial systems (Bibb et al., 1981, Anderson et al., 1981, Thalenfeld and Tzagoloff, 1980, Macino and Tzagoloff, 1980; Nobrega and Tzagoloff, 1980). The remaining eight unidentified reading frames (URF) are highly conserved in bovine and human mitochondrial DNA (Anderson et al., 1981), showing about 70% nucleotide and amino acid sequence homology. In addition, there is evidence of protein products corresponding to human mitochondrial DNA URFs 1, 3, 6 and A6L (Mariottini et al., 1983, Oliver et al., 1983; Chomyn et al., 1983). It is therefore believed that most, if not all, of these reading frames represent genes for mammalian mitochondrial proteins. There is only one region of mammalian mitochondrial DNA of significant size which contains no open reading frames, the displacement loop (D-loop). This region has been shown to contain the origin of heavy strand replication and sequences relevant to the promotion of RNA synthesis from each template strand (Montoya et al., 1982; Montoya et al., 1983).

In contrast, the yeast mitochondrial genome which is about five times the size of mammalian mitochondrial DNA, appears to be less economically organised. It codes for two ribosomal RNAs, 25 tRNAs and 16 proteins, 10 of which are of unassigned function at present. The genomic organisation of the yeast mitochondrial DNA is different to that of its mammalian counterpart, as shown by the fact that the large (21S) and small (16S) ribosomal RNA genes are 25,000 base pairs apart on the genome. In addition, the majority of tRNA genes are clustered on the H-strand, near the large ribosomal gene.

Finally, several yeast genes have been shown to contain intervening sequences (introns) in their structure, a feature absent from mammalian mitochondrial genes. The large ribosomal RNA gene has been demonstrated to contain one intervening sequence (Borst and Grivell, 1978)

which varies in length in different yeast strains. Cytochrome b gene in S.cerevisiae D273/10B contains two intron sequences (Nobrega and Tzagoloff, 1980) while evidence from Van Ommen et al. (1979), shows that this same gene in a different yeast strain contains four intervening sequences.

It can clearly be seen from these examples that there are marked differences in the overall organisation of genes in various eukaryotic mitochondria, as well as in different strains of the same organism.

1.1.3 Mitochondrial Genetic Code

The genetic code which is invariant from viruses to man is not as originally thought universal. Mitochondria have their own version which is subject to variation in different organisms. Mammalian mitochondria read AGA and AGG as stop signals rather than as codons for the amino acid arginine whereas in the mitochondria of Neurospora crassa (N.crassa) and Aspergillus nidulans (A.nidulans) these codons are translated as arginine. Additionally, mammalian mitochondria utilise the standard UAA as a termination codon. In many human mitochondrial messengers this latter termination codon is generated from the primary transcript by polyadenylation, since the transcript ends in either U or UA. Mammalian and yeast mitochondria translate AUA as methionine rather than isoleucine whereas in N.crassa and A.nidulans it is translated as isoleucine. In addition, AUA and AUU can serve as start signals instead of AUG in mammalian mitochondria. A further difference in the mitochondrial genetic code occurs in yeast which read the four codons beginning with CU as threonine rather than as leucine. Finally, to date the mitochondria of mammals, yeast, N.crassa and A.nidulans specify the amino acid tryptophan by the

'universal' stop codon UGA as well as by the usual UGG codon.

There are 61 codons in the mitochondrial genetic code which, according to the 'Wobble' hypothesis proposed by Crick in 1966, would require a minimum of 32 tRNAs to decipher them all, using a G:U 'Wobble'. Contrary to this hypothesis, mammalian mitochondria and several other lower eukaryotes appear to require substantially fewer tRNAs to read their entire genetic code. In the case of mammalian mitochondria only 22 tRNAs are necessary to fulfil such a function. Each of the 22 tRNAs in mammalian mitochondria is able to read a 'family' of either two or four synonymous codons. The tRNAs for the two codon families seem to have conventional G:U 'Wobble' anticodons whereas the tRNAs for four codon families have anticodons with a U in the 5' position. There are two possible mechanisms to explain the four codon families; either the U base in the 5' position of the anticodon pairs with all four of the 3' codons or the anticodons read only the first two bases and ignore the last one, a hypothesis proposed by Lagerkvist (1978). An interesting question is how some tRNAs with U in the 'Wobble' position are held to reading two codons rather than four. In the mitochondria of yeast and N.crassa and in many non-mitochondrial tRNAs, such recognition is prevented by chemical modification of the 'Wobble' U, but so far sequence analysis of mitochondrial tRNAs in animals has not revealed any modification at this position.

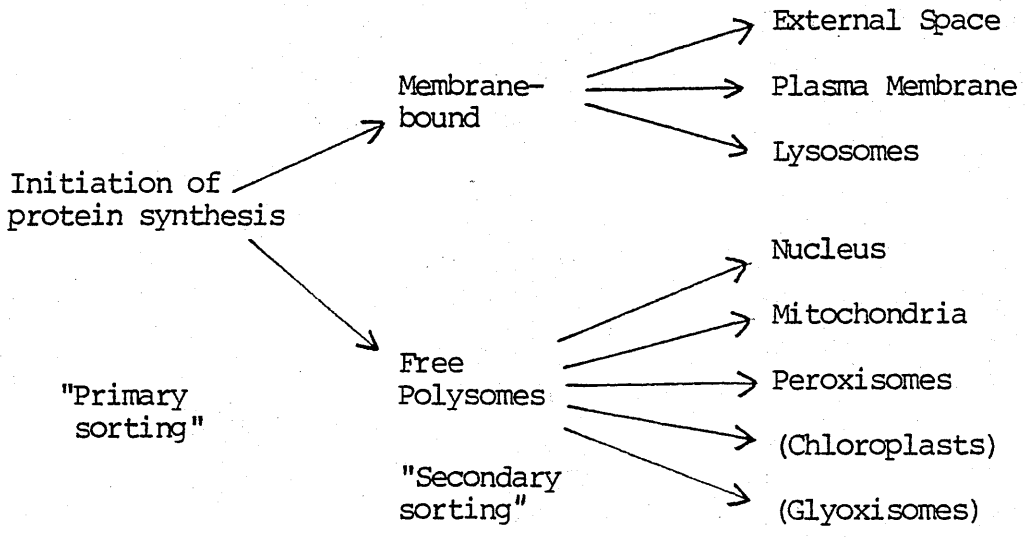
The mammalian mitochondrial tRNAs are also unusual in that most lack features common to all tRNAs. The most extreme case is that of the tRNA for serine which lacks the D arm (de Bruijn et al., 1980). In general, mitochondrial tRNAs are apparently stabilised by fewer tertiary interactions. These missing or different tertiary interactions may mean that the mammalian mitochondrial tRNAs have more freedom than cytoplasmic tRNAs reflecting a different interaction

with the ribosome.

1.2 Distribution of Secretory Proteins

1.2.1 Historical Background

Although the basic principles and molecular details of protein synthesis have long been established, one of the most intriguing problems to emerge in recent years has been to understand how newly-synthesised polypeptides, which are predominantly made in the cytosol, are distributed to their final locations in different sub-cellular compartments. The sub-cellular distribution of proteins in a eukaryotic cell can conceptually be divided into two 'sorting' events:



The present understanding of the mechanisms involved in the transport of polypeptides to the various sub-cellular compartments owes a great deal to the studies performed on the synthesis of secretory proteins in eukaryotic cells. The initial evidence on the mode of synthesis of secretory proteins was provided by electron microscopy studies and the discovery (Palade, 1955) that animal cells contain

membrane-bound ribosomes and ribosomes free in the cytoplasm. Subsequently, various secretory proteins were shown to be synthesised on the bound ribosomes while cytoplasmic proteins were formed predominantly on free ribosomes (reviewed by Palade, 1975). This distribution suggested that secreted proteins might cross membranes as growing chains rather than after completion of synthesis. Such a mechanism was supported by the observation of Sabatini and Blobel (1970) who showed that microsomes protected nascent chains of secretory but not of cytoplasmic protein from proteolytic degradation. The concept that the growing polypeptide chain is translocated through the membrane of the endoplasmic reticulum during protein synthesis was then recognised. The attachment of bound polysomes to membranes via the amino-terminal region of the growing polypeptide chain was first shown by Milstein et al. (1972) in their studies on the translation of immunoglobulin light chain mRNA in a cell-free system. They showed that a larger precursor containing extra amino acids at the amino-terminus was synthesised in the rabbit reticulocyte lysate but not in the presence of microsomal membranes. In 1975 (a,b), Blobel and Dobberstein presented the decisive experimental data which led to the formulation of the signal hypothesis with the following predictions:-

a) Initiation of translation of secretory proteins starts in the cytosol with the formation of an amino-terminal signal peptide that binds to the endoplasmic reticulum and initiates vectorial discharge of the growing polypeptide chain into the lumen of the endoplasmic reticulum.

b) Vectorial discharge must start before the chain reaches a certain length and be strictly coupled to protein synthesis (co-translational secretion).

c) Signal peptides are transient entities that are cleaved before chain termination.

1.2.2 Sequence and Structure of Signal Peptides

The signal hypothesis had a major impact on the development of this research area, leading to the determination of the sequences of numerous secretory proteins over the next few years. Initially, it was expected that the structure of signal peptides would be highly conserved, but this was found not to be the case, as these peptides varied in length from 15-30 amino acids (Kreil, 1981). There is little obvious homology among signal sequences, but closer examination reveals common primary structural features which may be crucial to the mechanism that leads proteins across membranes (Garnier et al., 1980). In all transient signal sequences studied so far there is a central region of hydrophobic residues with a minimum length of 9 residues. To demonstrate that translocation and processing of secretory proteins requires structural features determined by the primary amino acid sequence, Hortin and Boime (1980), investigated the effect of decreasing the hydrophobicity within this region. They incorporated the leucine analogue, β - hydroxyleucine into bovine preprolactin using a cell-free translational system and showed that the precursor was not processed to the mature protein, on addition of microsomal membranes.

Most signal peptides also contain residues with positively charged side chains, arginine and lysine, near the amino-terminus. Signal peptidase preferentially cleaves on the carboxyl-terminal side of the small, uncharged amino acids glycine, alanine, serine, cysteine and threonine, with alanine most commonly found.

In view of the lack of primary sequence homology among signal

peptides, attention was focused on the likely secondary structure of these sequences. In 1979, Austen employed empirical methods to predict the secondary structure of a variety of secretory proteins including prohormones and immunoglobulins. There was a tendency for α-helix formation in the central hydrophobic region of the signal sequence. Recently determined sequences and structural predications (Austen and Ridd, 1981) have confirmed that the central portion of all signal peptides exhibit a high potential for forming α-helix and to a lesser degree, extended or β-sheet conformation. In many cases β-turns, which would be expected to break this structure, were predicted to occur with high frequency close to the protease cleavage sites. The information gained from these structural studies has led to the design and synthesis of a consensus signal sequence by Austen et al. (1984). They studied the in vitro processing of nascent pre-prolactin, pre-forms of pancreatic digestive enzymes and pre-placental lactogen by microsomal vesicles in the presence of this consensus peptide. This peptide was shown to inhibit, in a concentration dependent manner, the processing of these secretory proteins. It would, therefore, appear that it is the three-dimensional structure and not the amino acid sequence of the signal peptide which is important for correct location of the nascent secretory proteins.

1.2.3 Molecular Aspects of Secretory Protein Synthesis

In recent years much attention has been directed towards a more detailed understanding of the mechanisms by which the signal peptide interacts with the rough endoplasmic reticulum. Warren and Dobberstein (1978) reported that when rough microsomes from canine pancreas were washed with high salt concentrations they lost the capacity to trans-

locate nascent immunoglobulin light-chains in a cell-free translational system. On re-addition of the salt extract, the microsomes were able to translocate this protein. Subsequently, the factor removed from the rough microsomes was shown to be a protein complex of M_r 250,000 (Walter and Blobel, 1980). It has a low affinity for ribosomes alone but a very high affinity for ribosomes synthesising secretory proteins. Addition of this protein complex to a cell-free translation system synthesising bovine preprolactin, blocked its synthesis as soon as the nascent chain was long enough to be recognised as a putative secretory protein (Walter and Blobel, 1981). The block remained in effect until salt-washed microsomes were added to the system. In 1982 Walter and Blobel illustrated that a 7S RNA species was also associated with this complex and subsequently named it the 'signal-recognition particle' (SRP). It functions by blocking translation of the nascent polypeptide chain and allows the protein to find the molecules required to mediate its membrane transfer.

The next step in the transfer must involve a system for removing the translational block and permitting transfer to proceed. A component required for the translocation of nascent secretory proteins was isolated from rough microsomes, using mild proteolysis in conjunction with high salt (Meyer and Dobberstein, 1980). The salt extract prepared from rough microsomes by such treatment was capable of restoring translocating activity to inactive membrane vesicles. Subsequent purification yielded a polypeptide of M_r 60,000 which represented the cytoplasmic domain of a larger membrane protein. To further characterize the membrane-bound molecule, Meyer et al. (1982a) raised antibodies to this polypeptide fragment. Immune-precipitation of solubilised, radiolabelled rough microsomal proteins, yielded a single polypeptide of M_r 72,000. On the basis of these findings, it

has been assumed to be the membrane SRP receptor. The protein functions by docking the SRP-ribosomal complex to the rough endoplasmic reticulum membrane and was, therefore, called the 'docking-protein' (Meyer et al., 1982b). There is very little information with regard to the mechanism of attachment of ribosomes to the membrane of the endoplasmic reticulum although it appears to involve two proteins, ribophorins I and II. Kreibich et al. (1978) demonstrated that these two proteins are only present in the rough endoplasmic reticulum.

Finally, the properties of the signal peptidase which cleaves the amino-terminal signal sequence prior to polypeptide chain completion are largely unresolved.

The current picture of the initial events involved in secretory protein synthesis are illustrated in Fig. 1.2.1. A summary of the molecular characteristics of the proteins involved in this system is shown in table 1.2.1.

1.3 Import of Proteins into Mitochondria

1.3.1 Introduction

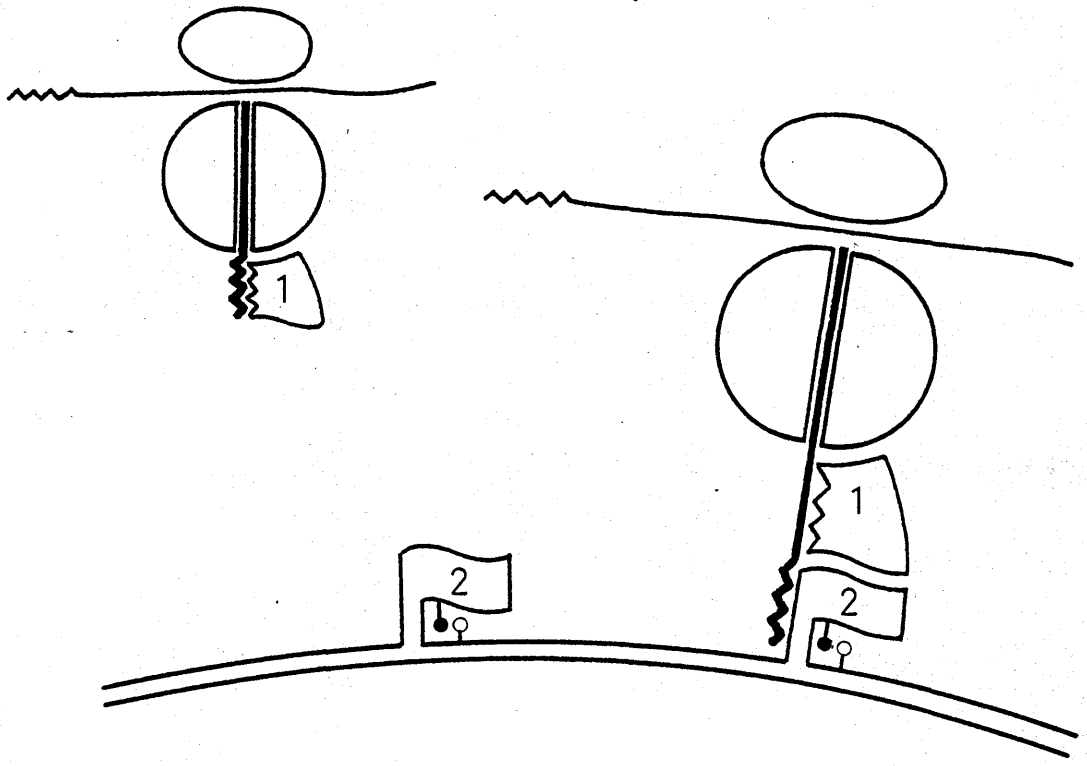
A special feature of the mitochondrion is the presence of a double membrane system which divides the organelle into two compartments: the intermembrane space and matrix bounded by the outer and inner mitochondrial membranes, respectively. These individual compartments each have their own special set of polypeptides (Ernster and Kuylenstierna, 1970) which are necessary for the normal functions of the organelle.

As previously discussed (Section 1.1), the mitochondrion has its own genetic material which has a very limited protein coding potential.

Fig. 1.2.1

CURRENT PICTURE OF THE INITIAL EVENTS IN SECRETORY PROTEIN SYNTHESIS

(Reproduced from Meyer et al., 1982b)



Sequence of events in ER-specific translocation of secretory proteins. Initiation begins in the cytoplasm on free ribosomes. Translation is blocked by SRP (component 1) after 70-80 amino acids have been polymerized and the signal sequence emerges from the large ribosomal subunit. This arrest of translation persists until contact is made with the 'docking protein' (component 2) which is a 72,000 M_r, ER-specific membrane protein. Translation then resumes and translocation proceeds.

TABLE 1.2.1

SUMMARY OF THE MOLECULAR AND FUNCTIONAL PROPERTIES OF PROTEINS INVOLVED IN SECRETORY PROTEIN SYNTHESIS

PROTEIN	MOLECULAR CHARACTERISTICS	FUNCTION
Signal Recognition Particle (SRP)	Sedimentation Coefficient of 11S 6 polypeptides M_r - 72,000, 68,000 54,000, 19,000, 14,000 and 9,000 Also contains 7S RNA species of unknown function	Interacts with the signal sequence extruding from large ribosomal subunit and arrests protein translation
Docking Protein (DP)	M_r of 72,000	Receptor for the SRP; may be required for translocation of nascent polypeptide chain across endoplasmic reticulum membrane
Ribophorins (I and II)	Glycoproteins with M_r of 65,000 and 63,000 respectively	Ribosome binding proteins
Signal Peptidase	?	Cleaves transient signal sequence from growing nascent polypeptide chain

Most mitochondrial proteins (greater than 95%) are nuclear-encoded and thus must be synthesised on cytoplasmic ribosomes before transport to the mitochondrion. Both inner and outer mitochondrial membranes present a barrier to the passage of most proteins. As a result, how do polypeptides destined for one of the inner mitochondrial compartments translocate across one or both membranes? The import process must also be highly specific since each compartment contains a defined set of protein components.

Incorporation of nuclear-encoded proteins into mitochondria can be divided conceptually into five steps:-

1) synthesis of the polypeptide itself, usually as a larger M_r precursor;

2) binding of the precursor to the cytoplasmic surface of the mitochondrial outer membrane;

3) translocation of the precursor across or into one or both mitochondrial membranes, depending on the final suborganeller location of the protein;

4) cleavage and/or other covalent modifications of the polypeptide chain to generate the mature protein;

5) assembly of subunits into functional proteins which may involve association with mitochondrially-encoded subunits.

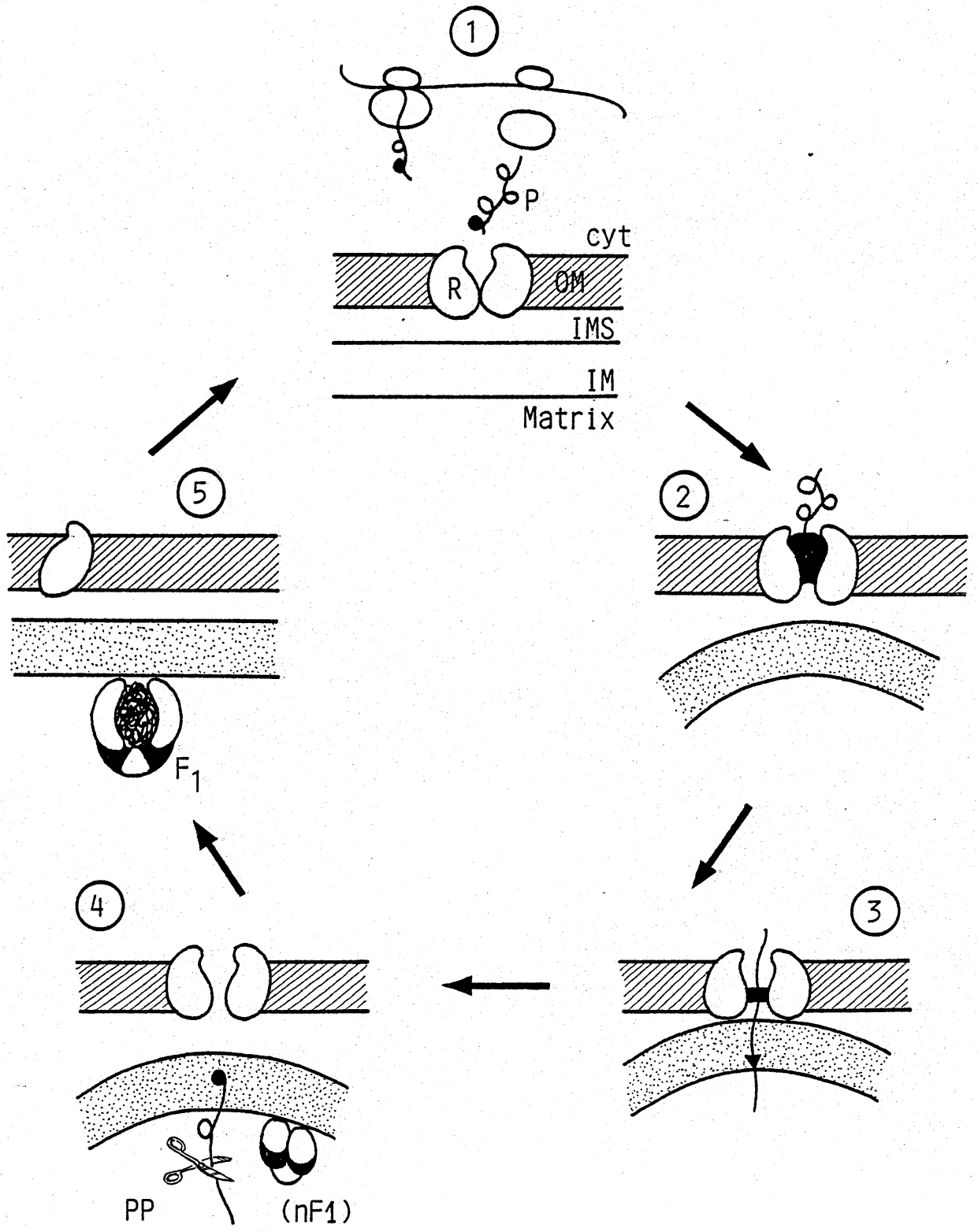
These steps are shown schematically in Fig. 1.3.1 for the β-subunit of F_1-ATPase and will now be discussed individually in more detail.

Fig. 1.3.1

SCHEME ILLUSTRATING THE STEPS INVOLVED IN THE SYNTHESIS, IMPORT AND ASSEMBLY OF THE β-SUBUNIT OF THE F_1-ATPase

(Hay et al., 1984)

A tentative scheme for import of the precursor to β-subunit of yeast F_1-ATPase. Step 1: a molecule of precursor (P) is translated on a cytoplasmic polysome and released into the cytosol (Cyt.). Step 2: the precursor binds by virtue of its amino-terminal recognition sequence (♥) to a receptor (R) on the outer mitochondrial membrane (OM). Step 3: permanent or transient contact between the mitochondrial membranes allows the precursor to utilize the transmembrane potential ($\Delta\bar{\mu}H^+$) of the inner membrane for translocation. Step 4: the amino-terminal recognition sequence is removed in the matrix by a chelator-sensitive processing protease (PP). Step 5: the processed precursor assembles with other subunits of the nascent F_1-ATPase complex (nF_1) to form the mature active holoenzyme (F_1).


1
P
cyt
R
OM
IMS
IM
Matrix
2
3
4
5
F1
PP
(nF1)

1.3.2 Mechanism of Synthesis of Nuclear-Encoded Mitochondrial Proteins

Determining the subcellular site of synthesis of mitochondrial proteins has been central to the question of how mitochondria take up proteins *in vivo*. Two possible mechanisms of synthesis could be considered:-

A) if protein import into mitochondria occurs by the same mechanisms as employed for secretory proteins then the following assumptions could be made:-

i) the cytosol should not contain any pools of mitochondrial proteins since they would be imported into the mitochondrion during translation;

ii) import of polypeptides into mitochondria should be strictly coupled to protein synthesis;

iii) no larger M_r value precursors to nuclear-encoded mitochondrial polypeptides should accumulate in intact cells, since vectorial translation usually involves the removal of the transient amino-terminal signal sequence prior to completion of the polypeptide chain.

B) On the other hand, mitochondrial proteins may be synthesised on free cytoplasmic ribosomes, existing initially as larger M_r precursors which are post-translationally taken up into the mitochondria.

These two possibilities have been distinguished from one another primarily by adopting two methods of experimental investigation:-

i) *in vitro* protein translation systems using either cell-free homogenates or reticulocyte lysates programmed with mRNA to perform protein synthesis;

ii) *in vivo* pulse-labelling studies of intact cells.

Original data from the laboratory of Butow and Kellems (1972), demonstrated the existence of mitochondrially-bound ribosomes in several strains of yeast. These ribosomes were shown by electron microscopy to be clustered in regions where the mitochondrial outer and inner membranes were in close proximity (Kellems *et al.*, 1975). These initial findings appeared to support the view of 'vectorial translation' of mitochondrial proteins.

In vivo studies on the synthesis of nuclear-encoded mitochondrial proteins, however, provided conflicting results. Hallermayer *et al.*, (1977), in an extensive study looked at the kinetics of labelling of newly-synthesised mitochondrial proteins in intact *N.crassa* cells. They demonstrated the existence of a lag period in the incorporation of radioisotope into mitochondrial proteins relative to total cellular, ribosomal, microsomal and cytosolic proteins. In addition, the delayed appearance of label was also found in immunoprecipitated mitochondrial matrix proteins, mitochondrial ribosomal proteins, carboxyatractyloside-binding protein and cytochrome c. The post-translational nature of the import mechanism was revealed by the fact that in the presence of cycloheximide, import of proteins into mitochondria still occurred. This suggested that newly-synthesised mitochondrial proteins exist in an extra-mitochondrial pool from which they are imported into mitochondria.

A similar study employing a cell-free homogenate, prepared from *N.crassa* cells, confirmed the above *in vivo* results (Harmey *et al.*, 1977) although the actual site of synthesis could not be determined (free or membrane-bound ribosomes).

The first insight into the site of synthesis of nuclear-encoded

mitochondrial proteins actually came from studies performed in the algae, Chlamydomonas reinhardtii. Dobberstein et al. (1977) showed that the chloroplast enzyme, ribulose-1, 5-bisphosphate carboxylase was synthesised on free polysomes, as a larger M_r precursor, in an in vitro translation system. In 1979, the field of mitochondrial protein import took on a new direction owing to the studies of Maccecchini et al. (1979). This group of workers looked at the mechanism of import of the three largest subunits (α, β and γ) of the yeast F_1-ATPase. The three F_1-ATPase subunits were synthesised either in vitro using a reticulocyte lysate programmed with yeast mRNA or in vivo employing pulse-labelled yeast spheroplasts. Under both experimental conditions, they were able to show that the α, β and γ subunits of the F_1-ATPase were synthesised as larger M_r precursors. Incubation of the in vitro synthesised precursors with isolated yeast mitochondria, in the presence of cycloheximide, resulted in their conversion to the mature proteins. This conversion was also demonstrated in the yeast spheroplasts by 'pulse-chase' experiments. In quick succession, several groups illustrated the existence of larger M_r precursor molecules for a number of mitochondrial proteins synthesised in vitro under the direction of free polysomes (Conboy et al., 1979, Neupert et al., 1979; Raymond and Shore, 1979).

However, Butow and Ades (1980) questioned the overall importance of free polysomes in mitochondrial protein synthesis. They isolated free polysomes and membrane bound polysomes associated with the mitochondria from yeast spheroplasts, which had been treated with cycloheximide to prevent polysome 'run off'. The proportion of α, β and γ subunits of the F_1-ATPase made by these two populations of polysomes was determined in a cell-free translation mixture. It was discovered that the

proportion of the subunits of F_1-ATPase was much greater among the products of the mitochondrially-bound polysomes than those synthesised on free polysomes. In a more detailed experiment, Suissa and Schatz (1982) repeated the above experiments with yeast adopting slightly different experimental conditions to extract the mRNA from the polysomal fractions. They showed that of the twelve mitochondrial polypeptides studied, none of the translatable mRNAs were predominately associated with mitochondrially-bound polysomes. The previous studies of Ades and Butow (1980) may have over-emphasised the function of mitochondrial-bound polysomes, as a result of inhibiting protein snythesis with cycloheximide prior to polysome preparation. This inhibition could result in the increased binding of the nascent polypeptide chain together with its attached polysome. In addition, it is very difficult to preserve the in vivo distribution of mRNAs between free and mitochondria-bound polysomes during breakage and fractionation of the cells.

In summary, it is generally believed that mitochondrial proteins are synthesised on free, cytoplasmic ribosomes as precursor molecules, which are post-translationally taken up by the organelle. The existence of mitochondrially-bound polysomes has been firmly established in yeast cells (Ades and Butow, 1980; Suissa and Schatz, 1982) but any role that they may play in mitochondrial protein import, does not appear to be obligatory for this process.

1.3.3 Molecular Characteristics of Precursors

In 1978, Korb and Neupert investigated the biogenesis of the mitochondrial intermembrane space protein, cytochrome c. These studies were carried out with a cell-free homogenate prepared from

N.crassa cells and a highly specific antiserum to detect the protein. In the absence of mitochondria, a precursor molecule was synthesised which migrated with the same M_r value as the authentic mature protein, on SDS-polyacrylamide gel electrophoresis. The precursor protein, apocytochrome c, lacked the haem group which was covalently-linked to the protein on addition of isolated N.crassa mitochondria. It was not until 1979 and the work of Maccecchini et al. that the existence of larger M_r precursor molecules was demonstrated. Employing in vitro and in vivo systems they were able to show precursors to the three subunits (α, β and γ) of the yeast F_1-ATPase that differed in M_r from their mature counterparts by 2000-6000.

Since these studies, the existence of larger M_r polypeptides to many cytoplasmically-synthesised mitochondrial proteins has been shown (Table 1.3.1). Their M_r sizes are between 500 and 10,000 daltons larger than those of the corresponding mature proteins. In a few cases, it has been directly shown that this size difference results from an amino-terminal extension of the polypeptide chain. The precursors to the yeast F_1-ATPase α, β and γ subunits, as well as precursors to each of the four cytoplasmically-made subunits of cytochrome c oxidase, are primary translation products whose amino-termini can be labelled with N-formylmethionine in vitro (Lewin et al., 1980; Mihara and Blobel 1980). There is no apparent correlation between the size of the amino-terminal extension and the final location of the mature protein within the mitochondrion.

To date, it has been proven difficult to isolate precursors in appreciable quantities to investigate the amino acid sequence of the amino terminal extension, directly. Recently, however, attention has focused on sequencing the genes for nuclear-encoded mitochondrial

TABLE 1.3.1

CYTOPLASMICALLY-SYNTHESISED MITOCHONDRIAL PROTEINS

ÞTEIN CATION	PROTEIN	ORGANISM	APPARENT MOLECULAR SIZE ($M_r \times 10^{-3}$)	
			MATURE	PRECURSOR
TRIX	Acetoacetyl CoA thiolase	Rat	38	41
	Acyl-CoA dehydrogenase	-	-	-
	General	Rat	39	41
	Long-chain	Rat	?	?
	Short-chain	Rat	36.5	41
	Adrenodoxin	Cattle	12	20
	Adrenodoxin reductase	Cattle	50	50
	S-Aminolevulinate synthase	Rat	45	51
		Chicken	63-65	75
	Aspartate aminotransferase	Chicken	44.5	47
	F_1-ATPase α-subunit	Yeast	58	64
	β-subunit	Yeast	54	56
	γ-subunit	Yeast	34	40
	Carbamoyl-phosphate synthetase	Rat	160	165
		Frog	160	160
	Citrate synthase	Yeast	47	50
		N.crassa	45	47
	Enoyl-CoA hydratase	Rat	25	29.5
	L-Glutamate dehydrogenase	Rat	54	60
	L-Hydroxyacyl-CoA dehydrogenase	Rat	29.5	33
	2 Isopropylmalate synthase	Yeast	65	65
	2-Ketoacyl-CoA thiolase	Rat	38	38

TABLE 1.3.1 (cont.)

OTEIN CATION	PROTEIN	ORGANISM	APPARENT MOLECULAR SIZE ($M_r \times 10^{-3}$)	
			MATURE	PRECURSOR
TRIX	Malate dehydrogenase	Rat	37	38
	Methylmalonyl-CoA mutase	Rat	77.5	80.5
	Ornithine aminotransferase	Rat	43	49
	Ornithine transcarbamylase	Rat	36-39	39.5-43
		Mouse	37	39.5
	Propionyl CoA carboxylase			
	α-subunit	Rat	70	74.5
	β-subunit	Rat	54	61.5
	RNA polymerase subunit	Yeast	45	47
	Mn^{2+}-superoxide dismutase	Yeast	24	26
NER MBRANE	Adenine nucleotide Translocator	N.crassa	32	32
		Rat	30	30
	F_1F_o-ATPase subunit IX - proteolipid DCCD-binding protein	N.crassa	8.2	14
	Carnitine acetyl transferase	Rat	67.5	69
	Cholesterol side-chain Cleavage cytochrome P-450	Cattle	49	54.5
	Cytochrome c oxidase	Yeast	-	-
	subunit IV	Yeast	14	17
	" V	Yeast	12.5	15
	" VI	Yeast	12.5	17-20

TABLE 1.3.1 (cont.)

TEIN ATION	PROTEIN	ORGANISM	APPARENT MOLECULAR SIZE ($M_r \times 10^{-3}$)	
			MATURE	PRECURSOR
ER BRANE	subunit VII	Yeast	5-7.5	5-7.5
	" IV	Rat	16.5	18-19.5
	" V	Rat	12.5	15.5
	D-β-Hydroxybutyrate dehydrogenase	Rat	32	37
	Ubiquinol cytochrome c reductase (bc, complex)			
	subunit I	Yeast	44	44.5
	" II	Yeast	40	40.5
	" V	Yeast	25	27
	" VI	Yeast	17	25
	" VII	Yeast	14	14
	" VIII	Yeast	11	11
	Cytochrome c_1	Yeast	31	37
	" I	N.crassa	50	47.5
	" II	N.crassa	45	47.5
	" V	N.crassa	25	28
	" VI	N.crassa	14	14
	" VII	N.crassa	11.5	12
	" VIII	N.crassa	11.2	11.6
	Cytochrome c_1	N.crassa	31	38

TABLE 1.3.1 (cont.)

'TEIN 'ATION	PROTEIN	ORGANISM	APPARENT MOLECULAR SIZE ($M_r \times 10^{-3}$)	
			MATURE	PRECURSOR
'ER- IBRANE CE	Adenylate kinase	Chicken	28	28
	Cytochrome c	N.crassa	12	12
		Rat	12	12
	Cytochrome c peroxidase	Yeast	33.5	39.5
	Flavocytochrome b_2 lactate dehydrogenase (-cytochrome b_2)	Yeast	58	68
	Sulfite oxidase	Rat	55	59
'ER IBRANE	Monoamine oxidase	Rat	59	59
	Porin	N.crassa	31	31
		Yeast	29	29
	14-Kilodalton protein	Yeast	14	14
	45-Kilodalton protein	Yeast	45	45
	70-Kilodalton protein	Yeast	70	70
	OMM-35	Rat	35	35.5

proteins, to overcome this problem. The sequences of the genes coding for the proteolipid subunit of the N.crassa ATPase (Viebrock et al., 1982) and the yeast cytochrome c peroxidase (Kaput et al., 1982), have been determined. The pre-sequence of cytochrome c peroxidase is an exclusively amino-terminal extension of 68 amino acids, which renders the precursor more basic and more hydrophilic. Similarly, the long signal sequence of 66 amino acids of the proteolipid subunit of mitochondrial ATPase, is very polar. It has been suggested that the presequence is specifically designed to solubilise the proteolipid for post-translational uptake into the mitochondria.

So far the emphasis has been placed on the properties of precursor proteins whose M_r value is greater than the corresponding mature mitochondrial proteins. A few mitochondrial proteins, on the other hand, are like cytochrome c, in that they have precursors with the same M_r as the mature protein (Table 1.3.1). There is evidence for some of these proteins, that although they lack a transient amino-terminal extension, they differ from the mature protein in conformation. For example, the extra mitochondrial form of the adenine nucleotide translocase binds to hydroxylapatite whereas the mature form does not bind (Zimmermann and Neupert, 1980). The precursor to the outer membrane protein, porin, of yeast is sensitive to proteinases but once it has become integrated into the membrane, it is insensitive to proteolysis, even in the presence of detergent (Gasser and Schatz, 1983). Further differences in the tertiary structures of cytosolic precursors, have been demonstrated by showing that they form large aggregates. The in vitro synthesised adenine nucleotide translocator forms aggregates of between 120,000 and 500,000 M_r, as estimated by gel filtration (Zimmermann and Neupert, 1980). It has been proposed

that the post-translational formation of precursor aggregates occurs either to protect them from proteolysis in the cytosol or for proper presentation to the mitochondrial surface.

1.3.4 Receptors Involved in the Recognition and Binding of Mitochondrial Precursors

After translation of the cytoplasmic, precursor forms of mitochondrial proteins, it is postulated that an essential step in the translocation process is the specific attachment of the precursors to the cytoplasmic face of the mitochondrial outer membrane. This interaction could involve specialised receptors or simply partitioning of the precursors into the lipid bilayer. There is no convincing evidence for specific receptors playing a role in the insertion of mitochondrial outer membrane proteins, although the process has some specificity. Gasser and Schatz (1983) demonstrated that the *in vitro* synthesised 29,000 M_r protein of the yeast mitochondrial outer membrane did not insert into yeast microsomes. However, evidence for the uptake of the cytoplasmic forms of mitochondrial proteins destined for the intermembrane space, inner membrane and matrix strongly implicate the participation of receptor molecules.

The most extensive data suggesting a mitochondrial receptor, has come from studies on the biosynthesis and import of cytochrome c, by Neupert and co-workers. As previously discussed (1.3.3) cytochrome c is synthesised in the cytosol as a protein with the same M_r as the authentic, mature polypeptide but lacks the haem group (Korb and Neupert, 1978). Covalent attachment of the haem group to apocytochrome c occurs after migration of the precursor to the inner membrane.

Hennig and Neupert (1981) examined the fate of the *in vitro*

synthesised precursor in a reconstituted import system prepared from N.crassa. This involved incubating the cell-free translation products with isolated mitochondria and individually immunoprecipitating the apocytochrome c and holocytochrome c forms from the mitochondrial and post-mitochondrial fractions with non-cross reacting antibodies. Radiolabelled apocytochrome c was rapidly cleared from the supernatant fraction and the mature holoenzyme appeared in parallel fashion in the mitochondrial fraction. There was no transient accumulation of the apoprotein within the mitochondrion, under these conditions. However, when deuterohemin, a reversible inhibitor of the haem attachment step, was added to the reconstituted import system, a large proportion of radiolabelled apocytochrome c was recovered with the mitochondria. To demonstrate that this binding was specific, Hennig and Neupert (1981) showed that the precursor could be released, in a dose-dependent manner from the mitochondria by incubation in the presence of excess chemically prepared apocytochrome c. This indicated reversible binding and competition between the two forms of apocytochrome c for sites on the mitochondrial surface. The binding was shown to be ligand-specific by further experiments in which only the apocytochrome c form and not the holocytochrome c form, competed with the uptake of radiolabelled precursor into mitochondria. Matsuura et al. (1981) subsequently demonstrated that the post-translational uptake of apocytochrome c into isolated rat liver mitochondria was inhibited by a specific segment ('addressing signal') of the polypeptide chain. This segment, generated by cyanogen bromide cleavage of apocytochrome c, extended from residue 66 to the carboxyl-terminus.

The existence of different receptors for precursor polypeptides entering other mitochondrial compartments has been implied from the following competition experiments. Excess apocytochrome c does not

inhibit the uptake of precursors to the adenine nucleotide translocase, the ATPase proteolipid subunit or cytochrome c_1, under analogous conditions to those employed above (Zimmermann et al., 1981; Teintze et al., 1982). These results taken together demonstrate many of the binding properties of receptors but do not show that this activity resides in a particular protein component of the outer membrane. To investigate the nature of the component(s) responsible for binding of precursors to mitochondria, Riezman et al. (1983) prepared outer membrane vesicles from yeast cells. In this system they were looking at the binding of precursor polypeptides in the absence of protein translocation and processing. When the outer membrane vesicles were treated with trypsin prior to binding, no precursor to cytochrome b_2 or F_1-ATPase β-subunit could be found to be associated with the vesicles. In contrast, Gasser and Schatz (1983) illustrated that the outer membrane protein, porin, could bind to these outer membrane vesicles, after such protease treatment. Ligand and membrane specificity of binding was demonstrated with these outer membrane vesicles. In addition, the existence of different receptors was suggested also from the finding that apocytochrome c failed to inhibit the binding of the precursor to cytochrome b_2.

It would appear from these studies on mitochondrial receptors, that there exists on the outer membrane a number of different protein receptors which interact with different precursor molecules. Until the outer membrane components responsible are identified in more detail and purified, this important stage in the import process remains largely unresolved at a molecular level.

Finally, a great deal of interest has recently been generated from the findings that a small cytosolic component is required for the functional post-translational import of the precursor to the rat liver

mitochondrial matrix protein, ornithine transcarbamylase (Argan et al., 1983). The component was retained by gel filtration of the reticulocyte lysate translational mixture on Sephadex G-25. Confirmation of these findings was provided by Ohta and Schatz (1984) when studying the import of the precursor to cytochrome b_2 into yeast mitochondria. However, they also showed that a cytosolic factor which was trypsin-sensitive and whose M_r was about 40,000, as judged by gel filtration, was required for the import of pure ATPase β-subunit precursor into isolated yeast mitochondria. This data suggested a role for soluble protein(s) of the cytoplasm in the import pathway. In addition, Firgaira et al. (1984) demonstrated that a cytoplasmic RNA moiety was necessary for the post-translational uptake of nuclear-encoded mammalian proteins, destined for the mitochondrial matrix. The ribonuclease-sensitive component showed many properties that are characteristic of ribonucleoproteins. This suggests the possibility that a similar 'signal-recognition particle' to that employed by secretory proteins (1.2.3) might function in the recognition and targeting of newly-synthesised proteins destined for the mitochondria. These findings also open up a completely new area of research with regard to mitochondrial import of cytoplasmically-synthesised proteins.

1.3.5 Translocation and Processing of Mitochondrial Precursors

1.3.5.1 Energy Dependence of Translocation

Detailed studies of the energy dependence of translocation have made use of an import assay in which in vitro synthesised radiolabelled precursors were incubated with isolated mitochondria. Translocation of polypeptides across the mitochondrial membranes was checked by their inaccessibility to externally-added trypsin. Proper proteolytic

processing of the precursors was assayed by immunoprecipitation and SDS-gel electrophoresis, to confirm that the imported proteins migrate with the same M_r as the mature mitochondrial polypeptide.

Early work by Nelson and Schatz (1979) utilising intact yeast spheroplasts, demonstrated that the import and cleavage of precursors of the F_1-ATPase α, β and γ subunits, as well as of two subunits of the ubiquinol: cytochrome c reductase complex, was energy-dependent. Addition of the uncoupler CCCP to respiring yeast spheroplasts or specific depletion of the level of matrix ATP in non-respiring yeast strains, blocked translocation and processing of the precursors. However, it was not possible from these observations to distinguish between a requirement for matrix ATP and an electrochemical potential across the inner membrane, since the addition of uncoupler makes the inner membrane permeable to protons and simultaneously stimulates the hydrolysis of ATP by the F_1-ATPase (Heytler, 1979). By using respiration deficient (rho^-) yeast strains which cannot form an appreciable membrane potential, Nelson and Schatz (1979) illustrated that import from the cytosol, still occurred. They concluded that matrix ATP was probably the immediate energy donor for translocation.

Definitive evidence to the contrary has come from experiments performed <u>in vitro</u>, using a similar import assay to the one described earlier in this section. To discriminate between the two possible sources of energy that can be utilised during translocation, the following types of inhibitors were added to the import assay mixture; specific inhibitors of the respiratory chain (KCN, antimycin A), of the F_1F_0-ATPase (oligomycin) and ionophores such as valinomycin. By using various combinations of these inhibitors, it was possible to maintain a high concentration of ATP in the mitochondrial matrix while the electrochemical gradient across the inner membrane was

depleted. Under these conditions, if the direct energy donor for translocation of proteins into mitochondria was the membrane potential, then the mitochondria would lose the ability to import precursor proteins despite a high concentration of matrix ATP.

Gasser et al. (1982a) investigated the effects of such inhibitors on the translocation and processing of the F_1-ATPase β-subunit into isolated yeast mitochondria. The precursor containing reticulocyte lysate was gel-filtered to remove endogenous energy sources and the isolated mitochondria treated with KCN to inhibit respiration of endogenous substrates. To ensure a high level of matrix ATP, the gel-filtered lysate was incubated in the presence of ATP. ATP can be transported into the mitochondrial matrix via the adenine nucleotide translocase for use either directly as an energy source or hydrolysed by the F_1F_0-ATPase to generate a proton gradient across the inner membrane. In the absence of other inhibitors, the mitochondria efficiently imported and processed the F_1-ATPase β-subunit precursor. However, when the mitochondria were also pre-treated with oligomycin, translocation was blocked completely. Under these conditions, the concentration of ATP in the matrix will be high while any electrochemical potential across the inner membrane will be very low. When KCN was absent from the incubation mixture and respiration re-stimulated by the addition of succinate, oligomycin no longer blocked import of the precursor. These findings showed that an electrochemical potential was essential for the import of matrix-located polypeptides.

Schleyer et al. (1982) further demonstrated the importance of an electrochemical potential difference across the mitochondrial inner membrane by following the import of F_1-ATPase subunit 9 from N.crassa into isolated mitochondria. In the presence of oligomycin and

antimycin A, import was inhibited but could subsequently be restored upon the addition of ascorbate and tetramethylphenylenediamine (TMPD), which donates electrons directly to cytochrome c oxidase, thus by-passing the antimycin A induced block of electron flow.

On a more detailed examination of the energy requirements for translocation into the individual mitochondrial compartments, large variations are to be found. Import of proteins destined for the mitochondrial outer membrane does not appear to require either ATP or an electrochemical potential across the mitochondrial inner membrane (Gasser and Schatz, 1983). On the other hand, proteins destined for the mitochondrial inner membrane or matrix require an electrochemical potential difference for successful transfer across the inner membrane (Gasser et al., 1982a; Schleyer et al., 1982; Gasser et al., 1982b).

The mitochondrial intermembrane space provides the greatest degree of diversity in its requirement of energy for translocation. An energised inner membrane is not required for the import of cytochrome c (Zimmerman et al., 1981) into the intermembrane space whereas translocation of cytochromes b_2 and c_1 are dependent on the membrane potential (Daum et al., 1982; Ohashi et al., 1982). In the case of both cytochromes b_2 and c_1, which are processed in two stages (see section 1.3.5.2), the first cleavage requires an energised inner membrane. However, for cytochrome b_2, the second processing step is probably not dependent on a membrane potential, since the addition of valinomycin and K^+ to isolated yeast mitochondria, does not block the conversion of the intermediate to mature form (Daum et al., 1982). The second processing step for cytochrome c_1, does appear to require an energised inner membrane (Teintze et al., 1982). The intermediate form of cytochrome c_1 was allowed to accumulate in intact N.crassa

cells during a [^{3}H]-leucine pulse at 8^{o}C. When the temperature was raised to 25^{o}C and the cells incubated for a further 5 min in the presence of CCCP, no conversion to mature cytochrome c_1 was observed. This indicated that the second processing step in the maturation of cytochrome c_1 was also energy-dependent. In addition, maturation of cytochrome c_1 from the intermediate form is haem-dependent (see section 1.3.5.3).

An electrochemical potential is also required for the translocation of polypeptides across the plasma membrane of Escherichia coli (Date et al., 1980; Enequist et al., 1981). In bacteria, however, the protein translocation occurs from negative to positive potential whereas the direction of translocation is opposite in mitochondria. Thus it is unlikely that the transmembrane potential serves in both cases as a simple electrophoretic gradient. The electrochemical potential required is likely to be quite small since the rho$^-$ mutant which lacks a functional ATPase complex as well as a respiratory chain, efficiently imports proteins.

1.3.5.2 Properties of the Yeast Mitochondrial Matrix Protease

After binding and translocation to the correct intra-mitochondrial compartment, the larger M_r precursor proteins are generally cleaved by a matrix-located protease. This cleavage results in the formation of a polypeptide with the same M_r value as the mature protein, as determined by SDS-gel electrophoresis.

The enzyme was first detected in a hypotonic extract of yeast mitochondria (Böhni et al., 1980) from its ability to cleave several precursors, in a specific manner, to their mature forms. This cleavage occurred in the absence of an energy source, such as ATP. Two groups

of workers have recently reported the further purification of the yeast enzyme (McAda and Douglas, 1981; Bohni et al., 1983). The protease which had a pH optimum of 7.5 was also strongly inhibited by a variety of chelating agents including EDTA, GTP and 1,10 - phenanthroline. This inhibition was reversed by the addition of Zn^{2+} or Co^{2+} ions. The enzyme was insensitive to the serine protease inhibitors and to sulphydryl modifying reagents such as N-ethylmaleimide and iodoacetamide.

These partially-purified preparations have been used to determine the substrate specificity of the enzyme and some of its physical characteristics. On gel filtration, the activity exhibits an M_r of 110,000 although SDS-polyacrylamide gel electrophoresis of the partially purified preparations reveals the presence of approximately ten Coomassie Blue stained bands. McAda and Douglas (1982) have reported that the activity correlates best with the presence of a 59,000 M_r subunit.

Incubation of an [^{35}S]-methionine labelled precursor polypeptide with the partially-purified protease, followed by specific immunoprecipitation and gel electrophoresis have been used to study the specificity of the protease. As previously observed with the crude submitochondrial extract (Böhni et al., 1980), the partially purified enzyme exhibits a narrow substrate specificity. It cleaves a variety of *in vitro* synthesised mitochondrial precursors but does not process non-mitochondrial proteins (Böhni et al., 1983). Cytochromes b_2 and c_1 located in the intermembrane space, are cleaved by the matrix enzyme to intermediate forms whose M_r values are between those of the corresponding precursor and mature forms. Cleavage is limited in that it proceeds no further than the mature form, even after long exposure to the enzyme. The *in vitro* processing results were confirmed

in vivo, by pulse-labelling yeast spheroplasts in the presence of 1,10-phenanthroline. Under these conditions, there was rapid accumulation of the precursor of the β-subunit of the F_1-ATPase (Böhni et al., 1983).

Cerletti et al. (1983) adopted a slightly different approach to illustrate that the precursors were correctly processed to their mature forms. This was achieved by looking at the effect of the partially-purified protease on the processing of cytochrome c oxidase subunit V precursor, by radiochemical sequence analysis of the processed amino-terminus of the mature protein. The amino-terminal region generated by this protease was in agreement with the amino-terminus of the mature polypeptide.

Finally, the yeast matrix protease is coded in the nucleus. This was illustrated by Böhni et al. (1983) who prepared a hypotonic extract from the mitochondria of a rho^- mutant strain of S.cerevisiae and showed that the extract could convert the precursor of the F_1-ATPase β-subunit to the mature size. The protease must be encoded by the nucleus and imported into mitochondria, since such mutant organelles have no functional mitochondrial protein-synthesising machinery (Schatz and Mason, 1974).

1.3.5.3 Processing of Mitochondrial Precursor Polypeptides

Mitochondrial precursor proteins of larger M_r are proteolytically processed to generate the mature-sized polypeptide. The processing step(s) can involve any one of three different mechanisms. These mechanisms have largely been determined by using the in vitro import assay (described in section 1.3.5.1) and confirmed in vivo by pulse-labelling intact cells for very short times, detecting initial products

of translation by immunoprecipitation.

Protein precursors destined for the mitochondrial outer membrane are synthesised in the cytosol as products with the same M_r as the mature polypeptides, with one possible exception being the 35,000 M_r polypeptide from rat liver mitochondria (Shore et al., 1981) (see table 1.3.1). In their case, no proteolytic processing is required to complete maturation. Several other nuclear-encoded, mitochondrial proteins localised in the inner membrane, intermembrane space and matrix also do not appear to undergo proteolytic cleavage, after translocation. These include cytochrome c of the intermembrane space, the adenine nucleotide translocase of the inner membrane and 2-isopropylmalate synthase of the mitochondrial matrix (Korb and Neupert, 1978, Zimmerman and Neupert, 1980; Hampsey et al., 1983). Cytochrome c does, however, require the addition of haem for complete maturation (see section 1.3.3). Interestingly, of all the mitochondrial matrix proteins studied to date, 2-isopropylmalate synthase is the only example to show a lack of proteolytic processing during its maturation. The remaining mitochondrial proteins, studied so far, are synthesised in the cytosol as larger M_r precursors which undergo one of two proteolytic processing mechanisms after correct translocation. A major role is played by a matrix-located protease in both of these processing mechanisms. The properties of the partially-purified yeast matrix protease have been described previously (see section 1.3.5.2).

The first of these processing mechanisms involves a 'one-step' proteolytic cleavage and accounts for the majority of proteins listed in table 1.3.1. Endoproteolytic removal of the transient amino-terminal sequence of the larger M_r precursor is accomplished by proteolytic cleavage at one site and generates the mature protein.

The final mechanism of processing involves cleavage of the precursor to the mature protein in two discreet stages. Cytochrome b_2 and cytochrome c_1, located in the intermembrane space and outer surface of the inner membrane respectively, have clearly been shown to be processed by this 'two-step' mechanism (Teintze et al., 1982, Reid et al., 1982b, Gasser et al., 1982b, Ohashi et al., 1982, Daum et al., 1982; Böhni et al., 1983) (see section 1.3.5.1 for details of first proteolytic cleavage).

To investigate the location of the intermediate-sized polypeptide generated by the matrix protease, Daum et al. (1982) studied the in vitro import of cytochrome b_2 into isolated yeast mitoplasts. When import of the precursor was allowed to proceed at low temperatures, the intermediate polypeptide remained attached to the mitoplasts whereas most of the precursor and mature forms of the protein were found in the supernatant fraction. In addition, the intermediate protein was fully accessible to externally-added protease under conditions where the bulk of matrix-located polypeptide was inaccessible. It was suggested from these findings, that the intermediate was bound to the inner membrane with the major portion exposed to the intermembrane space. Therefore, the second proteolytic cleavage, which converts the intermediates to their mature forms, would take place on the outer surface of the inner membrane.

In yeast, the enzyme responsible for the second cleavage is different from the matrix protease, as it is resistant to chelating agents such as 1,10-phenanthroline, although attempts to extract it from the mitochondria have proved to be unsuccessful (Daum et al., 1982).

In the case of cytochrome c_1, covalent haem attachment to the intermediate form is required before the second cleavage can take place

(Ohashi et al., 1982). In haem-deficient yeast mutants, the intermediate accumulates and is only converted to the mature protein upon supplementation of the strain with the compound required to restore haem biosynthesis. Maturation of cytochrome b_2 from its intermediate species does not require haem attachment before proteolysis (Gasser et al., 1982b; Ohashi et al., 1982). A diagram summarizing the import and processing of these proteins by the 'two-step' mechanism is shown in Fig. 1.3.2.

More recently, Sadler et al. (1984), sequenced the gene coding for the precursor to the yeast cytochrome c_1. The deduced amino acid sequence of the precursor revealed an unusually long transient amino-terminal pre-sequence of 61 amino acids. This pre-sequence consisted of a strongly basic amino-terminal region of 35 amino acids, a central region of 19 uncharged residues and an acidic carboxy-terminal region of 7 amino acids. They have suggested from this sequence data, a model for the orientation of the precursor across the mitochondrial inner membrane which can explain the 'two-step' processing mechanism. The mechanism of processing of the matrix located enzyme, ornithine transcarbamylase is intriguing. Conboy et al. (1982), demonstrated that the rat liver protein was initially made as a larger precursor of M_r 39,000 in vitro. Subsequent import and processing by isolated rat liver mitochondria not only resulted in the production of the mature polypeptide (M_r 36,000) but also an intermediate species of M_r 37,000 (Morita et al., 1982a,b). The intermediate-sized polypeptide could also be formed by incubating the 39,000 M_r precursor form with a partially-purified rat liver matrix protease (Miura et al., 1982), but could not be detected in vivo in isolated hepatocytes (Mori et al., 1981). Conboy et al. (1982) by adding low concentrations of Zn^{2+} to a matrix fraction of rat liver mitochondria were able to suppress production of the intermediate-

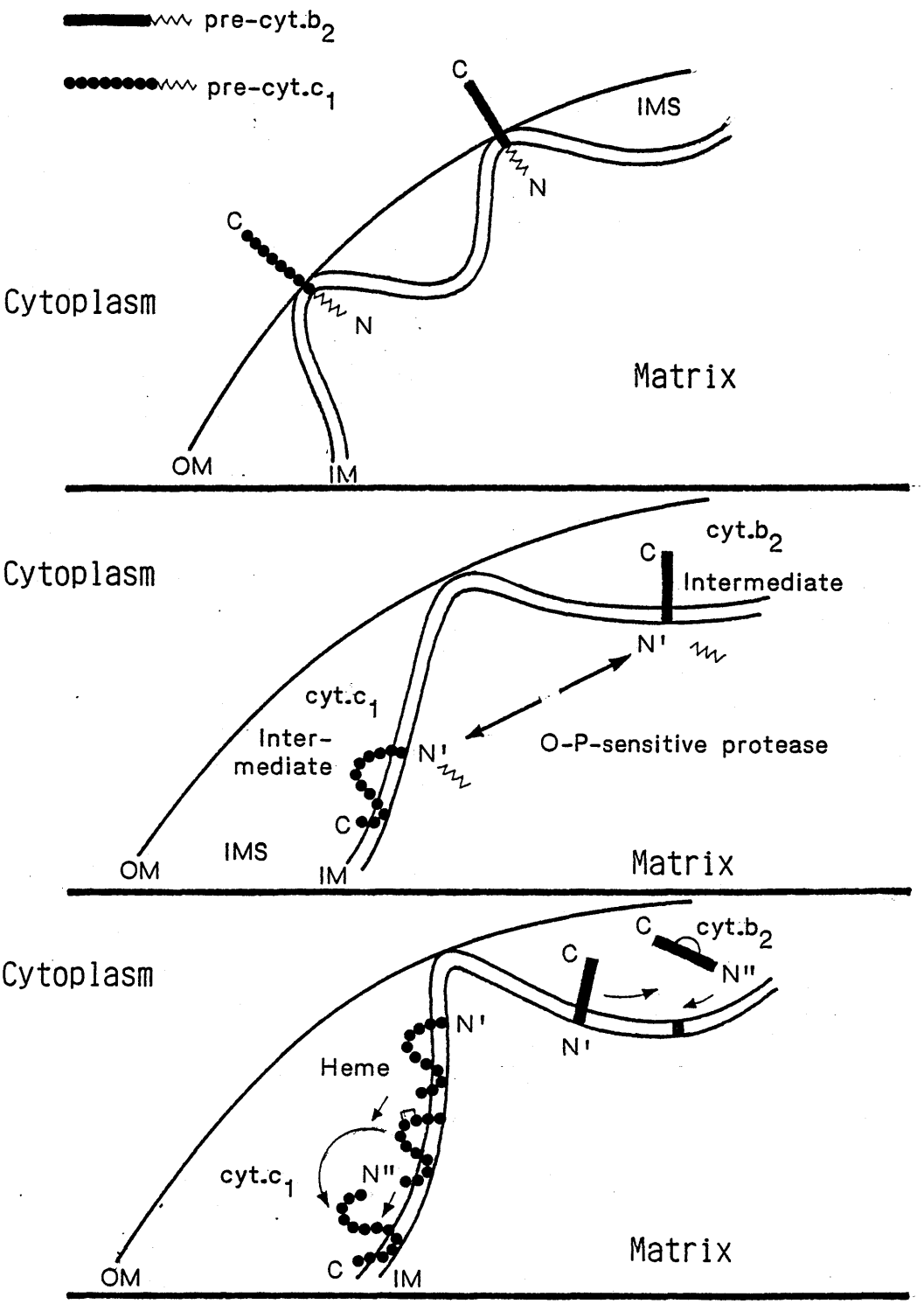
Fig. 1.3.2

'TWO-STEP' IMPORT AND PROCESSING MECHANISM FOR CYTOCHROMES c_1 AND b_2

(Reproduced from Hay et al., 1984)

Precursors to cytochromes b_2 and c_1 of yeast mitochondria are depicted as undergoing two separate proteolytic cleavages after entering the mitochondrion. Panel 1: each precursor utilises the inner membrane electrochemical potential to assume a transmembrane conformation. Panel 2: initial cleavage (arrows), mediated by the matrix protease yields a membrane-bound intermediate with a new amino-terminus (N'). Panel 3: the second cleavage thought to involve a protease at the outer face of the inner membrane generates the mature amino-terminus (N''). Mature cytochrome b_2 is released in soluble form (with a noncovalently bound heme group (semi-circle) into the intermembrane space. Covalent attachment of heme to the intermediate form of cytochrome c_1 (box) precedes the second cleavage.

'Two-step' import pathway model



sized form and to convert pre-ornithine transcarbamylase to its mature form, *in vitro*. This result suggested that the intermediate species and the mature processed forms were generated by two distinct, competing enzyme activities and that the 37,000 M_r polypeptide was not a *bona fide* intermediate *in vivo*.

The overall findings to date on the mechanism(s) of import of cytoplasmically-synthesised polypeptides are summarized in table 1.3.2.

1.3.6 Assembly of Processed Proteins into Functional Units

The least well characterized step in the import of nuclear-encoded mitochondrial proteins is the assembly of the processed proteins into functional units. A major problem so far has been to show experimentally that after *in vitro* import, the mitochondrial polypeptides assume the structure and physical properties of the native protein. To confirm that a nuclear-encoded mitochondrial polypeptide has been correctly assembled, the following criteria should be met:-

1) an imported protein should be shown to have reached its correct submitochondrial compartment;

2) in some instances the protein should have associated with identical or non-identical subunits to form the structure of the native molecule;

3) imported and assembled subunits should be functional.

To date, only a few experiments have addressed these problems. In 1982 (a), Gasser *et al.* investigated the specificity of localisation of mitochondrial precursor proteins by preparing from isolated yeast

TABLE 1.3.2

SUMMARY OF THE MECHANISM(S) OF IMPORT OF CYTOPLASMICALLY-SYNTHESISED MITOCHONDRIAL PROTEINS

OCHONDRIAL LOCATION	RECEPTOR BINDING	ENERGY REQUIREMENT	PROTEOLYTIC CLEAVAGE PROCESS
er Membrane	Binding does not show a sensitivity to Trypsin	None	Zero-step
er-Membrane space	Binding is Trypsin sensitive	Existence of an inner membrane potential	Two-step
er Membrane	Binding is Trypsin sensitive	Existence of an inner membrane potential	One-step
rix	Binding is Trypsin sensitive	Existence of an inner membrane potential	One-step

ochondrial polypeptides whose import pathway differs with regard to any of the points above are discussed in Sections .2 - 1.3.5 and/or listed in Table 1.3.1.

mitochondria, a soluble intermembrane space fraction, a soluble matrix fraction and a membrane fraction (inner and outer). Prior to sub-fractionation, an in vitro import assay was performed by incubating a labelled reticulocyte lysate with isolated yeast mitochondria. They discovered that the labelled membrane proteins (cytochrome c_1 and cytochrome c oxidase subunit V) were exclusively located in the membrane fraction whereas soluble matrix (2-isopropylmalate synthase) and intermembrane space proteins (cytochrome b_2) were recovered to a large extent in their respective soluble compartment and in the membrane fraction. It was not, however, determined whether these membrane-associated cytochrome b_2 or 2-isopropylmalate synthase forms represented precursors trapped 'in transit' across the membranes or non-specific adsorption.

In a study on the import of carbamoyl phosphate synthetase into the mitochondria of foetal rat liver (Campbell et al., 1982), radio-labelled carbamoyl phosphate synthetase was recovered in a soluble fraction when mitochondria were subjected to fractionation following an in vitro import assay. After import of the precursor into foetal rat liver mitochondria, an increase in carbamyl phosphate synthetase activity was demonstrated. The precursor itself showed no enzymatic activity which implied that not only import into the correct compartment but also assembly of the polypeptide into an active holo-enzyme (two identical subunits), had taken place. However, it was not ruled out that another copurifying factor may have stimulated endogenous enzymatic activity.

The correct orientation of an imported protein within the mitochondrion has been suggested from findings on two membrane proteins. Porin, an outer membrane protein in mitochondria, has been extensively studied in yeast (Gasser and Schatz, 1983; Mihara et al., 1982)

and N.crassa (Freitag et al., 1982). The native protein is insensitive to protease even in the presence of detergents, whereas the in vitro synthesised product, although of the same M_r as the mature protein, is highly sensitive to protease. Upon incubation with isolated mitochondria, the newly-imported porin was shown to be resistant to externally added protease, even in the presence of detergent, implying correct orientation within the membrane. In a similar study, Zimmerman and Neupert (1980) were able to demonstrate that the imported N.crassa inner membrane adenine nucleotide translocase, resembled the native protein in that it was excluded from hydroxylapatite. In contrast, in vitro synthesised precursor binds to hydroxylapatite although it is of the same M_r as the native protein. These two studies illustrated that the precursor and native forms of a protein exist in different conformations which can markedly affect the properties of the protein.

Finally, as yet, no one has directly demonstrated the proper assembly of newly-imported subunits into complex holoenzymes in mitochondria, although Lewin and Norman (1983) have strong evidence in the case of the yeast F_1-ATPase. They studied the assembly of the F_1-ATPase complex in isolated mitochondria by initially incubating yeast mitochondria with radiolabelled precursors to the α, β and γ subunits. Newly-assembled F_1-ATPase was detected by autoradiography of the isolated enzyme, the criterion being that only polypeptide subunits which had been synthesised in vitro and imported into the isolated mitochondria, could be radioactive. Incorporation of labelled subunits did not occur in the presence of uncouplers or a divalent metal ion chelator, nor did it occur in submitochondrial particles. They also showed that incorporation of labelled ATPase subunits into the enzyme could be competed by unlabelled subunits, provided the unlabelled

proteins were added before mitochondria were incubated with radioactive precursor. The latter two findings suggest that precursors are being assembled into new F_1-ATPase complexes rather than exchanging with pre-existing subunits.

1.4 Mitochondrial Phosphate Transport Protein

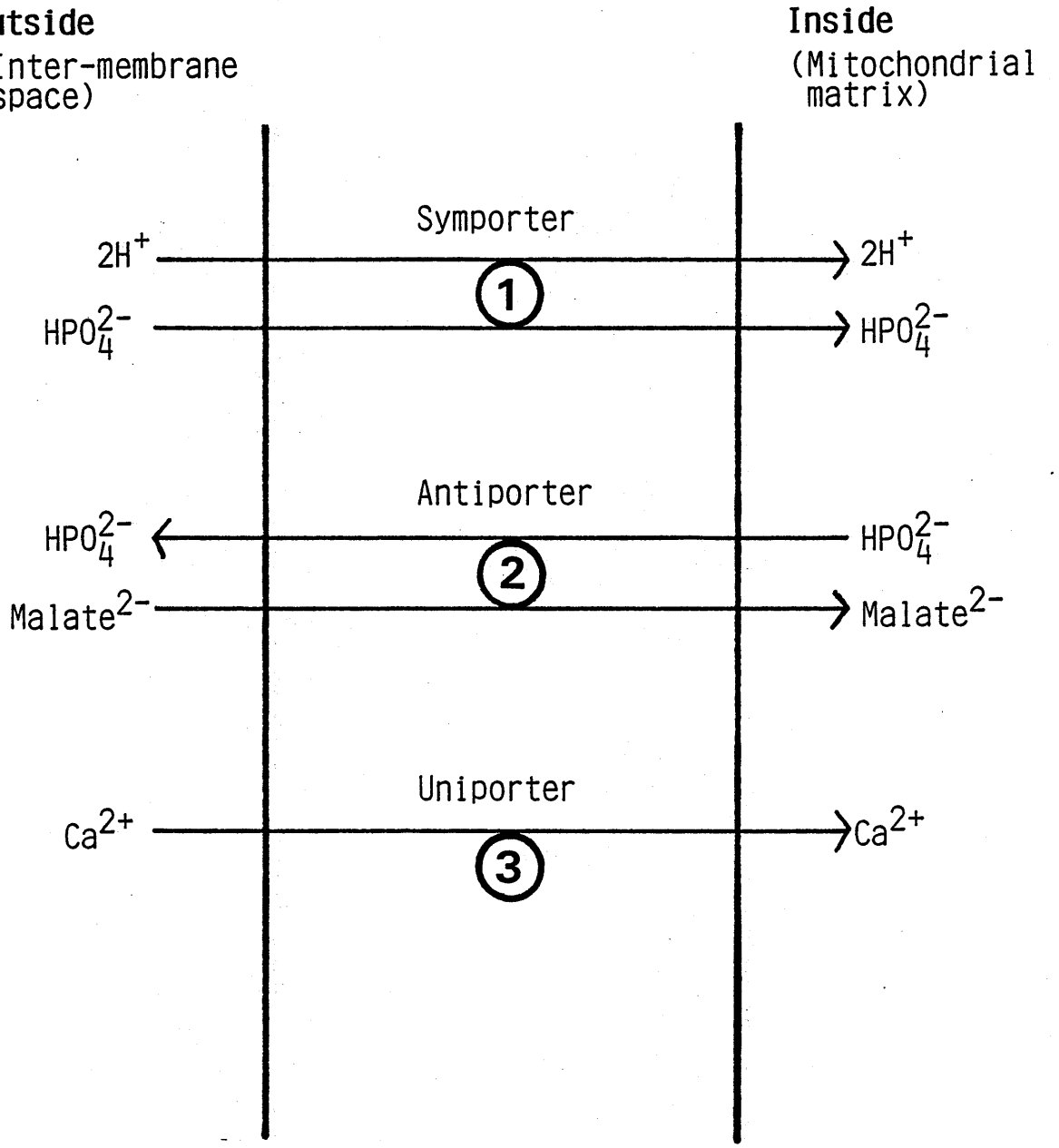
1.4.1 Introduction

A special feature of the mitochondrion is the presence of a double membrane system in which the outer membrane displays an unusual permeability, allowing non-specific access to molecules up to approx. 4,000 M_r (Pfaff et al., 1968). The inner membrane represents the physical permeability barrier to the passage of small molecules into the organelle.

Many of the most important functions of the mitochondrion (Krebs cycle oxidations, fatty acid oxidation, ATP synthesis) occur in the matrix and substrates and co-factors for these reactions are separated from the enzymes by the inner membrane permeability barrier. This segregation of reactions in mitochondria suggests that a series of carriers must exist for the transport of the substrates and products across the inner membrane.

There are two main types of transporters in mitochondria; anti-porters, where a single ion or substrate is moved into the matrix in exchange for one of similar charge and symporters where the transport of the anion is also electroneutral, as it is accompanied by a proton to achieve electrical balance. Also present are uniporters where a single ion is transported into the matrix (see Fig. 1.4.1). A transport system of particular importance for maintaining mitochondrial functions

Fig. 1.4.1



1. Phosphate transport protein
2. Phosphate/dicarboxylic acid carrier protein
3. Ca^{2+} transport protein

is the inorganic phosphate carrier system. Transport of inorganic phosphate into the mitochondrial matrix can occur via two distinct carrier system; an electro-neutral phosphate/dicarboxylic acid exchange carrier and a phosphate/hydroxyl ion transport protein, which catalyses the influx of about 90% of mitochondrial phosphate. These two transport systems are particularly important because:

i) they supply the inorganic phosphate which is essential for maintaining the steady-state oxidative phosphorylation of ADP in the organelle.

ii) the latter carrier is directly linked to the pH gradient generated by the electron transport chain in the inner mitochondrial membrane.

iii) they allow flux of dicarboxylates and tricarboxylates via the phosphate/dicarboxylic exchange carrier.

1.4.2 Identification of the Protein

An interesting property of the two phosphate carrier systems is their differing sensitivity to sulphydryl group reagents. The phosphate/hydroxyl ion antiporter can be inhibited by low concentrations of N-ethylmaleimide, mersalyl and p-mercuribenzoate (Fonyo and Besman, 1968, Tyler, 1969; Coty and Pedersen, 1974a). In contrast, the phosphate/dicarboxylate carrier can be blocked by mersalyl and p-mercuribenzoate but is insensitive to N-ethylmaleimide (Meyer and Tager, 1969, Meijer et al., 1970, Quagliariello and Palmieri, 1972; Coty and Pedersen, 1974a). The phosphate/dicarboxylate carrier can also be inhibited by substrate analogues such as n-butylmalonate

(Hoek et al., 1971; Coty and Pedersen, 1974a).

In 1974(b), Coty and Pedersen using the selective inhibitors N-ethylmaleimide and n-butylmalonate demonstrated that the overall phosphate transport activity could be resolved into two components; an N-ethylmaleimide sensitive component, which catalysed the transport of about 90% of the phosphate i.e. the phosphate/pH gradient-linked carrier system and an n-butylmalonate sensitive transport protein i.e. the phosphate/dicarboxylate exchange carrier.

Identification of phosphate/hydroxyl ion carrier protein (phosphate transport protein) has been greatly facilitated by its sensitivity to N-ethylmaleimide. Coty and Pedersen (1975), on treating rat liver mitochondria with a concentration of N-[^{3}H]-ethylmaleimide to produce maximum inhibition of phosphate transport, labelled at least 10 polypeptide components of the inner membrane. A marked increase in the specificity of N-ethylmaleimide for components of the phosphate transport system, was attained by first protecting with the reversible sulphydryl group inhibitor p-mercuribenzoate as illustrated in Fig. 1.4.2. Using this technique N-[^{3}H]-ethylmaleimide, labelled five proteins, one of which, a 32,000 M_r value protein, contained 40% of the bound radioactivity.

In 1978, Wohlrab and Greaney Jr. studied the N-[^{3}H]-ethylmaleimide-labelling pattern of proteins in sub-mitochondrial particles, prepared by sonication of mitochondria purified from the flight muscles of the blowfly, Sarcophaga bullata. The reasons for studying phosphate carrier protein(s) in mitochondria from the flight muscle of the blowfly were:

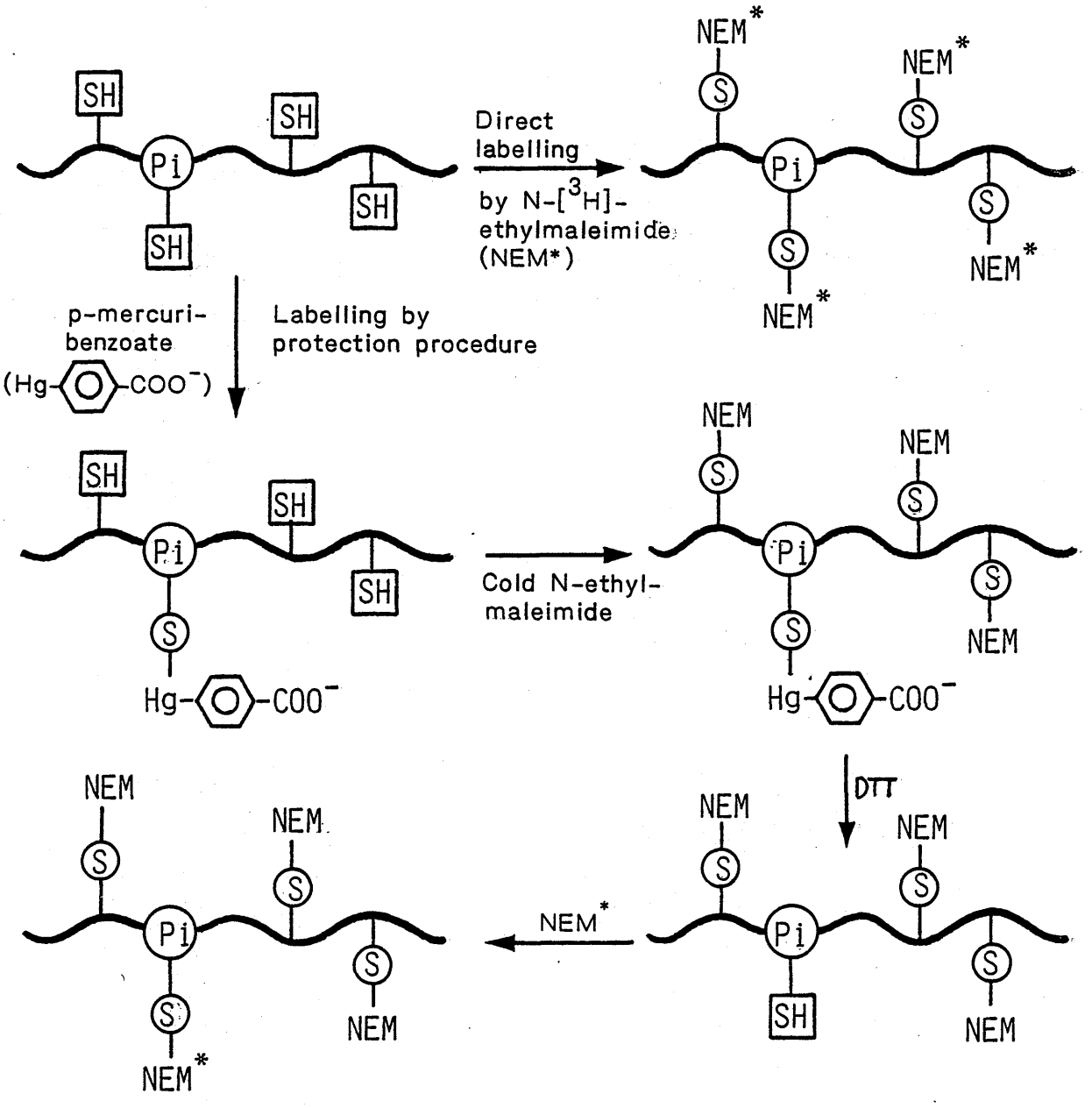
i) mitochondria have a high metabolic capacity which implies a high concentration of phosphate transport systems;

Fig. 1.4.2

THE EFFECT OF p-MERCURIBENZOATE PROTECTION ON THE SPECIFITY OF LABELLING OF THE PHOSPHATE TRANSPORT SYSTEM WITH N-[^{3}H]-ETHYLMALEIMIDE

(Reproduced from Coty and Pedersen 1975)

This diagram compares the labelling of the mitochondrial phosphate transport system and other membrane protein with N-[^{3}H]-ethylmaleimide using two different procedures: direct labelling (upper reaction) and labelling after p-mercuribenzoate protection and blocking with N-ethyl-maleimide (lower scheme). If phosphate transport is inhibited by direct addition of N-[^{3}H]-ethylmaleimide, a number of membrane proteins are labelled in addition to the N-ethylmaleimide binding component of the phosphate transport system. The p-mercuribenzoate protection procedure, however, provides means of reducing the extent and increasing the specificity of labelling. This procedure consists of the following four steps: (a) Protection of the phosphate transport system -SH group with p-mercuribenzoate. (b) Blocking of accessible membrane -SH groups with excess 'cold' N-ethylmaleimide. (c) Removal of p-mercuribenzoate protecting group with DTT, followed by washing of the mitochondria to remove excess reagent. (d) Reinhibition of phosphate transport with N-[^{3}H]-ethylmaleimide.


SH
Pi
Direct labelling
by N-[3H]-ethylmaleimide (NEM*)
NEM*
S
p-mercuri-benzoate
(Hg-⬡-COO⁻)
Labelling by protection procedure
Hg-⬡-COO⁻
Cold N-ethyl-maleimide
NEM
DTT

ii) mitochondria appear to possess only one phosphate transport system (Van den Bergh and Slater, 1962; Wohlrab, 1976) and thus represent a unique membrane for the study of phosphate transport.

Seven major labelled proteins were observed under the conditions employed, only two of which, 32,000 and 45,000 M_r value species were common to the rat liver mitochondria (Coty and Pedersen, 1975).

Further confirmation as to the identity of the protein(s) responsible for phosphate transport was provided by Wohlrab (1978), again using mitochondria prepared from the flight muscle of the blowfly. By comparing the alkylation of proteins in the submitochondrial particles with the inhibition of phosphate transport, Wohlrab found that the extent of labelling of proteins of M_r value 45,000 and 32,000 could be correlated closely with the degree of inhibition of transport.

Finally, Wohlrab (1979) presented evidence which strongly suggested that only 32,000 M_r protein constituted the phosphate transporter. By comparing the N-[^{3}H]-ethylmaleimide labelling patterns of sub-mitochondrial particles prepared from rat heart with those of the blowfly flight muscle, Wohlrab (1979) illustrated that the 45,000 M_r protein was absent from heart sonic submitochondrial particles. Since both types of mitochondria are energetically active and possess a phosphate carrier system, this finding strongly suggested a role for the 32,000 M_r protein in phosphate transport.

1.4.3 Purification Studies on the Phosphate Transport Protein

Wohlrab (1980), having identified the protein responsible for phosphate transport in mitochondria, partially purified the phosphate transport protein from beef heart mitochondria, by taking advantage of its low affinity for hydroxylapatite. The purified fraction consisted of the

phosphate transport protein and the carboxyatractyloside binding protein (ADP-ATP translocase protein). SDS-polyacrylamide gel electrophoresis analysis demonstrated that the phosphate transport protein had an M_r of 34,000. Reconstitution experiments, in which the phosphate transport protein was incorporated into liposomes, suggested a primary role in mitochondrial phosphate transport. However, the reconstituted system catalysed phosphate-phosphate exchange, as determined by ^{32}Pi efflux or uptake, with a maximal exchange rate which was about 2% of the rate in isolated mitochondria (Coty and Pedersen, 1974b). This phosphate-phosphate exchange was shown to be sensitive to N-ethylmaleimide, mersalyl and p-mercuribenzoate but insensitive to the dicarboxylate carrier inhibitor, n-butylmalonate or the adenine nucleotide translocase inhibitor, carboxyatractyloside.

In 1981, Kolbe et al. successfully separated the pig heart mitochondrial phosphate transport protein from the adenine nucleotide translocase, by passing the hydroxylapatite eluate through a Celite column. Reconstitution experiments in which this hydroxylapatite/Celite eluate was incorporated into liposomes, demonstrated that the rate of exchange of ^{32}Pi was slightly higher than in the hydroxylapatite eluate but still only represented about 2% of the activity in vivo. The low activity of phosphate transport reported in these two studies did not rule out the possibility that a secondary, contaminating protein was responsible for this activity and not the primary phosphate transport protein.

Kolbe et al. (1981) in the same study as described above, demonstrated that the Celite purified protein could be resolved into four separate components by SDS-polyacrylamide gradient gel electro-

phoresis (M_r range 35,000-31,500). Three possible explanations were proposed to interpret this result:-

i) the phosphate carrier of mitochondria occurs as a complex of up to four different polypeptide chains;

ii) some of the four proteins represent proteolytically or otherwise modified forms of the same protein;

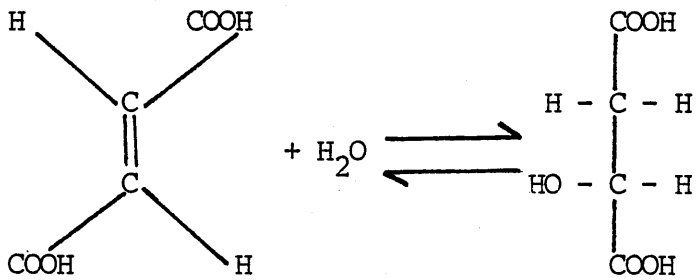
iii) the phosphate carrier is composed of one protein, the other proteins represent different functional proteins, not related to phosphate transport.

In recent years, a great deal of attention has been directed towards solving this problem and will be discussed in chapter three.

1.5 Fumarase or Fumarate Hydratase (EC 4.2.1.2)

1.5.1 Introduction

Fumarase or fumarate hydratase (EC 4.2.1.2) catalyses the reversible hydration of fumarate to L-malate according to the following reaction:



Fumarate L-malate

The hydrogen and hydroxyl groups of water are exchanged stereospecifically during the conversion of fumarate and L-malate.

Due to its fundamental role as an essential enzyme of the tricarboxylic acid cycle (Fig. 1.5.1), fumarase is found in a wide variety of organisms including bacteria (de Mello Ayres and Lara, 1962; Lamartiniere et al., 1970), yeast (Favelukes and Stoppani, 1958), moulds (Jacobson, 1931), plants (Shih and Barnett, 1968), invertebrates (Clutterbuck, 1928) and mammals (Kuff, 1954; Shepherd, et al., 1955).

In 1952, fumarase was first successfully crystallised from pig heart muscle by Massey, who demonstrated that it crystallised from ammonium sulphate solutions in an essentially homogeneous form. Subsequent methods for improving the yield from pig heart muscle, were developed by Kanarek and Hill (1964), enabling many of its molecular and catalytic properties to be studied in detail.

The M_r value of the native enzyme as determined by sedimentation equilibrium studies was 194,000 (Kanarek and Hill 1964). However, the following experimental findings indicated that the native enzyme was composed of four identical subunits (Kanarek et al., 1964; Robinson et al., 1967):

i) In the presence of 6M urea the M_r was judged to be 48,500 by sedimentation equilibrium methods.

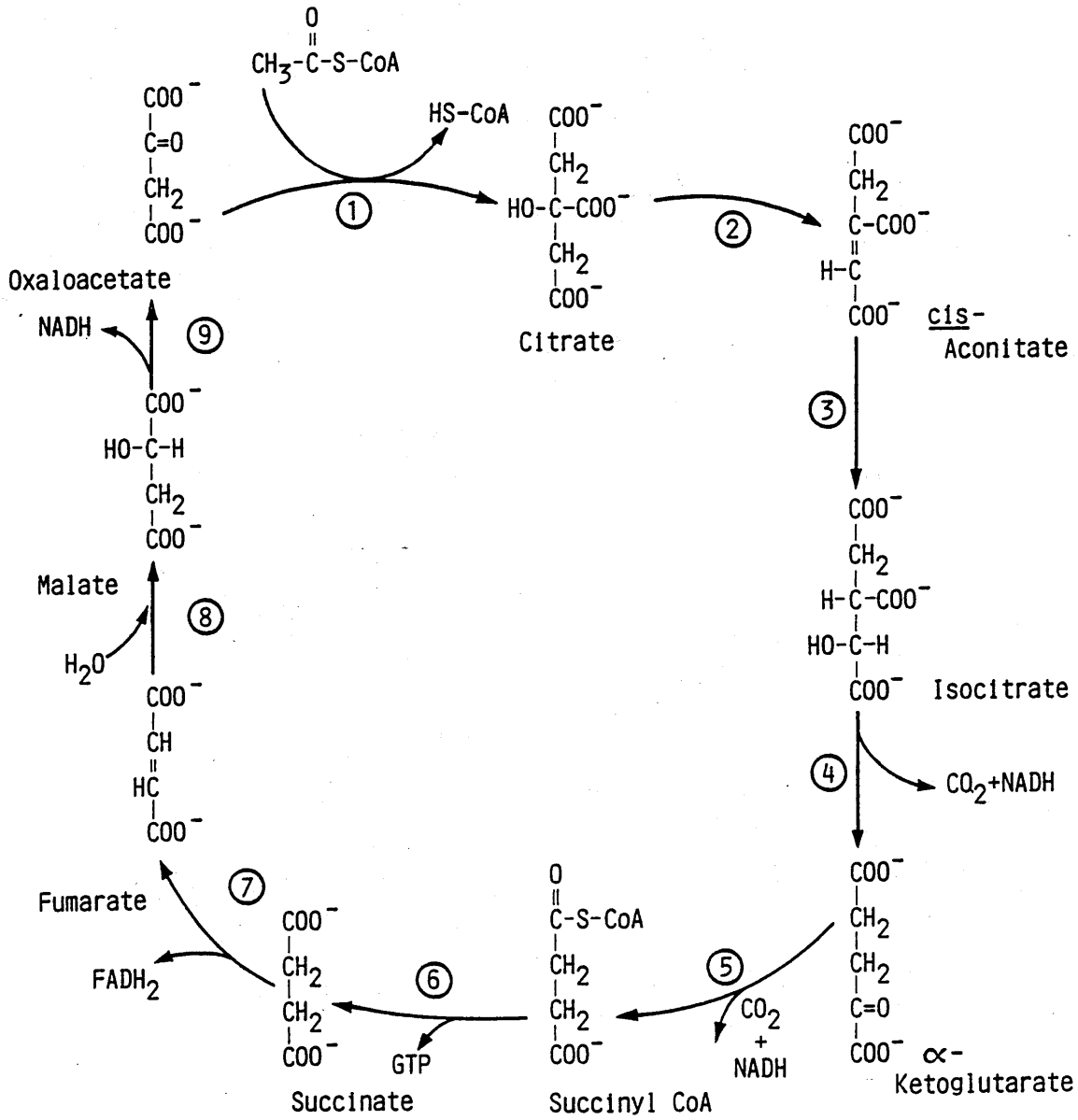
ii) Amino-terminal end group analysis revealed 3.6 residues of alanine per molecule assuming an M_r of 194,000.

iii) Tryptic peptide maps of fumarase revealed about one-quarter of the expected number of peptides based on the lysine and arginine content of the enzyme.

The enzymatically-active form of fumarase appears to be the tetrameric structure since, in general, any condition which results

Fig. 1.5.1

THE TRICARBOXYLIC ACID CYCLE



Fumarase catalyses reaction (8) of the citric acid cycle.

in dissociation of the enzyme leads to a loss of enzymic activity. In the presence of urea or guanidine hydrochloride the enzyme is fully dissociated and inactivated at concentrations greater than 2.0M (Robinson et al., 1967; Teipel and Hill, 1971). However, removal of these agents by dialysis against phosphate buffer or phosphate buffer containing malate, restores over 90% of the fumarase activity.

For many years it was thought that the substrate specificity of fumarase was quite rigid and that the enzyme only reacted with its natural substrates, fumarate and L-malate. However, several artificial substrates have been discovered, the structures of which revealed two general requirements for fumarase substrates:-

a) Two negatively charged carboxyl groups are required for a substrate, in agreement with earlier findings that this was also a structural requirement for competitive inhibitors (Massey 1953).

b) The hydroxyl groups in all substrates must have the same configuration as found in L-malate.

The reversible binding of fumarase with its substrates, fumarate and L-malate and with two competitive inhibitors, citrate and trans-aconitate has been examined by equilibrium dialysis (Teipel and Hill 1968). These studies revealed that there were four substrate or inhibitor binding sites per molecule of enzyme or an average of one site per polypeptide chain subunit. At low concentrations of substrate the hydration of fumarate and dehydration of malate follow Michaelis-Menten kinetics but at concentrations more than about five times Km, substrate activation is observed (Taraszka and Alberty 1964). The substrate activation can be explained by one of the following mechanisms:-

i) A second molecule of substrate can bind at a site(s) other than the active site and thereby increase the reactivity of the active site.

ii) Fumarase possesses two or more identical binding sites for substrate which display co-operative interactions such that binding of substrate to the first site(s) decreases the affinity of substrate for the latter site(s). This mechanism is known as 'negative co-operativity' and was originally proposed by Conway and Koshland Jr. to explain the kinetic behaviour of glyceraldehyde-3-phosphate dehydrogenase (Conway and Koshland Jr. 1968).

iii) The enzyme possesses two or more independent but non-identical binding sites, or, alternatively, there are two or more forms of the enzyme present which have different affinities for substrate.

1.5.2 Compartmentalisation of Fumarase

It has long been established that fumarase is located in the mitochondrial matrix where it is an essential enzyme of the tricarboxylic acid cycle. However, fumarase has also been reported to be involved in many metabolic processes which occur in the cell cytoplasm (Ratner et al., 1953, Ravdin and Crandall, 1951; Knox and Lemay-Knox, 1951):-

i) In the urea cycle fumarate is generated by the enzyme argininosuccinase which cleaves argininosuccinate into arginine and fumarate.

ii) During tyrosine-oxidation, fumarate is generated by the hydrolysis of 4-fumarylacetoacetate.

iii) In purine nucleotide biosynthesis, fumarate is derived from adenylsuccinate.

In all cases, an extramitochondrial fumarase would be able to link these cycles to the mitochondrial matrix tricarboxylic acid cycle, by converting fumarate to malate, which could subsequently be taken up as a permeant substrate.

In 1976, Nakashima et al. demonstrated that there was as much fumarase activity localised in the rat liver cytosol as in the mitochondria. This intracellular distribution of hepatic fumarase was confirmed in analogous studies in mouse, rabbit, dog, chicken, snake, frog and carp (Akiba et al., 1984). In the same report, these workers looked at the subcellular distribution of the enzyme in rat kidney, brain, heart and skeletal muscle and in hepatoma cells (AH-109A). Among these tissues, the brain was the only exception having no fumarase activity in the cytosolic fraction, all the other tissues showing a bimodal distribution of the enzyme.

In recent years, extensive research has been directed towards gaining an insight into the structural and physical properties of these two rat liver fumarase activities. The first successful purification of the mitochondrial and cytosolic fumarases was achieved by Kobayashi et al. (1981). They purified the two fumarases from isolated rat liver mitochondrial and cytosolic fractions respectively, employing the method of Kanarek and Beeckmans (1977), which involved affinity chromatography on a pyromellitic acid column. The mitochondrial fumarase was resolved into two fractions by Whatman DE-52 column chromatography, a non-absorbed and an absorbed fraction whereas the cytosolic fraction largely passed through this column. The fumarase activity contained in these three fractions was then crystallised

with ammonium sulphate. Subsequent studies to characterize the physicochemical and catalytic properties of the two separate fumarases are summarised in table 1.5.1. It can clearly be seen from this table that the two forms of the enzyme are very similar with regard to their properties. In addition, the amino acid composition of the cytosolic fumarase was shown to be very similar to that of the mitochondrial enzyme. Furthermore, immunochemical techniques could not differentiate the two enzyme forms from one another, since a single precipitin band was formed between the rabbit antiserum against the cytosolic fumarase and the cytosolic or mitochondrial fumarase. To obtain more conclusive evidence on the identity of the cytosolic and mitochondrial fumarases, Kobayashi and Tuboi (1983) examined the terminal amino acid residues. The carboxyl-terminal amino acid of both enzymes was identified as leucine by using carboxypeptidase A. However, the amino-terminal amino acid residue of each fumarase could not be identified by classical methods, suggesting that they were masked. The N-termini of the mitochondrial and cytosolic fumarases were subsequently identified as pyroglutamic acid and N-acetylalanine, respectively. In the same study, these workers also compared the primary structures of the enzymes in detail by digesting either with an arginine-specific protease or with cyanogen bromide. Electrophoretic profiles of the digests were indistinguishable from each other.

As would be expected from these observations on the molecular properties of the mitochondrial and cytosolic fumarases purified from rat liver, it has until recently been impossible to separate them from their mixture. However, Hiraga et al. (1984) illustrated that the mitochondrial and cytosolic fumarases of rat liver could be

TABLE 1.5.1

MOLECULAR PROPERTIES OF RAT LIVER MITOCHONDRIAL AND CYTOSOLIC FUMARASES

	MITOCHONDRIAL		CYTOSOLIC
	NON-ABSORBED	ABSORBED	
-Polyacrylamide Gel Electrophoresis	M_r 49,000	M_r 49,000	M_r 49,000
rose Density Gradient Centrifugation ecular Sieve Chromatography (Sephadex G200)	M_r 200,000	M_r 200,000	M_r 200,000
ss-Linking Experiments (Glutaraldehyde)	No. of Subunits 4	No. of Subunits 4	No. of Subunits 4
arent K_m for Fumarate			
Without PO_4	0.013	0.015	0.013
+ 1mM PO_4	0.056	0.056	0.062
+ 10mM PO_4	0.333	0.333	0.286
arent K_m for Malate			
Without PO_4	0.18		0.14
+ 10mM PO_4	0.59		0.60

separated from each other by Bio-gel hydroxylapatite column chromatography. This showed that there are some conformational differences between the native proteins which have been indistinguishable from their physicochemical, catalytic and immunochemical properties.

Finally, it has been suggested that the cytosolic and mitochondrial fumarases are products of the same gene (Tolley and Craig, 1975; McKusick and Ruddle, 1977). The molecular findings to date would certainly confirm such a suggestion, since the two fumarase activities have been shown to be very closely related to one another. If the mitochondrial and cytosolic fumarases are derived from the same gene, the mechanism by which fumarase synthesised on cytoplasmic ribosomes is distributed into two different subcellular compartments is an extremely important question that has still to be resolved.

GENERAL AIMS

When I started my research in October, 1981, I set out with the following objectives:

i) to purify the phosphate transport protein from rat liver mitochondria and to further characterize the isolated protein by employing its known sulphydryl group sensitivity properties;

ii) to raise a polyclonal, monospecific antiserum to the isolated phosphate transport protein which I could use as a specific tool to follow its biosynthesis in vivo, employing mammalian tissue-culture cell lines;

iii) to raise a polyclonal, monospecific antiserum to pig heart fumarase and to subsequently utilise this antiserum to gain an insight into the mechanism of bimodal distribution of the enzyme, in vivo, as above (see ii).

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Biological Materials

Animals

Female rats of the Wistar strain were bred and obtained from the Departmental Animal House.

New Zealand white rabbits (4 months old) were purchased from MRC accredited sources.

Cell Lines

Buffalo rat liver (BRL) cells were a kind gift from Dr. J. Pitts, Beatson Institute for Cancer Research, Garscube Estate, Glasgow, U.K.

Pig kidney (PK-15) cells were supplied by GIBCO Europe, Paisley, U.K.

Bovine kidney (NBL-1) cells were purchased from Flow Laboratories Ltd., Irvine, Ayrshire, U.K.

Yeast

The following yeast strains were the kind gift of Dr. G. Reid, Department of Microbiology, University of Edinburgh:-

Wild type *Saccharomyces cerevisiae* (*S.cerevisiae*) strain D273-10B (α, ATCC 25657) and the corresponding rho$^-$ mutant D273-10B-1

2.1.2 Chemicals for Polyacrylamide Gel Electrophoresis

Acrylamide monomer, N,N' methylene bis-acrylamide (specially purified for gel electrophoresis), ammonium persulphate and sodium dodecyl sulphate (SDS) were supplied by BDH Chemicals, Poole, Dorset, U.K.

N,N,N',N' tetramethylethylenediamine (TEMED) and Coomassie Brilliant Blue R250 were purchased from Sigma (London) Chemical Company, Poole, Dorset, U.K.

Pyronin Y dye was supplied by George T. Gurr Ltd., London, U.K.

Low M_r standards were purchased from Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Central Milton Keynes, Buckinghamshire, U.K.

2.1.3 Radiochemicals

L-[^{35}S]-methionine (approx. 1100 Ci/mmol) and carrier free Na^{125}I (approx. 100 mCi/ml) were purchased from Amersham International plc, White Lion Road, Amersham, U.K.

N-[2-^{3}H]-ethylmaleimide (approx. 50 Ci/mmol) was supplied by New England Nuclear, Du Pont (U.K.) Ltd., Southampton, U.K.

2.1.4 Chemicals for Fluorography

Dimethyl sulphoxide (DMSO) and 2,5-diphenyloxazole (PPO) were obtained from Koch-light Laboratories Ltd., Colnbrook, U.K.

Salicylic acid was supplied by Aldrich Chemical Co. Ltd., Gillingham, U.K.

2.1.5 Photographic Materials

Plastic intensifying screen holders (Plast-X cassettes) were purchased from Anthony Monk (ENG) Ltd., Sutton-in-Ashfield, U.K.

Du Pont Cronex intensifying screens (18 x 24 cm) were supplied by MAS, Springkerse Industrial Estate, Stirling, U.K.

Kodak X-Omat S film, Kodak XAR-S film, Kodak LX-24-X-ray developer and Kodak FX-40 X-ray liquid fixer were obtained from Kodak

(U.K.) Ltd., London, U.K.

2.1.6 Immunochemicals

Freund's adjuvants were obtained from Difco Laboratories Ltd., Colnbrook, U.K.

Nitrocellulose paper (0.45μm pore size) was supplied by Anderman and Co. Ltd., Laboratory Supplies Division, Kingston-upon-Thames, Surrey, U.K.

Heat-inactivated horse serum was purchased from GIBCO Europe, Paisley, U.K.

Anti-rabbit gamma-globulin conjugated to horse radish peroxidase was supplied by Miles Laboratories Ltd., Stoke Poges, Slough, U.K.

Falcon 3912 Microtest III™ Flexible assay plates were purchased from Flow Laboratories Ltd., Irvine, Ayrshire, U.K.

Goat serum and normal rabbit serum were obtained from the Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.

Pansorbin (Staphylococcus aureus Cowan I strain (S.aureus) cell suspension) was supplied by Calbiochem-Behring, Cambridge Bioscience, Hardwick, Cambridge, U.K.

Protein A isolated from S.aureus Cowan I strain was purchased from Sigma (London) Chemical Co. Ltd., Poole, Dorset, U.K.

2.1.7 Tissue Culture Materials

Sterile reagents to prepare Glasgow modification of Eagle's medium were supplied by the Tissue Culture Unit, Department of Biochemistry, University of Glasgow, with the exception of non-essential amino acids (NEAA), (-) methionine Eagle's medium and glutamine which

were all purchased from GIBCO Europe, Paisley, U.K.

Yeast

Bacto-peptone and yeast extract were purchased from Difco Laboratories Ltd., Colnbrook, U.K.

2.1.8 Chromatographic Media

Hydroxylapatite (Bio-gel HTP) was purchased from BIO-RAD Laboratories Ltd., Watford, Hertfordshire, U.K.

Celite type 535 was supplied by Uniscience Limited, Cambridge, U.K.

Procion Red A was obtained from Amicon Ltd., Scientific Systems Division, Upper Mill, Stonehouse, Gloucestershire, U.K.

Sephadex G-50 was purchased from Pharmacia Ltd., Pharmacia House, Midsummer Boulevard, Central Milton Keynes, Buckinghamshire, U.K.

2.1.9 Enzymes

The following enzyme preparations were purchased from Sigma (London) Chemical Co., Poole, Dorset, U.K.:-

Fumarase (EC 4.2.1.2) from porcine heart

Citrate synthetase (EC 4.1.3.7) from porcine heart

2.1.10 General Chemicals

Oxaloacetic acid (OAA), acetyl CoA, 2,4 dinitrophenol, digitonin, L-malic acid, bovine serum albumin (BSA), 1,10-phenanthroline, 5,5'-dithio(bis)2-nitrobenzoic acid (DTNB), Triton X-100, sodium deoxycholate (DOC), polyethylene sorbital mono-laureate (Tween 20),

carbonyl cyanide m-chlorophenylhydrazone (CCCP), D-mannitol, o-phenylene diamine, 4-morpholinopropanesulfonic acid (Mops), concanavalin A (con A) and wheat germ agglutinin (WGA), acetyl-L-leucyl-L-leucyl-L-argininal (leupeptin), iodoacetamide, p-amino-benzamidine were all supplied by Sigma (London) Chemical Co., Poole, Dorset, U.K.

EGTA, 2-mercaptoethanol and Folin and Ciocalteu's phenol reagent were purchased from BDH Chemicals Ltd., Poole, Dorset, U.K.

Phenylmethylsulfonylfluoride (PMSF) and Tris were obtained from Boehringer Mannheim, Boehringer Corporation (London) Ltd., Lewes, East Sussex, U.K.

Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) was purchased from Aldrich Chemical Co., Ltd., Gillingham, U.K.

Sodium azide was purchased from Hopkin and Williams, Chadwell Heath, Essex, U.K.

Dithiothreitol (DTT) was supplied by Koch-light Laboratories, Colnbrook, U.K.

Iodogen™ was purchased from Pierce Chemical Co., Rockford, Illinois, U.S.A.

All the other fine chemicals and reagents used were of the highest available commercial grade.

2.2 GENERAL METHODS

2.2.1 Protein Estimations

The amount of protein in a sample was measured either by the method of Lowry et al. (1951) incorporating the modification of Markwell et al. (1976) or by the Biuret method (Gornall et al., 1949).

Calibration curves were constructed from a standard stock solution of BSA (10mg/ml).

2.2.2 Enzyme Assays

A) Citrate synthetase (EC 4.1.3.7)

The activity of citrate synthetase was measured by the method of Srere (1969). The assay contained in a total vol. of 1ml, 0.4M Tris-HCl pH 8.0, 0.5mM DTNB solution, 3mM acetyl CoA and 50-100µg of sample protein. Initiation of the reaction was achieved by the addition of 5mM OAA and the formation of 5-thio-2-nitrobenzoate anion monitored at 412nm.

B) Fumarase (EC 4.2.1.2)

The activity of fumarase was measured by the method of Hill and Bradshaw (1969) by monitoring the formation of fumarate from malate at 240nm. The procedure for performing the assay, in a total vol. of 1ml, was as follows:

0.05M L-malic acid solution (dissolved in 0.1M potassium phosphate buffer pH 7.6) was equilibrated to 25°C.. The reaction was started by the addition of 1-5µg of purified fumarase or 50-100µg of mitochondrial extract protein.

2.2.3 Radioiodination of Proteins

In general, iodination of proteins with $Na^{125}I$ was performed by the method of Salancinski et al. (1981). 1mg of protein dissolved in 1ml of 20mM Tris-HCl buffer pH 7.2 containing 150mM NaCl was added to a small glass vial, the bottom surface of which was coated with a

thin layer of Iodogen™ (1mg). Radiolabelling was initiated by the addition of 300-500μCi of $Na^{125}I$ (100m Ci/ml) before allowing the reaction to proceed for 15 min at room temperature. Incorporation was terminated by transferring the mixture to a centrifuge tube and spinning at 1000g for 5 min to pellet any residual Iodogen™. Free [^{125}I] iodide was removed by subjecting the supernatant fraction to chromatography on a Sephadex G-50 column (6.5 x 1.4cm), which had been equilibrated with 20mM Tris-HCl buffer pH 7.2 containing 150mM NaCl. Void volume aliquots (1ml) were analysed on an LKB Wallac 1275 mini-gamma counter and those containing the ^{125}I-labelled protein pooled, dispensed (2 x 10^6 cpm/fraction) and stored at -20°C. Bio-rad low M_r standards and protein A were labelled in a similar manner. The lectins, con A and WGA were labelled with $Na^{125}I$ by the method described in section 3.2.2.

2.2.4 SDS-Polyacrylamide Gel Electrophoresis

2.2.4.1 Preparation of Gels-Electrophoretic Conditions

Polyacrylamide gel electrophoresis was carried out in a discontinuous system, according to Laemmli (1970) in the presence of 0.1% (w/v) SDS. Slab gels (19 x 9.5cm) were cast between two glass plates separated by 1.5mm thick teflon spacers in a perspex box designed to accommodate four pairs of glass plates at any one time. To prepare the separating gel, acrylamide stock solution, running gel buffer and ammonium persulphate (listed in table 2.2.4.1) were mixed and degassed under vacuum on a water pump. Polymerisation was initiated by the addition of TEMED to the mixture, the gel solution poured immediately and overlaid with isopropanol. When the separating gel

TABLE 2.2.4.1

Composition and volumes of solutions used to cast SDS-polyacrylamide gels

Stock solution	Separating gel		Stacking gel
	10% (w/v) Vol. (ml)	12.5% (w/v) Vol. (ml)	4.5% (w/v) Vol. (ml)
Acrylamide 29.2% (w/v) and bis-acrylamide 0.8% (w/v)	60	75	9.0
Running gel buffer 0.75M Tris-HCl pH 8.8, 0.2% (w/v) SDS	90	90	-
Stacking gel buffer 0.17M Tris-HCl pH 6.8, 0.14% (w/v) SDS	-	-	41
H_2O	-	-	7.75
0.3% (w/v) ammonium persulphate	30	15	-
10% (w/v) ammonium persulphate	-	-	-
TEMED	0.045	0.045	0.02

had set the isopropanol layer was removed and stacking gel solution (see table 2.2.4.1) placed on top of the separating gel. Sample wells were formed by inserting a teflon comb into the stacking gel solution (24 wells per gel) prior to polymerisation. Variations on this method of casting gels will be described in the appropriate section.

The reservoir buffer contained 0.025M Tris-HCl pH 8.3, 0.192M glycine and 0.1% (w/v) SDS. Electrophoresis was performed at 60mA per gel until the tracking dye had reached the bottom edge of the gel. The gel was maintained at 10-15^{o}C by a circulating cold water system which was attached to the electrophoretic apparatus.

2.2.4.2 Preparation of Protein Samples for Electrophoresis

In general, samples to be resolved by SDS-polyacrylamide gel electrophoresis were precipitated by the addition of 4 vol. of acetone and the protein collected by centrifugation at 1500g for 10 min on a Beckman model TJ-6 benchtop centrifuge. The pellet was dissolved in Laemmli sample buffer (0.0625M Tris-HCl pH 6.8, 2% (w/v) SDS, 10% (w/v) sucrose, 5% (v/v) 2-mercaptoethanol and 0.001% (w/v) Pyronin Y, to act as tracker dye), by boiling for 2 min at 100^{o}C.

2.2.4.3 Staining of Protein(s)

Protein(s) were visualised after electrophoresis by immersing the gel in a solution containing; 0.04% (w/v) Coomassie Brilliant Blue R250, 25% (v/v) isopropanol and 10% (v/v) acetic acid for 8-12h at room temperature. Destaining of the gel to reveal protein(s) was achieved by washing the gel several times in a solution containing 25% (v/v) isopropanol and 10% (v/v) acetic acid.

Generally, if radiolabelled proteins were being investigated the gels were fixed in a solution of 25% (v/v) isopropanol, 10% (v/v) acetic acid for 8-12h at room temperature before further processing.

2.2.4.4 Estimation of the Relative Molecular Mass (M_r) of a Protein

To calculate the M_r value of a protein on an SDS-polyacrylamide gel, the electrophoretic mobility (R_f) must be defined:-

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

The R_f value for a protein can be calculated either by directly measuring the distance on the stained gel or, more accurately, after densitometric scanning of the gel with an LKB 2202 Ultroscan laser densitometer. The method has to be calibrated with a set of standard proteins of known M_r values. A plot of R_f value versus log M_r for these marker proteins enables the subunit M_r of the sample protein to be determined directly.

A list of the standard marker proteins used and their M_r values is given below:-

Phosphorylase b 94,000 ; BSA 67,000 ; ovalbumin 43,000 ; carbonic anhydrase 30,000 ; soyabean trypsin inhibitor 20,100 ; α-lactalbumin 14,400.

2.2.4.5 Autoradiography

Polyacrylamide slab gels containing ^{125}I-labelled proteins were dried down on Whatman 3MM filter paper using a Flexi-dry™ freeze-drier (FTS Systems Inc., Stone Ridge, New York, U.S.A.). Detection of

radiolabelled proteins was carried out at -80°C using Kodak X-Omat S film, employing Du Pont Cronex intensifying screens to enhance the sensitivity of the procedure.

2.2.4.6 Fluorography

Two independent methods, which varied in their use of chemical fluor, were utilised to detect [^{3}H] or [^{35}S]-labelled proteins. The sensitivity of the two techniques was comparable.

A) This procedure adopted the method of Bonner and Laskey (1974) and utilised a water insoluble fluor. After fixing (2.2.4.3), the gel was washed twice for 1h at room temperature with DMSO to remove any water, then placed in a solution of DMSO containing the fluor PPO 20% (w/v) . After a 3h incubation at room temperature, the gel impregnated with PPO was washed continuously for 30 min under running water, resulting in the precipitation of the fluor in the gel.

B) A water soluble fluor, sodium salicylate was incorporated into the gel by closely following the method of Chamberlain (1979). After fixing, (2.2.4.3) the gel was washed several times with distilled water to remove any acetic acid, then immersed in a solution of 1M sodium salicylate and incubated for a further 30 min at room temperature. In both instances the gels were dried down on Whatman 3MM paper before exposure to X-ray film as described in section 2.2.4.5.

2.2.5 Subcellular Fractionation Techniques

2.2.5.1 Isolation of Rat Liver Mitochondria

Mitochondria were prepared by the method of Chance and Hagihara

(1963). Female rats of the Wistar strain (180-200g), which had been starved overnight to eliminate glycogen storage particles, were killed by cervical dislocation, the livers excised and rinsed 2-3 times with ice-cold isolation medium (0.225M mannitol, 0.075M sucrose, 500μM EGTA, 2mM Mops buffer pH 7.2). After washing, the livers were chopped finely with a pair of scissors before cell breakage with a tight-fitting teflon pestle (clearance 0.004-0.006μm) on a Potter-Elvenhjem homogeniser (5 passes). Blood, unbroken cells and nuclei were sedimented by centrifugation of the suspension at 800g for 7 min. The resulting supernatant fraction was decanted into fresh tubes for centrifugation at 6500g for 15 min to pellet the mitochondria. Residual blood and nuclei were removed from this pellet by resuspending it in isolation medium and centrifuging at 800g for 7 min. Finally, mitochondria were pelleted from the above supernatant fraction by centrifugation at 6500g for 7 min before being washed once in isolation medium to remove polysomal and microsomal contamination.

All the centrifugation steps were performed at $4^{o}C$ and the final mitochondrial pellet stored at $-20^{o}C$ until further use.

2.2.5.2 Preparation of Rat Liver Mitoplasts (Mitochondria with Outer Membrane Removed)

Purified mitochondria, isolated from rat liver (2.2.5.1) were resuspended in 10mM Tris-phosphate buffer pH 7.5 (swelling medium), at a protein concentration of 10mg/ml before incubating on ice for 5 min. The mitochondria were then shrunk by the addition of a solution containing 1.8M sucrose, 2mM ATP and 2mM $MgSO_4$. After a further 2 min incubation on ice, mitoplasts were pelleted by centrifugation at 6500g

for 10 min. The mitoplasts were resuspended in isolation medium (2.2.5.1) and washed twice.

2.2.5.3 Isolation of Subcellular Fractions from Cultured Mammalian Cells

Crude subcellular fractions were prepared from the three mammalian cell lines, BRL, PK-15 and NBL-1 by the method of Attardi and Ching (1979). To isolate subcellular fractions from cultures, cells were grown as monolayers in 20 oz. roller bottles at 37°C (2.4.2). On reaching confluence, growth medium was decanted, the cell monolayer washed 3X with a solution containing 0.13M NaCl, 0.005M KCl, before harvesting in 6 vol. of 0.01M Tris-HCl buffer pH 6.7 containing 0.01M KCl and 0.15mM $MgCl_2$. After incubating for 2 min on ice, the cell suspension was disrupted with a Potter-Elvenhjem homogeniser, using a sufficient number of strokes (6 or 7) to break greater than 95% of the cells. Cell breakage was measured by using a light microscope. On addition of 0.25M sucrose, the homogenate was centrifuged at 1100g for 3 min to sediment nuclei and unbroken cells. A small fraction of the supernatant was retained at this stage to represent cell extract, the remainder being subjected to centrifugation at 6500g for 10 min to pellet the mitochondria. The post-mitochondrial supernatant fraction from this spin represented the cytosol of the cell. The mitochondrial pellet was resuspended in 0.01M Tris-HCl buffer pH 6.7 containing 0.25M sucrose and 0.15mM $MgCl_2$ before repeating the low speed centrifugation step to remove any residual nuclei. Finally, this supernatant fraction was centrifuged at 6500g for 10 min to pellet the mitochondria which were subsequently stored in 0.01M Tris-acetate buffer pH 7.0 containing 0.25M sucrose for further analysis.

2.3 IMMUNOLOGICAL TECHNIQUES

2.3.1 Preparation of Antisera

Commercially available pig heart fumarase (EC 4.2.1.2) was used as an antigen. Purity was estimated to be greater than 95% by densitometric scanning of a Coomassie Blue stained polyacrylamide gel. Fumarase (1mg) dissolved in 0.9% (w/v) NaCl was mixed thoroughly with an equal vol. of Freund's complete adjuvant prior to injection subcutaneously at various sites in the neck, back and thighs of the rabbit. This treatment was repeated at 2-3 week intervals in the presence of Freund's incomplete adjuvant. Antiserum was collected by bleeding the rabbit from a marginal ear vein, 10-14 days after the 4th injection of antigen. After allowing the blood to clot overnight at $4^{o}C$, the antiserum was removed with a pasteur pipette, aliquoted (1ml fractions) and stored at $-20^{o}C$. In some instances the antiserum had to be clarified by centrifugation of 2500g for 10 min to remove contaminating red blood cells. Before subsequent bleedings, rabbits were injected 10-12 days previously with 0.5-1.0mg of fumarase in incomplete adjuvant.

A similar regime was followed for the production of the antiserum against the phosphate/hydroxyl ion antiport protein.

2.3.2 Characterization of the Antisera

2.3.2.1 Immune-blotting (Immune-replica analysis)

A) Transfer of proteins to nitrocellulose

After SDS-polyacrylamide gel electrophoresis (2.2.4.1) the

resolved proteins were transferred electrophoretically on to nitrocellulose paper using the BIO-RAD Trans-Blot™ apparatus. A modification of the method of Towbin et al. (1979) was adopted to perform the transfer at 400mA for 3-4h. The electrode buffer consisted of 0.025M Tris-HCl pH 8.3, 0.192M glycine, 20% (v/v) methanol supplemented with 0.02% (w/v) SDS.

B) Immunological detection of proteins on nitrocellulose paper

The procedure of Towbin et al. (1979) was closely followed although a modification suggested by Batteiger et al. (1982) was incorporated into the methodology. After electrophoretic transfer of the proteins to nitrocellulose, additional binding sites on the paper were saturated by immersing for 60 min at 37°C or overnight at 4°C in a buffer containing 20mM Tris-HCl pH 7.2, 150mM NaCl, 0.5% (v/v) Tween 20 and 0.5mg/ml sodium azide (Buffer A). Antiserum, diluted 1 in 40 in buffer A supplemented with 5% (v/v) heat-inactivated horse serum was then incubated with the blot for 90 min at room temperature, after which time the nitrocellulose paper was washed free of excess antibody by rinsing it five times with 60ml of buffer A over a period of 30 min. Antigen-antibody complexes were then decorated with ^{125}I-labelled protein A (2.2.3), by incubating the blot for 90 min at room temperature in buffer A plus the labelled protein A (2×10^6 dpm/50ml). Excess ^{125}I-labelled protein A was removed by washing the paper, as described above. The blot was dried at room temperature overnight, before exposure to autoradiography, as described in section 2.2.4.5

2.3.2.2 Preparation of Cell Lysate for Immune-Precipitation Studies

L-[^{35}S]-methionine-labelled tissue culture cells (see section 5.2.1), were washed 3X in ice-cold phosphate-buffered saline (PBS) solution (20mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.9% (w/v) NaCl) prior to solubilisation in 1ml of buffer containing 0.1M Tris-HCl pH 8.2, 0.1M KCl, 5mM $MgCl_2$, 1% (v/v) Triton X-100 and the protease inhibitor PMSF (final concentration 1mM). Nuclei were removed by centrifugation at 850g for 10 min , DOC and SDS dissolved in above Triton X-100 buffer plus 1,10-phenanthroline (3D-TKM) were added to the post-nuclear supernatant fraction to final concentrations of 1% (w/v) and 0.5% (w/v) respectively. Insoluble debris was removed by high speed centrifugation (19,000g for 30 min), to yield a clear supernatant fraction which was stored at -80°C. Prior to storage a 5ul aliquot of the supernatant fraction was counted in scintillation fluid (65% (v/v) toluene, 35% (v/v) Triton X-100, 0.023M PPO) on a Beckman LS6800 liquid scintillation counter.

All the buffers utilised above were sterilised by passing through a 0.2µm Nalgene filter (Nalge Company, Division of Sybron Corporation, Rochester, New York, U.S.A).

2.3.2.3 Immune-Precipitation

10-20µl of antiserum was added to an aliquot (10-20 x 10^6 cpm/ fraction) of radiolabelled cell lysate (2.3.2.2) and the mixture incubated for 1h at room temperature, then overnight at 4°C. Antigen-antibody complexes were then collected by the addition of 20-40µl of a heat-killed, formalin-fixed S.aureus cell suspension 10% (v/v) . After incubating for 1h at room temperature the bacteria were sedimented by centrifugation at 14,000g for 2 min on an MSE Eppendorf Micro-

Centaur centrifuge (MSE scientific instruments, Manor Royal, Crawley, Sussex, U.K.). Specifically-precipitated radiolabelled material was recovered by washing the bacteria, three times with 3D-TKM (2.2.3.2), once with Triton X-100 buffer (2.2.3.2) and once with 10mM Tris-HCl pH 7.4 before resuspending in 50µl of Laemmli sample buffer (2.2.4.2). After boiling for 2-5 min at 100°C the bacteria were pelleted by centrifugation at 14,000g for 2 min.

A 5µl aliquot of the supernatant fraction was counted in scintillation fluid, as previously described (2.3.2.2). The immune precipitated proteins (20-30µl) were subsequently analysed by SDS-polyacrylamide gel electrophoresis (2.2.4.1) and fluorography (2.2.4.6).

2.3.2.4 The Enzyme-linked Immunosorbant Assay (ELISA)

To measure the titres of respective antisera, the antigen in 100µl of PBS, was bound to the bottom of a well of a Falcon microtitre plate by incubating overnight at 4°C. Excess antigen was removed by immersing the plate in washing buffer (PBS, 0.05% (v/v) Tween 20). Residual binding sites were blocked by adding 200µl of washing buffer containing 5% (v/v) goat serum to the wells, prior to incubating for 1h at room temperature or overnight at 4°C. The plate was rinsed again with buffer as before, then serial dilutions of antiserum ($^1/100$ to $^1/100{,}000$) in 100µl of washing buffer placed in the wells. After incubating for 2h at 4°C, the plate was washed in a similar manner before adding 100µl of goat anti-rabbit gamma-globulin conjugated to horse radish peroxidase (diluted $^1/500$ with washing buffer) to each well and incubating for a further 1h at 4°C. Excess conjugate was removed by immersing the plate in washing buffer. The quantity of peroxidase activity bound to the plate was determined

spectrophotometrically using 100µl per well of the following assay solution:-

0.04% (w/v) o-phenylene diamine (OPD)

0.04% (v/v) H_2O_2

36mM citric acid

128mM Na_2HPO_4

After 20 min incubation in the dark at room temperature, the reaction was stopped by the addition of 50µl of 4N H_2SO_4. The absorbance at 492nm was determined for each well using a Titertek multiscan spectrophotometer.

2.4 TISSUE CULTURE TECHNIQUES

2.4.1 Growth Media for Cell Lines

BRL, PK-15 and NBL-1 cell lines were grown routinely in Glasgow modification of Eagle's medium supplemented with 1% (v/v) NEAA, 100×10^3 units/1 penicillin, 37×10^3 units/1 streptomycin and either 10% (v/v) calf serum (BRL), 5% (v/v) foetal calf serum (PK-15) or 10% (v/v) foetal calf serum (NBL-1).

2.4.2 Routine Maintenance of Cells

Cell lines were maintained in their respective media (2.4.1) as monolayers at 37°C in Roux or 20 oz. roller bottles in an atmosphere of 5% CO_2, 95% air. Cells were subcultured 1 in 5 every 3-5 days by rinsing the cell sheets once with versene solution (5mM EDTA in PBS) and then placing in a small vol. of trypsin solution 0.25% (w/v) trypsin, 0.105M NaCl in 0.01M tri-sodium citrate adjusted to pH 7.8

with NaOH). After incubating for a few minutes at 37°C, 20ml of warm growth medium was added and a single cell suspension obtained by pipetting gently up and down several times. For radiolabelling studies, the cells were grown in small plastic Petri dishes and placed in a humidified incubator at 37°C containing a 5% CO_2/95% air atmosphere.

All the operations described above were carried out under asceptic conditions.

2.5 CHARACTERIZATION OF THE PHOSPHATE/HYDROXYL ION ANTIPORT PROTEIN (PHOSPHATE TRANSPORT PROTEIN)

2.5.1 Measurement of Phosphate Transport by Passive Isotonic Swelling

The transport of phosphate into mitochondria or mitoplasts (2.2.4.1 and 2) was carried out by the method of Coty and Pedersen (1975). Mitochondria or mitoplasts were diluted to a final concentration of 10mg protein/ml in isolation medium (2.2.5.1) and inhibitors of transport, where appropriate. After a suitable incubation at 0°C, an 0.1ml aliquot was withdrawn and transferred to a cuvette containing 0.9ml of swelling medium (0.1M $(NH_4)_2HPO_4$, 1mM EGTA and 5mM K^+-Hepes pH 7.4). After mixing rapidly, passive swelling was monitored at 750nm on a Uvichem spectrophotometer linked to a Servoscribe chart recorder.

In control experiments to monitor the specificity of inhibitors for phosphate transport, the swelling media contained 0.125M of either NH_4Cl, $NH_4OOC\,CH_3$ or NH_4HCO_3 in place of $(NH_4)_2HPO_4$.

2.5.2 N-[2-^{3}H]-Ethylmaleimide Labelling of the Phosphate Transport Protein

Rat liver mitochondrial protein was precipitated by the addition of 4 vol. of acetone and was subsequently collected by centrifugation at 1500g for 10 min. The pellet was resuspended in a small vol. of 20mM Tris-HCl buffer pH 7.2 containing 2% (w/v) SDS before initiation of radiolabelling by the addition of 40µCi of N-[2-^{3}H]-ethylmaleimide (50 Ci/mmol). After a 30 min incubation at room temperature the reaction was terminated by the addition of 2-mercaptoethanol, to a final concentration of 5% (v/v). Thereafter, the protein was precipitated by the addition of acetone, as described above and the pellet solubilised in Laemmli sample buffer (2.2.4.2). To determine the extent of incorporation of the label, a 5µl aliquot of the above sample was counted, as described previously (2.3.2.3). Radiolabelled proteins were separated on an SDS-polyacrylamide gel (2.2.4.1) and subsequently detected by fluorography (2.2.4.6). Additional protein fractions from the purification procedure (3.2.1) were labelled in an analogous manner. Intact rat liver mitochondria were resuspended in isolation medium (2.2.5.1) prior to labelling, as described above.

2.5.3 Performic Acid Oxidation of the Isolated Phosphate Transport Protein

To estimate the cysteine and methionine content of the protein, these residues were oxidised to cysteic acid and methionine sulphone using performic acid.

The performic acid was prepared by mixing 1ml of 30% (w/v) H_2O_2 with 9ml of 88% (v/v) formic acid and incubating at room temperature for 1h before use. After cooling for 15 min on ice, 1ml of performic acid was added to the lyophilised protein and the reaction continued for 4h at 0^{o}C. To stop the reaction, 20ml of distilled water was

added and the sample lyophilised. This procedure of resuspension in distilled water followed by lyophilisation was repeated. The protein sample was now ready for determination of its amino acid composition.

2.5.4 Amino Acid Analysis

3ml of distilled water was added to the lyophilised protein which was then split into 6 equal fractions and placed in 16.5 x 0.75cm test tubes. To each tube was added 10 nmoles of the internal standard norleucine and 0.5ml of Aristar HCl, before degassing the mixtures with nitrogen. The samples were frozen in dry ice prior to the tubes being heated and drawn out for sealing. After evacuating, the tubes were sealed, then placed on an aluminium block set at $110 \pm 1^{\circ}C$. Samples were removed after 24, 48 and 72h of hydrolysis (in duplicate) and their contents dessicated. Finally, the samples were analysed on an LKB 4400 amino acid analyser operated by Mr. J. Jardine of the Department of Biochemistry, University of Glasgow.

CHAPTER THREE

PURIFICATION AND CHARACTERIZATION OF THE PHOSPHATE TRANSPORT PROTEIN FROM RAT LIVER MITOCHONDRIA

3.1 INTRODUCTION

In recent years, considerable attention has been directed towards a better understanding of the operation of the mitochondrial inner membrane phosphate transport system. The phosphate carrier plays a major role in cellular metabolism since inorganic phosphate must be transported continuously into the mitochondrial matrix to maintain the steady-state oxidative phosphorylation of ADP. Specific uptake of this anion occurs via two distinct transport systems; an electro-neutral inorganic phosphate/dicarboxylic acid exchange carrier and an inorganic phosphate/pH gradient-linked carrier which catalyses the influx of about 90% of the mitochondrial phosphate.

Identification of the phosphate transport protein has been greatly facilitated by its sensitivity to the sulphydryl group reagent, N-ethylmaleimide, as previously discussed (Section 1.4.2). Since 1979 when Wohlrab presented strong evidence on the identity of the phosphate transport protein, several groups have attempted to purify the protein from a variety of tissues including beef heart (Wohlrab, 1980) and pig heart mitochondria (Kolbe et al., 1981). However, controversy has surrounded the purity of the final preparation since the discovery by Kolbe et al. (1981) that the pig heart protein consisted of four to five major Coomassie-Blue staining bands of similar M_r value, when resolved on SDS-polyacrylamide 14-20% (w/v) gradient gels. These controversial findings will be discussed in detail at a later stage in this chapter.

Since the exact nature of the phosphate transport protein(s) had not been fully elucidated at that time, an initial aim of my project was concerned with isolating and further characterizing the rat liver mitochondrial protein, as this represented an interesting area of

research in itself.

3.2 METHODS

3.2.1 Procedure(s) for the Purification of the Phosphate Transport Protein from Rat Liver Mitochondria

Method (a)

In general terms, a modification of the method of Kolbe et al. (1981) was utilised to purify the phosphate transport protein from rat liver mitochondria.

Initially, mitochondria were prepared from the livers of four rats which had been starved overnight, as described in section 2.2.5.2. The mitochondrial pellet was resuspended in a buffer containing 20mM LiCl, 0.1mM EDTA, 0.5mM DTT, 20mM H_3PO_4 adjusted to pH 7.0 with LiOH and 0.5% (v/v) Triton X-100, at a protein concentration of 10mg/ml. After incubating on ice for 15 min the mitochondrial suspension was centrifuged at 105,000g for 1h at $4^{o}C$. The resultant pellet was subsequently solubilised in the above buffer containing 8% (v/v) Triton X-100 at a similar protein concentration and incubated for 15 min on ice. Prior to centrifugation at 105,000g for 1h at $4^{o}C$, the extract was diluted 1:1 with ice-cold distilled H_2O. Detergent-solubilised mitochondrial protein was then subjected to adsorption chromatography on hydroxylapatite followed by a similar treatment with Celite. The extracted material from the second centrifugation step described above, was mixed initially with hydroxylapatite at 0.5g hydroxyl-apatite per ml of solubilised membrane. This slurry was incubated on ice for 15 min before centrifugation at 33,000g for 15 min. The supernatant fraction was retained at this stage while the pellet

was washed once with extraction buffer containing 4% (v/v) Triton X-100. Subsequent treatment of this suspension, in an analogous manner to the initial hydroxylapatite incubation, yielded a second supernatant fraction which was bulked with the first hydroxylapatite eluate. Clarification of the hydroxylapatite eluates was achieved by centrifugation at 1100g for 10 min. All centrifugation steps were performed at $4^{o}C$.

The hydroxylapatite eluate was finally subjected to adsorption chromatography on Celite-type 535 in a similar fashion to that described for hydroxylapatite chromatography. The only difference was that the Celite was washed with hot water before incubation with the hydroxylapatite eluate. The final Celite eluate was stored at $-20^{o}C$ until further use.

Method (b)

An alternative procedure has been developed which copurifies both the phosphate transport protein and the adenine nucleotide translocase.

Initially mitochondria, prepared as described previously (2.2.5.1) were resuspended in 20mM Mops buffer pH 6.8 containing 0.25M sucrose, 0.5mM EDTA, 2.5mM $MgCl_2$ and incubated with 40µM carboxyatractyloside for 10 min on ice to stabilise the adenine nucleotide translocase. After this incubation, mitochondria were pelleted by centrifugation at 8,000g for 10 min before pre-extracting with 20mM Mops buffer pH 7.2 containing 0.4M NaCl and 0.5% (v/v) Triton X-100 by incubating for 10 min on ice. After centrifugation for 1h at 105,000g the resulting pellet was solubilised in a 10mM Mops buffer pH 7.2 containing 0.5M NaCl and 4% (v/v) Triton X-100 by incubation for 10 min on ice. Residual, insoluble material was removed by centrifugation at 105,000g

for 1h. The supernatant fraction was subjected to hydroxylapatite chromatography on a 1.75 x 25cm column which had been pre-equilibrated with a 0.5% (v/v) Triton X-100, 0.1M NaCl, 10mM Mops pH 7.2 buffer solution. The non-absorbed fraction from this column was collected and dialysed against 20mM Tris-HCl pH 7.2 buffer containing 0.5% (w/v) Triton X-100, before application to a Procion Red A column (8 x 3 cm) equilibrated in the same buffer. Differential elution of the two proteins from this column was utilised to separate them; the phosphate transport protein being eluted with 0.25M NaCl whereas the adenine nucleotide translocase required 3M NaCl for complete elution. Both eluates were stored at -20^{o}C until required.

In both procedures, fractions were retained for analysis at each stage in the purification. Proteins were precipitated by the addition of 4 vol. of ice-cold acetone and finally prepared for analysis by SDS-polyacrylamide gel electrophoresis, as described in sections 2.2.4.1.

3.2.2 Iodination of Concanavalin A (con A)

Tyrosine residues of con A were radio-labelled with Na^{125}I by incubation with iodogen as follows:-

2mg con A dissolved in 0.5ml of 20mM Tris-HCl buffer pH 8.0 containing 0.2M methyα-glucoside was added to a glass vial, the surface of which had been coated with 200µg of iodogen. Incorporation of the isotope was initiated by the addition of 300µCi Na^{125}I (carrier-free) and the reaction allowed to proceed for 30 min at room temperature. After terminating the reaction by dilution with 20mM Tris-HCl pH 8.0, the incubation mixture was removed to another vessel and non-radioactive NaI added to give a final concentration of 25mM.

This solution was dialysed subsequently against 20mM Tris-HCl pH 8.0 buffer, before application to a (8.5 x 1.5 cm) Sephadex G50 column equilibrated with dialysis buffer. ^{125}I-labelled con A was eluted from the column by washing with 20mM Tris-HCl pH 8.0 buffer containing 0.2M methyα-glucoside. Finally, this eluted con A was dialysed against 20mM Tris-HCl pH 8.0 and stored at -20^{o}C in small aliquots (0.5ml).

3.2.3 Lectin Overlay Analysis of the Phosphate Transport Protein

After SDS-polyacrylamide gel electrophoresis, the resolved proteins were fixed by incubating the gel overnight in 10% (v/v) acetic acid and 25% (v/v) isopropanol. Prior to incubation with the ^{125}I-labelled lectin, the gel was thoroughly washed by rinsing several times in lectin overlay buffer consisting of 20mM Tris-HCl pH 8.0, 0.12M KCl, 1mM $CaCl_2$, 1mM $MnCl_2$, 1% (v/v) Tween 20, 0.1% (w/v) haemoglobin and 0.05% (w/v) sodium azide. Incubation of the gel with ^{125}I-labelled con A (see section 3.2.2) was then initiated by the addition of the lectin to the gel soaked in overlay buffer (approx. 10 x 10^6 cpm ^{125}I-labelled con A). After 2 days of incubation at room temperature this solution was removed and the gel subsequently washed for 3-5 days in lectin-free overlay buffer (5-6 changes), to reduce the background level of radiation. Autoradiography was finally carried out on the dried gel, as described previously (see section 2.2.4.5).

3.2.4 Preparation of SDS-polyacrylamide 14-20% (w/v) Gradient Gels

SDS-polyacrylamide gradient gels (18 x 16 cm) were prepared at 4^{o}C using a Bio-Rad slab gel apparatus (Model No. Protean 16cm) and a gradient gel mixer which consisted of two compartments (40ml capacity/

compartment) with a connecting flow-line to allow mixing of the two stock acrylamide solutions listed in table 3.2.1.

The two acrylamide solutions were degassed under vacuum before polymerisation was initiated by the addition of TEMED (6µl) to each acrylamide stock solution. Immediately on addition of TEMED, the main gel was poured in the following manner:-

A piece of rubber tubing connected to the gradient gel mixer was placed between the two glass plates and the flow rate of acrylamide solution controlled by a peristaltic pump (90 ml/h). The gel was carefully overlayed with isopropanol before polymerisation was complete. After polymerisation of the separating gel, a stacking gel containing the sample wells (15 wells/gel) was placed on top using the previously described procedure (see section 2.2.4.1).

3.3 RESULTS AND DISCUSSION

3.3.1 SDS-polyacrylamide Gel Electrophoresis Analysis of the Isolated Phosphate Transport Protein

The Coomassie Blue staining profile of different stages in the purification of the phosphate transport protein from intact rat liver mitochondria, after separation on an SDS-polyacrylamide gel 10% (w/v) is illustrated in Fig. 3.3.1a. A single, apparently homogeneous band can be visualised after both hydroxylapatite and Celite chromatography. The electrophoretic mobilities of a set of marker proteins of known M_r value were measured in this gel system and used to construct standard curves of electrophoretic mobility against log subunit M_r value (Fig. 3.3.2). Comparison of the electrophoretic mobility of the purified phosphate transport protein with these markers gave a value of 34,000.

Table 3.2.1

COMPOSITION AND VOLUMES OF SOLUTIONS USED TO CAST SDS-POLYACRYLAMIDE GRADIENT GELS 14-20% (W/V)

Stock solution	Right-hand compartment 14% (w/v) acrylamide (Vol. added)	Left-hand compartment 20% (w/v) acrylamide (Vol. added)
29.2% (w/v) acrylamide 0.8% (w/v) bis-acrylamide	9.3 ml	13.3 ml
Running gel buffer	5.0 ml	5.0 ml
15% (w/v) sucrose	5.7 ml	-
70% (w/v) sucrose	-	1.7 ml
0.03% (w/v) ammonium persulphate	60 µl	60 µl

The subunit M_r value of the protein is in agreement with the findings of other groups who have purified the beef heart (Wohlrab, 1980) and pig heart (Kolbe et al., 1981) mitochondrial phosphate transport protein, respectively. However, the finding that only the phosphate transport protein did not adsorb to hydroxylapatite is in marked contrast to their data. In both instances, the adenine nucleotide translocase (M_r 30,000) was shown to copurify with the phosphate transport protein after hydroxylapatite chromatography. However, Kolbe et al. (1981) demonstrated that adsorption chromatography on Celite effected quantitative removal of the translocase.

Recently, Wehrle and Pedersen (1983) attempted to purify the phosphate transport protein from rat liver mitochondria, essentially following the method of Wohlrab (1980). If intact mitochondria were utilised as the initial starting material, Triton X-100 extraction followed by hydroxylapatite chromatography resulted in purification of the phosphate transport protein, the adenine nucleotide translocase and a protein of M_r 68,000. This additional 68,000 M_r protein was suggested to represent a subunit of an enzyme found only in liver mitochondria, since rat liver mitochondria are more metabolically complex than both bovine heart and blowfly flight muscle mitochondria, used in earlier studies. As a result of this finding, Wehrle and Pedersen (1983) purified the phosphate transport protein from mitochondrial inner membrane vesicles which had been treated with urea. In this case, the 68,000 M_r protein was removed but the two transport proteins still copurified during hydroxylapatite chromatography. They have attempted subsequently to remove the adenine nucleotide translocase by Celite chromatography without success. It is obvious that these findings are in marked contrast to our own data, despite the identity of the tissue sources, namely rat liver mitochondria.

3.3.2 Lectin Overlay Analysis of the Phosphate Transport Protein

Membranes of eukaryotic cells usually contain between 2% and 10% carbohydrate by weight in the form of glycoproteins and glycolipids. In the plasma membrane of many cell types including red blood cells (Nicolson and Singer, 1974) and lymphocyte membranes (Hayman and Crumpton, 1972), this carbohydrate is asymmetrically distributed with the sugar moieties protruding out into the extracellular environment. It has also been demonstrated that many of the glycoproteins are transmembrane proteins, which are exposed to both external and internal environments providing a means of communication across the bilayer, eg. band 3 or the anion channel protein of the red cell which makes it selectively permeable to HCO_3 and Cl^- and glycophorin A also of the red cell plasma membrane whose function is unknown.

It has been reported that isolated mitochondria contain 1-2% carbohydrate by weight (De Bernard et al., 1971) and that covalently-associated saccharide moieties are present on the cytoplasmic surfaces of both the inner and outer membranes (D'Souza and Lindsay, 1981). In this connection, it was decided to investigate the possibility that the integral inner membrane phosphate transport protein from rat liver mitochondria was glycosylated. This was achieved by using the lectin overlay method described in section 3.2.3 and ^{125}I-labelled con A to permit detection of covalently-bound mannose, N-acetylglucosamine and/or glucose residues. Con A was chosen to determine whether the phosphate transport protein was glycosylated or not because it had previously been shown in this laboratory that about 80% of rat liver mitochondrial glycoproteins bound to con A-Sepharose columns. Comparison of the protein staining pattern (Fig. 3.3.1a), in which the phosphate transport protein appears to be homogeneous with the corresponding

^{125}I-labelled con A overlay autoradiograph (Fig. 3.3.1b), clearly illustrates the existence of numerous low and high M_r value glycoprotein contaminants. However, there is no bound label in the region of the phosphate transport protein (M_r 34,000) which suggests that it is unlikely to be glycosylated.

An improved purification procedure was devised to remove contamination with glycoproteins by extracting the mitochondria initially with a buffer containing 0.5% (v/v) Triton X-100 before selective extraction of the phosphate transport protein (see section 3.2.1 method a). These findings are depicted in Figs. 3.3.3(a) and 3.3.3(b) which represent the Coomassie Blue and ^{125}I-labelled con A overlay profiles of the purified phosphate transport protein after separation on an SDS-polyacrylamide gel 12.5% (w/v). It is apparent that the pre-extraction step solubilises about 80% of the mitochondrial protein, including the bulk of contaminating glycoproteins.

Fig. 3.3.3(a), track 4 demonstrates that the 0.25M KCl eluate from the Procion Red A column (see section 3.2.1 method b) migrates with an identical M_r value of 34,000 when compared with the final Celite product (see section 3.2.1 method a).

3.3.3 SDS-Polyacrylamide Gradient Gel Electrophoresis of the Purified Protein

In recent years, a great deal of controversy has surrounded the nature of the protein component(s) that constitute the phosphate carrier system. This controversy was initiated in 1981 by Kolbe et al. who purified the transport protein from pig heart mitochondria and separated the protein component(s) on an SDS-polyacrylamide gradient gel 14-20% (w/v). They discovered that the Celite purified protein had been

resolved into four separate components which ranged in M_r from 35,000 to 31,000. Three possible explanations were proposed to interpret this result:-

i) the phosphate transporter of mitochondrial inner membrane is composed of four different polypeptide chains;

ii) some of the four proteins represent proteolytically or otherwise modified forms of the same protein;

iii) the phosphate transporter is one protein, the other proteins represent contaminants which copurify with the transport protein.

A subsequent report by Kolbe et al. (1982) which investigated the nature of these additional bands in more detail, led to the proposal of a model in which proteolytic processing was suggested to account for their existence (Fig. 3.3.4). The phosphate transport protein was purified from beef heart and pig heart mitochondria respectively. Purification involved using slightly different procedures to the original protocol including two successive extractions of mitochondria with Triton X-114 instead of Triton X-100. The model was based on the findings of three very similar proteins which differed slightly in M_r (34,500, 34,000 and 33,000), amino acid composition and could explain the contradictory observations of the study. It was assumed in this model that the native carrier is composed of two subunits. The occurrence of the active phosphate transport protein as a dimer of two identical subunits would be in accordance with other membrane transport proteins which were found to consist of two equal subunits in their active form; eg. the adenine nucleotide translocase (Klingenberg et al., 1978).

As a consequence of these findings, the purified rat liver and beef

heart mitochondrial phosphate transport proteins were subjected to SDS-polyacrylamide gradient gel electrophoresis 14-20% (w/v) and their Coomassie Blue staining profiles compared (Fig. 3.3.5). The beef heart protein was purified from mitochondria in an analogous manner to the rat liver mitochondrial polypeptide (section 3.2.1 method (a)). It can clearly be seen that there is a major difference between the protein product(s) at the hydroxylapatite stage in the two preparations. One major band is observed in the rat liver sample which migrates with an M_r value of 34,000, whereas two major bands with M_r of 34,000 and 31,000 respectively are detectable in the beef heart preparation. Wohlrab (1980) previously demonstrated this latter result when purifying the phosphate transport protein from the same tissue source and suggested that the 31,000 M_r polypeptide band was likely to be the adenine nucleotide translocase. It has subsequently been illustrated that there are three possible ways to remove this 31,000 M_r protein involving either Celite chromatography (Kolbe et al., 1981), mersalylultrogel chromatography (Touraille et al., 1981) or Triton X-114 extraction of mitochondria (Kolbe et al., 1982). As depicted in track (6), this was shown to be correct when the beef heart hydroxylapatite eluate was subjected to adsorption chromatography on Celite.

In marked contrast to the findings of Kolbe et al. (1982), however, there is no evidence for the existence of four to five Coomassie Blue staining bands in either preparations hydroxylapatite or Celite eluates. If the proteolytic processing model proposed by these workers to explain their results with the beef heart and pig heart mitochondrial phosphate transport proteins is correct, then the rat liver and beef heart mitochondria used in our purifications are less sensitive to proteolytic attack.

Confirmation as to the purity of the rat liver mitochondrial protein,

separated on the above gel system, was provided by densitometric scanning of the gel using an LKB Ultroscan 2202 laser densitometer. The purity of the Coomassie Blue stained protein of M_r 34,000 was estimated to be about 95%. Finally, it should be noted that the 0.25M KCl eluate from the Procion Red A column of the alternative procedure (method b, section 3.2.1), demonstrated the existence of only one polypeptide band when separated and stained with Coomassie Blue on the above gradient gel system (track 4). The SDS-polyacrylamide gradient gel 14-20% (w/v) was prepared as described in section 3.2.4.

3.3.4 CHARACTERIZATION OF THE PHOSPHATE TRANSPORT PROTEIN

3.3.4.1 Effect on N-ethylmaleimide on Phosphate Transport into Isolated Mitochondria

Initially our aim was to confirm that the protein isolated from rat liver mitochondria constituted the phosphate transport system by established procedures. It has been well documentated (Fonya and Besman, 1968, Tyler, 1969; Coty and Pedersen, 1974a), that this transport system is inhibited by very low levels of the sulphydryl group reagent, N-ethylmaleimide. It is also generally accepted that N-ethylmaleimide reacts specifically with an essential sulphydryl group of the phosphate transport system rather than non-specifically altering membrane permeability. Preliminary experiments were carried out to confirm the susceptibility of this system to N-ethyl-maleimide. This property can simply be demonstrated by measuring the transport of phosphate into isolated mitochondria utilising the fact that mitochondria passively swell in solutions of isotonic ammonium phosphate buffer (Chappell and Crofts, 1966).

The swelling of mitochondria in solutions of ammonium salts depends

on the ability of the anion present to penetrate the mitochondrial inner membrane, as illustrated in Fig. 3.3.6 (Chappell, 1968). A rapid qualitative means of measuring the swelling process is to monitor the decrease in absorbance at 750nm, when mitochondria are placed in such ammonium salt buffers. Fig. 3.3.7(a) confirms the earlier findings of Chappell and Crofts (1966), in that it describes the effect of incubating rat liver mitochondria in isotonic solutions of ammonium chloride, acetate, bicarbonate and phosphate, respectively. It can clearly be seen that there is a marked decrease in the absorbance at 750nm of the mitochondrial suspension, when incubated in ammonium acetate, bicarbonate or phosphate buffers. This indicates that swelling has occurred when mitochondria are placed in these respective media. However, no swelling is induced in the presence of ammonium chloride buffer. These results demonstrate that the three anions, phosphate, bicarbonate and acetate can penetrate the mitochondrial inner membrane whereas the chloride ion cannot enter into the matrix. To confirm that phosphate uptake can be inhibited by very low levels of the sulphydryl group reagent, N-ethylmaleimide (Coty and Pedersen, 1974b), isolated rat liver mitochondria were pre-incubated with this inhibitor prior to monitoring their passive swelling in isotonic ammonium phosphate buffer (Fig. 3.3.7b). It is clear that there is no decrease in absorbance over the time-course of the experiment, suggesting that N-ethylmaleimide has inhibited phosphate uptake. Pre-treatment with N-ethylmaleimide did not significantly affect chloride, acetate or bicarbonate induced swelling of mitochondria. Since bi-carbonate and acetate ions penetrate the mitochondrial inner membrane by simple diffusion, this finding rules out the non-specific alteration of membrane permeability by N-ethylmaleimide.

The swelling studies were performed according to the method of

Coty and Pederson (1974b), as described in section 2.5.1.

3.3.4.2 N-[^{3}H]-Ethylmaleimide Labelling Studies of the Phosphate Transport Protein

In the past, the phosphate transport protein has been identified by several groups as a result of its sensitivity to N-ethylmaleimide. Incorporation of N-[^{3}H]-ethylmaleimide into mitochondrial inner membrane proteins at concentrations known to inhibit phosphate transport into isolated mitochondria, was the criterion used to achieve such an identification (Coty and Pederson, 1975, Wohlrab, 1978; Wohlrab, 1979). By comparing the N-[^{3}H]-ethylmaleimide labelling patterns of blowfly flight muscle mitochondria with rat heart sonic submitochondrial particles, Wohlrab (1979) was able to identify a protein of M_r 32,000 as constituting the phosphate transport protein. A more detailed account as to the identification of the protein can be found in section 1.4.2. In this section, this property of the phosphate transport system was utilised to confirm the identity of the isolated rat liver mitochondrial protein of M_r 34,000.

It has been established (section 3.3.4.1) that N-ethylmaleimide can inhibit the transport of phosphate into intact rat liver mitochondria as measured by the 'ammonium swelling technique' of Chappell and Crofts (1966). It was also shown that this effect was specifically due to interaction with a free sulphydryl group on the transport protein and not due to non-specific alteration of membrane permeability (Fig. 3.3.7b). However, when intact rat liver mitochondria or the 8% (v/v) Triton X-100 extracted material are labelled with N-[^{3}H]-ethylmaleimide, several polypeptides can be visualized after fluorography of the resolving SDS-polyacrylamide gel (12.5% (w/v) Fig. 3.3.8b). This result is in agreement with the earlier findings of several groups including Coty

and Pedersen (1975), who showed that at least ten polypeptide components of the rat liver mitochondrial inner membrane could be labelled in an analogous manner. After subsequent hydroxylapatite chromatography (track 3), only a protein of M_r 34,000 incorporates the label. This is in marked contrast to the results of Wohlrab (1980) and Kolbe et al. (1981), who purified the transport protein from beef heart and pig heart mitochondria, respectively. They discovered that not only could this transporter be labelled with N-[^{3}H]-ethylmaleimide, after adsorption chromatography on hydroxylapatite, but that the adenine nucleotide translocase (M_r 30,000) also co-labelled with the isotope. The result does, however, confirm our earlier observations that after treatment of the Triton X-100 extract with hydroxylapatite, only a polypeptide band of M_r 34,000 stains with Coomassie Blue. Celite chromatography, as expected, results in the labelling of one band of M_r 34,000 in agreement with the data of Kolbe et al. (1981), who showed that this treatment removed the adenine nucleotide translocase protein from their pig heart mitochondrial preparation. Fig. 3.3.8(a) demonstrates the initial Coomassie Blue staining patterns of the proteins prior to fluorography.

The above data was confirmed in an experiment where intact rat liver mitochondria were initially incubated with N-[^{3}H]-ethylmaleimide and the phosphate transport protein subsequently purified, as described in section 3.2.1 method (a). This experiment was performed to rule out the possibility that labelling of the protein of M_r 34,000 was the result of a conformational change during its isolation and that in the native state the polypeptide reacted with N-[^{3}H]-ethylmaleimide. A similar labelling pattern to that achieved in the previous experiment (Fig. 3.3.8b) was attained in that only a protein of M_r 34,000 could be detected on the fluorograph after hydroxylapatite and Celite chromato-

graphy, respectively (Fig. 3.3.8c).

The evidence presented in this section, as to the nature of the isolated polypeptide of M_r 34,000, illustrates many of the previously recognised features of the phosphate transport protein. All N-[^{3}H]-ethylmaleimide labelling experiments were performed according to the procedure described in section 2.5.2.

3.3.4.3 IMMUNOLOGICAL STUDIES

3.3.4.3.1 Effect of an Anti-Phosphate Transport Protein Serum on Phosphate Transport

Recent studies to illustrate the role of the purified polypeptide of M_r 34,000 have concentrated on reconstitution experiments. In these experiments the purified protein was incorporated into liposomes and the rate of phosphate-phosphate exchange measured by ^{32}Pi efflux or uptake. Wohlrab (1980) and Kolbe et al. (1981), both demonstrated that the reconstituted system prepared from the purified beef heart and pig heart mitochondrial proteins respectively, catalysed phosphate-phosphate exchange. However, the maximal exchange rate was only about 2% of the rate in isolated mitochondria (Coty and Pedersen 1974b). Kolbe et al. (1982) have subsequently demonstrated that if the phospholipid cardiolipin is included in the purification procedure, the rate of exchange of phosphate can be enhanced 30-fold.

As a result of the generally poor reconstitution exchange rates and the fact that I was particularly interested in studying the biosynthesis of this protein in vivo, which required the use of a specific antiserum, further confirmation as to the identity of the protein isolated was provided by the following experiments; phosphate transport

into intact mitochondria and mitochondrial inner membrane vesicles (mitoplasts) was studied in the presence of a rabbit, polyclonal antiserum directed against the polypeptide of M_r 34,000 (see section 2.3.1). Mitochondria or mitoplasts were pre-incubated for 1h at 4°C before monitoring phosphate transport, as described in section 2.5.1. Fig. 3.3.9(a) demonstrates that the antiserum directed against the purified protein of M_r 34,000 inhibits phosphate uptake into mitoplasts by about 50%, whereas control serum, as expected, has no such effect on swelling. In addition, although not shown here the anti-phosphate transport protein serum had no effect on the swelling of mitochondria in NH_4HCO_3, NH_4COOCH_3 or NH_4Cl. Partial inhibition of phosphate uptake into mitoplasts can be explained by the fact that the substrate for the transport protein is a small molecule which may still have access to its binding site even in the presence of antiserum and thus cause swelling. Additionally, the following factors may contribute to only observing a partial inhibition of mitoplast swelling:

i) Since the antiserum raised against the purified protein is polyclonal in nature, many of the resulting antibody molecules may be recognising antigenic determinants (epitopes) of the protein which do not constitute the phosphate binding site and thus do not interfere with phosphate binding and mitoplast swelling.

ii) Secondly, a small amount of the outer membrane may still be attached to these inner membrane vesicles, particularly at the contact points of the two membranes. If the phosphate transport protein is located at or near to these junctions then antibody access would be limited by the outer membrane.

As an additional control, phosphate uptake into intact rat liver mitochondria which had been pre-incubated with either the antiserum

directed against the polypeptide of M_r 34,000 or control serum, was monitored. In this case (Fig. 3.3.9b), no inhibition of swelling was observed, as one would expect, since with intact mitochondria immunoglobulin molecules are too large to penetrate the outer membrane and thus block antigenic sites on the transport protein, deeply embedded in the inner membrane. Mitoplasts were prepared from intact mitochondria by the method described in section 2.2.5.2.

In summary, the findings of the last two sections (3.3.4.2 and 3.3.4.3) strongly suggest that the protein isolated from rat liver mitochondria with an M_r value of 34,000, is the phosphate transport protein.

3.3.4.4 Amino Acid Analysis of the Phosphate Transport Protein

The amino acid composition of the purified rat liver mitochondrial phosphate transport protein, which had been treated with performic acid prior to analysis, was performed according to the method described in section 2.5.4. To account for the loss of serine and threonine as a result of acid hydrolysis the values found at 24, 48 and 72h were extrapolated to zero-time. Values for valine and isoleucine were estimated from samples taken at 72h, since the recovery of these resisues is not complete until after 72h of acid hydrolysis. When the number of residues was calculated from the original amino acid composition data of the rat liver mitochondrial phosphate transport protein, an M_r value of about 17,000 was obtained. If the number of amino acid residues is then doubled, the M_r of the phosphate transporter is in accord with the previously reported values (M_r 34,000). The amino acid composition of the rat liver protein was subsequently expressed as moles amino acid percentage of total composition of the protein. Table 3.3.1 compares the amino acid compositions of the rat liver and beef heart mitochondrial

phosphate transport proteins. The composition of the beef heart transport protein was determined by Kolbe et al. (1982). Clearly, the compositions of the two phosphate transport proteins are in general very similar although there are major differences in the contents of cysteine and tyrosine. The rat liver mitochondrial phosphate transport protein contains at least twice as much cysteine as the beef heart protein. Additionally, the rat liver phosphate transport protein does not appear to contain tyrosine whereas the beef heart protein contains about 10 residues of this amino acid assuming a subunit M_r of 34,000 for the protein. The lack of tyrosine in the rat liver mitochondrial phosphate transport protein partially confirms the purity of the protein. If the final preparation of the protein was contaminated with other polypeptides, it is unlikely that these additional polypeptides would not also contain tyrosine residues.

Fig. 3.3.1 (a) and (b)

LECTIN OVERLAY ANALYSIS OF THE PURIFIED PHOSPHATE TRANSPORT PROTEIN FROM INTACT RAT LIVER MITOCHONDRIA

The phosphate transport protein was purified from intact rat liver mitochondria, as described in section 3.2.1 method (a) and initially resolved on a 10% (w/v) SDS-polyacrylamide gel (gels were cast between 14 x 8cm plates). After electrophoretic separation and detection of the protein(s) with Coomassie Blue (see section 2.2.4.3), the gel was subjected to lectin overlay analysis (see section 3.2.3) with ^{125}I-labelled con A.

a) Coomassie Blue staining profile of different stages in the purification of the phosphate transport protein; (1) initial 8% (v/v) Triton X-100 extract, (2) hydroxylapatite fraction, (3) Celite-adsorbed fraction, (4) low M_r standard proteins.

b) Tracks (5 - 8) ^{125}I-labelled con A overlay profiles of tracks (1 - 4).

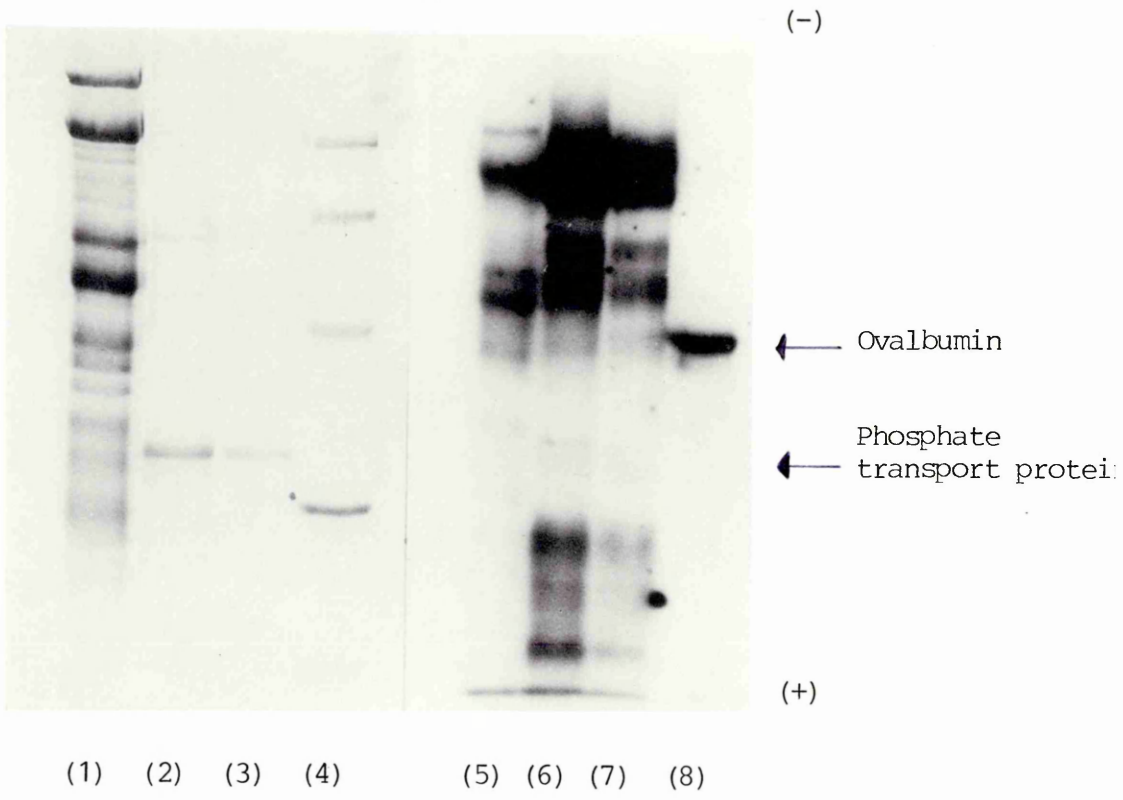
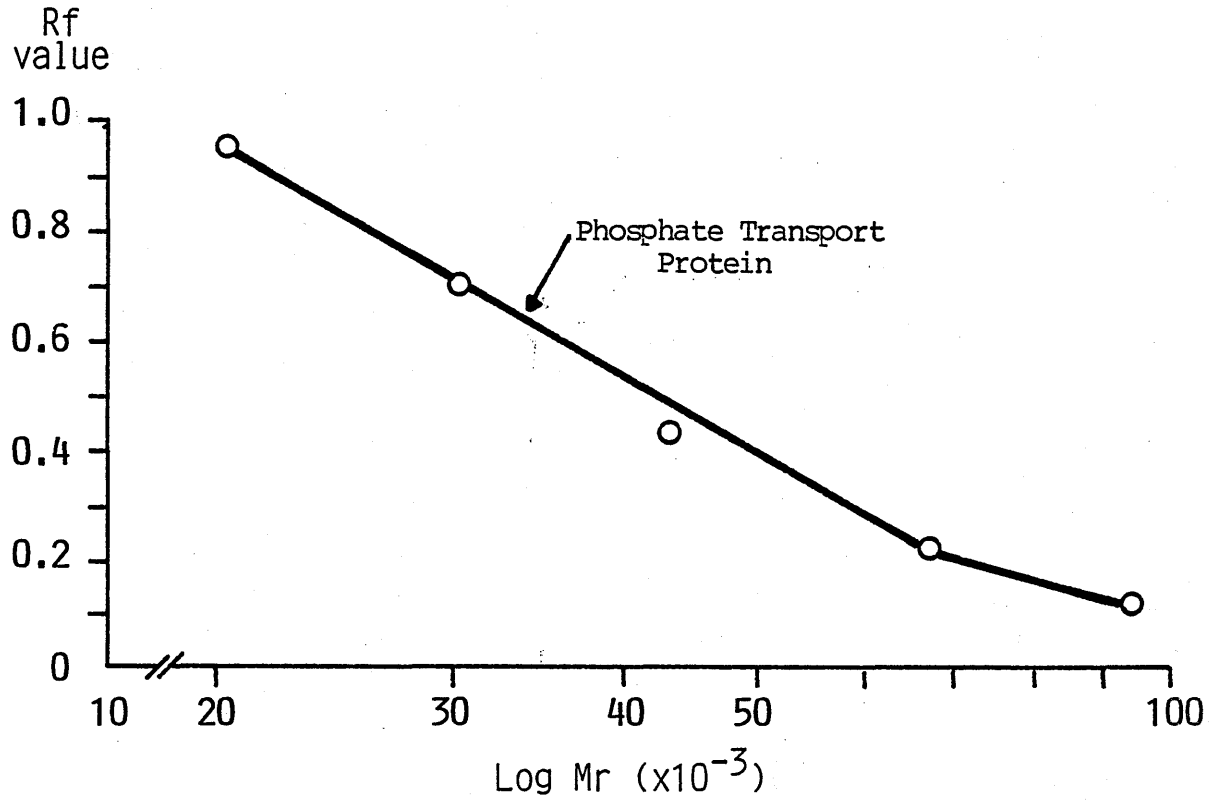

a)
b)
(-)
Ovalbumin
Phosphate
transport protei
(+)
(1) (2) (3) (4) (5) (6) (7) (8)

Fig. 3.3.2

STANDARD CURVE OF ELECTROPHORETIC MOBILITY OF MARKER PROTEINS VERSUS LOG SUBUNIT M_r VALUE

To estimate the M_r value of the purified rat liver mitochondrial phosphate transport protein on an SDS-polyacrylamide gel (10% (w/v)), a set of standard proteins of known M_r were resolved on the same gel. By plotting the electrophoretic mobility (R_f value) of the standard proteins versus log subunit M_r value, the M_r of the phosphate transporter could then be determined. The low M_r standard proteins used in this calculation are listed in section 2.2.4.4.


Rf
value
1.0
0.8
0.6
0.4
0.2
0
Phosphate Transport
Protein
10
20
30
40
50
100
Log Mr (x10^{-3})

Figs. 3.3.3 (a) and (b)

LECTIN OVERLAY ANALYSIS OF THE PURIFIED PHOSPHATE TRANSPORT PROTEIN FROM TRITON X-100 0.5% (v/v) PRE-EXTRACTED RAT LIVER MITOCHONDRIA

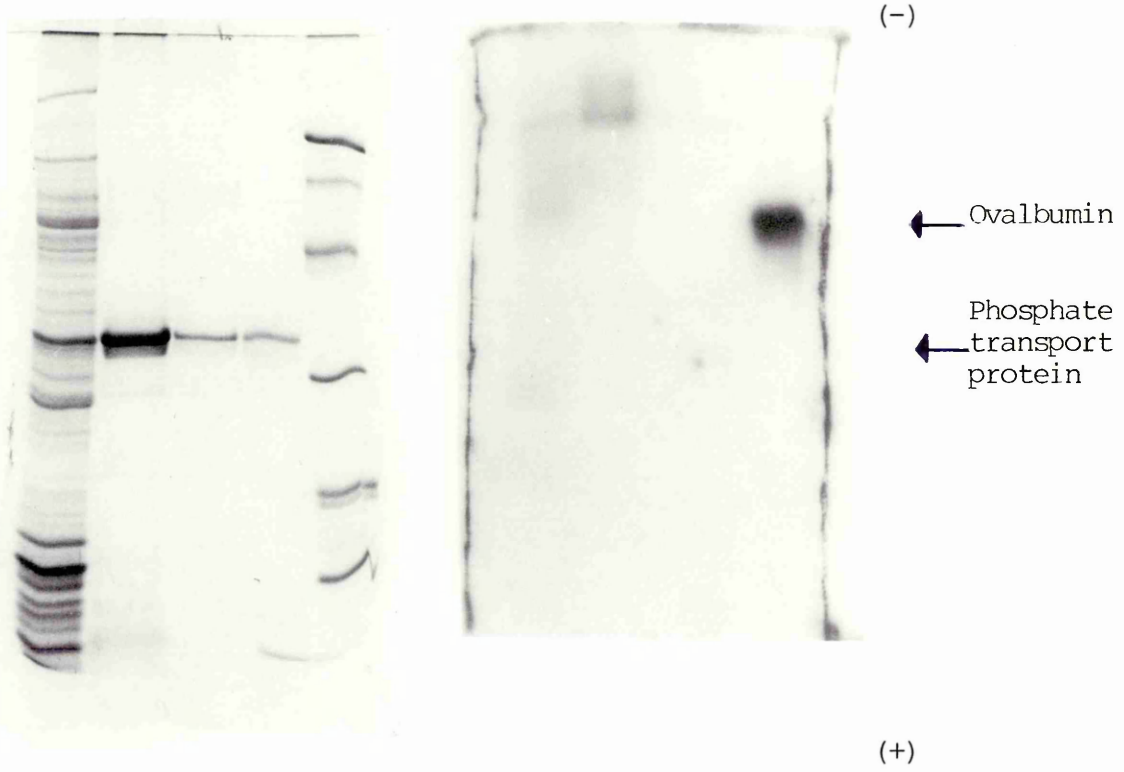
Rat liver mitochondria were initially pre-extracted with a 0.5% (v/v) Triton X-100 buffer prior to solubilization in an 8% (v/v) Triton X-100 buffer and further purification of the phosphate transport protein (see section 3.2.1 method a). The phosphate transporter was then subjected to analysis by SDS-polyacrylamide gel electrophoresis 12.5% (w/v) and subsequent lectin overlay analysis (see section 3.2.3). The gels were cast between 14 x 8cm glass plates in a perspex box designed to accommodate four pairs of plates.

a) Coomassie Blue staining profile of (1) 8% (v/v) Triton X-100 extract (2) hydroxylapatite eluate (3) Celite-adsorbed fraction (4) 0.25M KCl eluate from Procion Red A column (5) low M_r standard proteins.

b) Lanes (6-10) ^{125}I-con A overlay profiles of (1-5).

a)

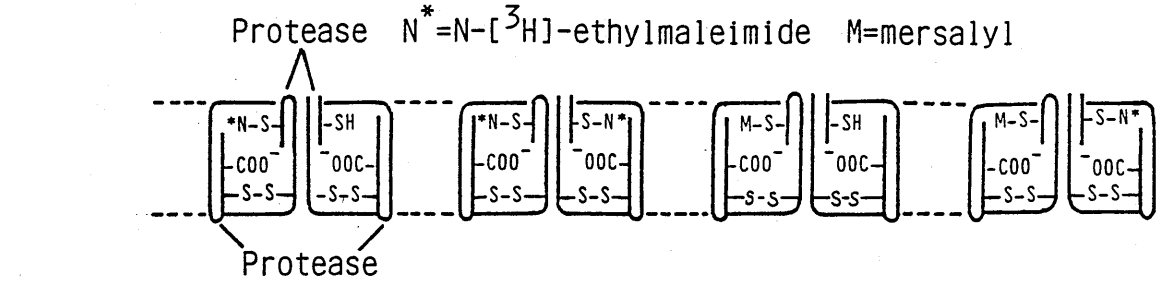
b)



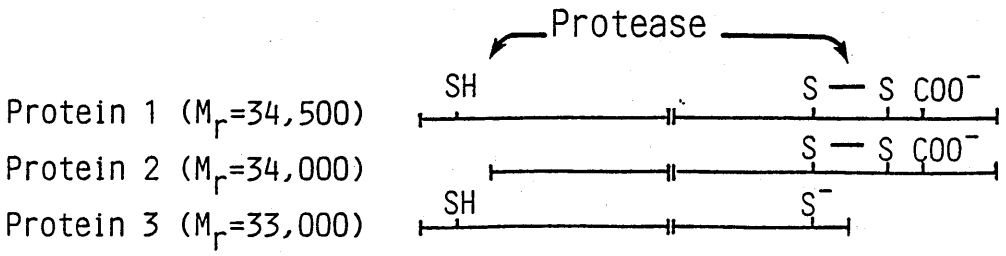
(1) (2) (3) (4) (5) (6) (7) (8) (9) (10)

Fig. 3.3.4

Model of the mitochondrial phosphate carrier



Transport:	active	inactive	active	inactive
SDS-PAGE:	labelled	labelled	unlabelled	unlabelled



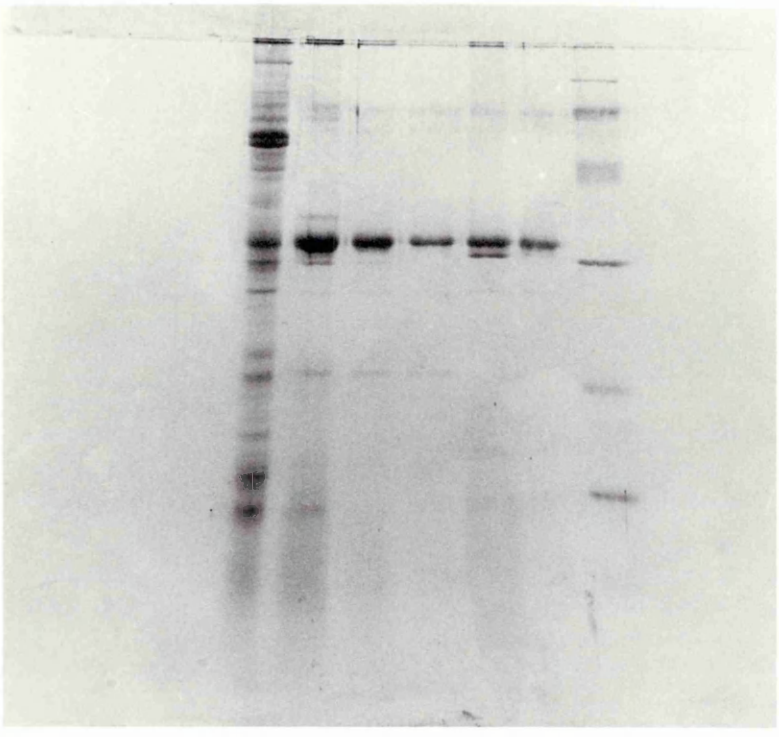
The model is based on the finding of three very similar proteins differing slightly in M_r and amino acid composition. It assumes that the native transporter is composed of two subunits and that proteolytic cleavage of the native protein occurs at two positions close to the amino and carboxyl terminal ends of the polypeptide chain during isolation of mitochondria. The cleaved polypeptides remain associated to the protein due to hydrophobic, ionic and/or hydrogen bond interactions. The model explains why no labelled N-ethylmaleimide was found to be bonded to the phosphate transporter fraction in the presence of protecting concentrations of mersalyl, although under these conditions the transport of phosphate was inhibited. (Reproduced from Kolbe et al., 1982).

Fig. 3.3.5

SDS-POLYACRYLAMIDE GRADIENT GEL ANALYSIS 14-20% (w/v) OF PURIFIED PHOSPHATE TRANSPORT PROTEIN FROM RAT LIVER AND BEEF HEART MITOCHONDRIA

Coomassie Blue profile of different stages in the purification of the phosphate transport protein (see section 3.2.1 methods a and b). From rat liver mitochondria; (1) 8% (v/v) Triton X-100 extract, (2) Hydroxylapatite eluate, (3) Celite-adsorbed fraction, (4) 0.25M NaCl eluate from Procion Red A column. From beef heart mitochondria, (5) Hydroxylapatite eluate, (6) Celite-adsorbed fraction, (7) Marker proteins 94,000-14,400 M_r.

(-)



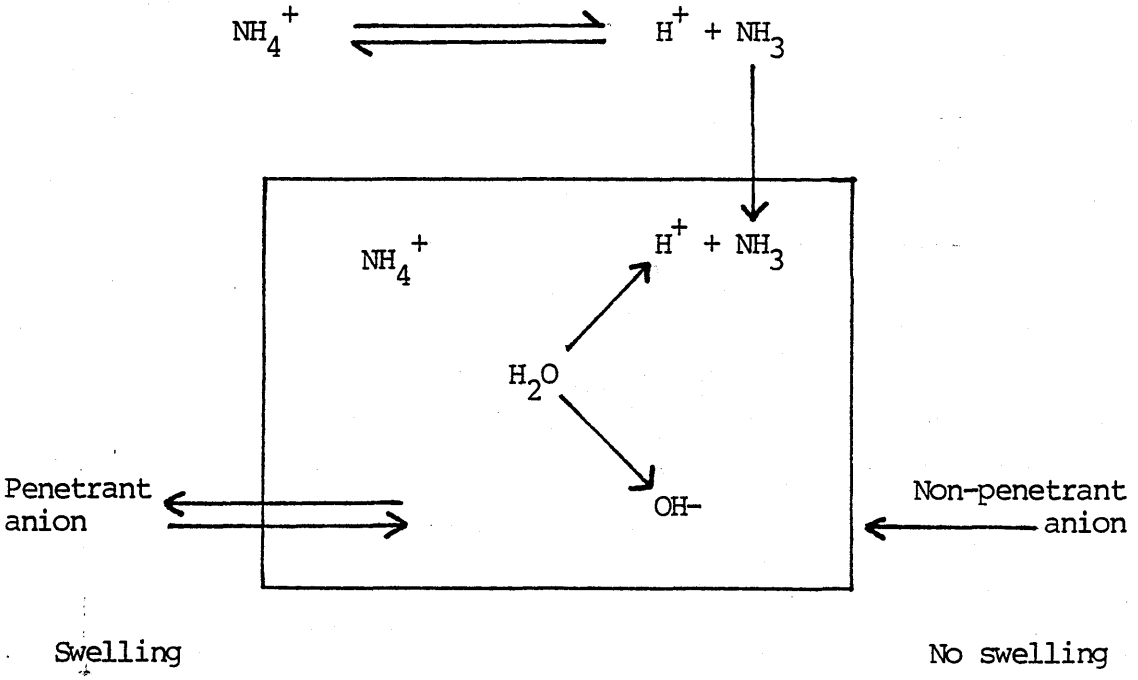
Phosphate transport protein (M_r 34,000)

(+)

(1) (2) (3) (4) (5) (6) (7)

Fig. 3.3.6

POSTULATED MECHANISM OF MITOCHONDRIAL SWELLING IN SOLUTIONS OF ISOTONIC AMMONIUM SALTS



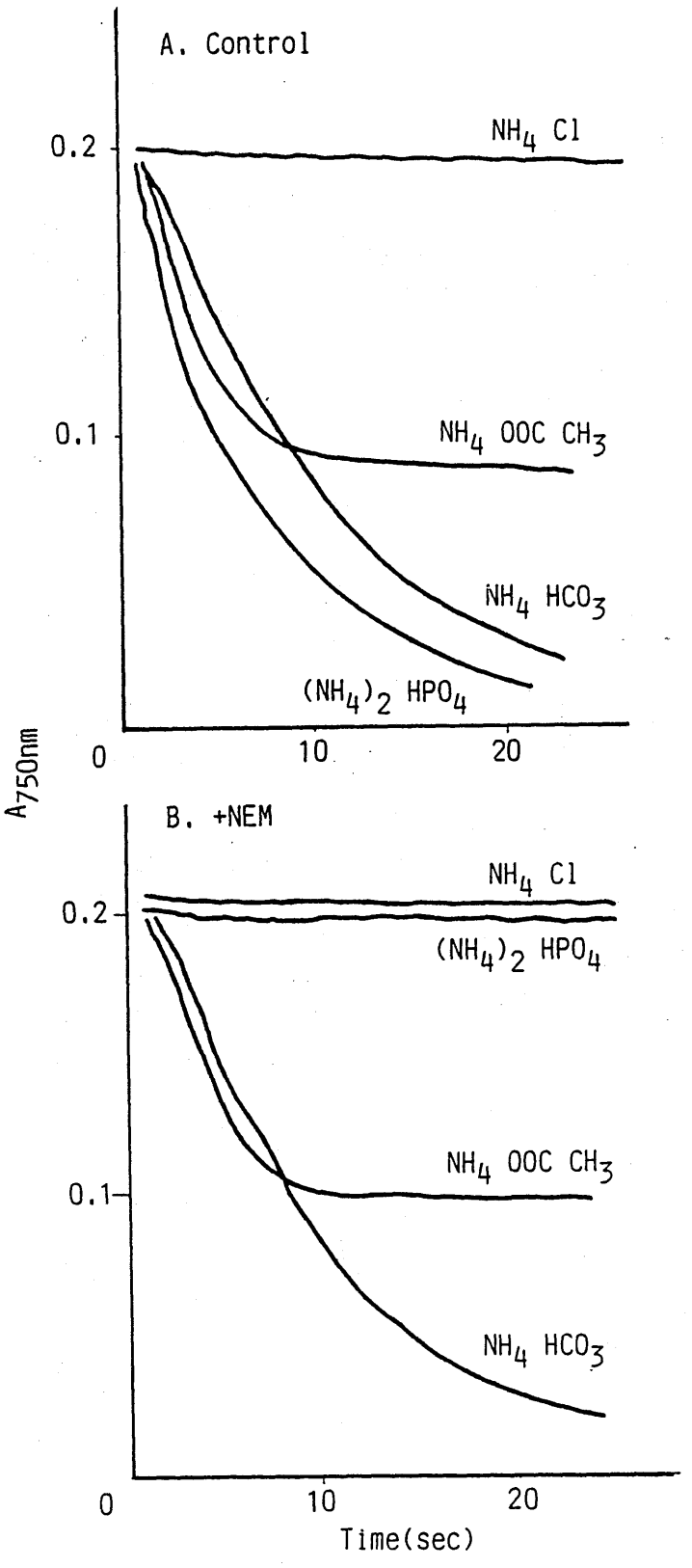
Figs. 3.3.7 (a) and (b)

INHIBITION OF MITOCHONDRIAL SWELLING IN ISOTONIC $(NH_4)_2HPO_4$ MEDIUM IN THE PRESENCE OF N-ETHYLMALEIMIDE

Rat liver mitochondria (prepared as described in section 2.2.5.1) were initially resuspended in isolation medium (section 2.2.5.1) to a protein concentration of 10mg protein/ml. N-ethylmaleimide (50μM) was added to the suspension and incubated on ice for 2 min prior to the swelling of mitochondria in isotonic $(NH_4)_2HPO_4$ medium (see section 2.5.1). In control experiments to monitor the specificity of inhibitors for phosphate transport, the swelling media contained 0.125M of either NH_4Cl, $NH_4OOC\ CH_3$ or NH_4HCO_3 in the place of $(NH_4)_2HPO_4$.

a) Mitochondrial swelling in the absence of N-ethylmaleimide.

b) Mitochondrial swelling in the presence of N-ethylmaleimide.


A. Control
NH_4 Cl
0.2
0.1
NH_4 OOC CH_3
NH_4 HCO_3
$(NH_4)_2$ HPO_4
0
10
20
A_{750nm}
B. +NEM
NH_4 Cl
0.2
$(NH_4)_2$ HPO_4
NH_4 OOC CH_3
0.1
NH_4 HCO_3
0
10
20
Time(sec)

Figs. 3.3.8 (a), (b) and (c)

N-[^{3}H]-ETHYLMALEIMIDE LABELLING STUDIES OF THE PHOSPHATE TRANSPORT PROTEIN FROM RAT LIVER MITOCHONDRIA

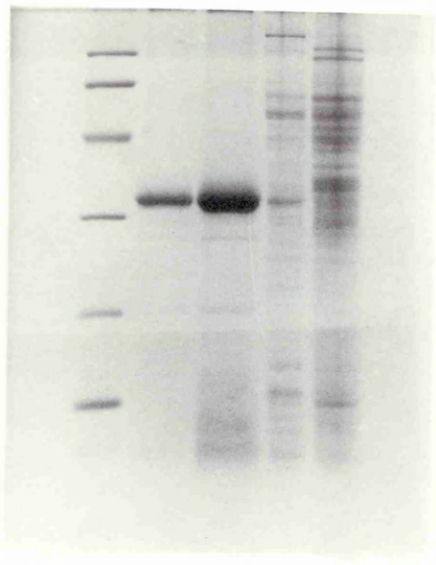
The phosphate transport protein was either labelled with N-[^{3}H]-ethylmaleimide in intact mitochondria and then purified (Fig. 3.3.8c) or labelled at the various stages of its purification with N-[^{3}H]-ethylmaleimide (Fig. 3.3.8b). Incorporation of N-[^{3}H]-ethylmaleimide into the phosphate transport protein was performed according to the method described in section 2.5.2.

a) Original Coomassie Blue staining profile of various fractions from the purification of the phosphate transporter; (1) low M_r standards, (2) Celite-adsorbed fraction, (3) hydroxylapatite fraction, (4) 8% (v/v) Triton X-100 extract, (5) intact rat liver mitochondria.

b) Fluorographic pattern of (2 - 5) after labelling with N-[^{3}H]-ethylmaleimide (6 - 9).

c) N-[^{3}H]-ethylmaleimide labelling pattern of the phosphate transport protein when labelled in intact rat liver mitochondria prior to purification; (1) intact rat liver mitochondria, (2) hydroxylapatite eluate, (3) Celite-adsorbed fraction.

a)

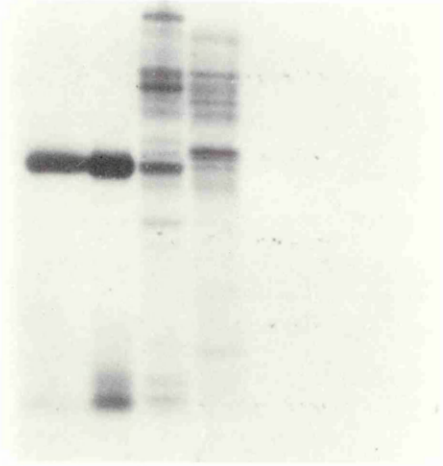


(-)

(+)

(1) (2) (3) (4) (5)

b)

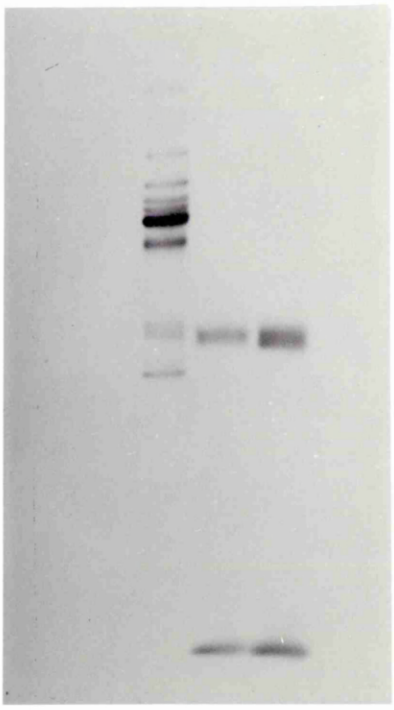


(-)

(+)

(6) (7) (8) (9)

c)



(-)

Phosphate transport protein

(+)

(1) (2) (3)

Figs. 3.3.9 (a) and (b)

PARTIAL INHIBITION OF PHOSPHATE UPTAKE IN RAT LIVER MITOPLASTS PRE-INCUBATED WITH A SPECIFIC ANTISERUM TO THE PHOSPHATE TRANSPORT PROTEIN AS MEASURED BY THE NH_4^+-ION SWELLING TECHNIQUE

Rat liver mitoplasts, prepared as described in section 2.2.5.2, or mitochondria (see section 2.2.5.1), were initially resuspended in isolation medium (section 2.2.5.1) at a protein concentration of 10mg/ml. An equal volume of anti-phosphate transport protein serum or control serum was then added to each suspension and pre-incubated for 1h on ice. The passive swelling of the suspensions in isotonic $(NH_4)_2HPO_4$ medium (see section 2.5.1) was then monitored at 750nm.

a) Swelling of mitoplasts in $(NH_4)_2HPO_4$ medium, in the presence of anti-phosphate transport protein serum or control serum.

(b) Swelling of mitochondria in $(NH_4)_2HPO_4$ medium, in the presence of anti-phosphate transport protein serum or control serum.

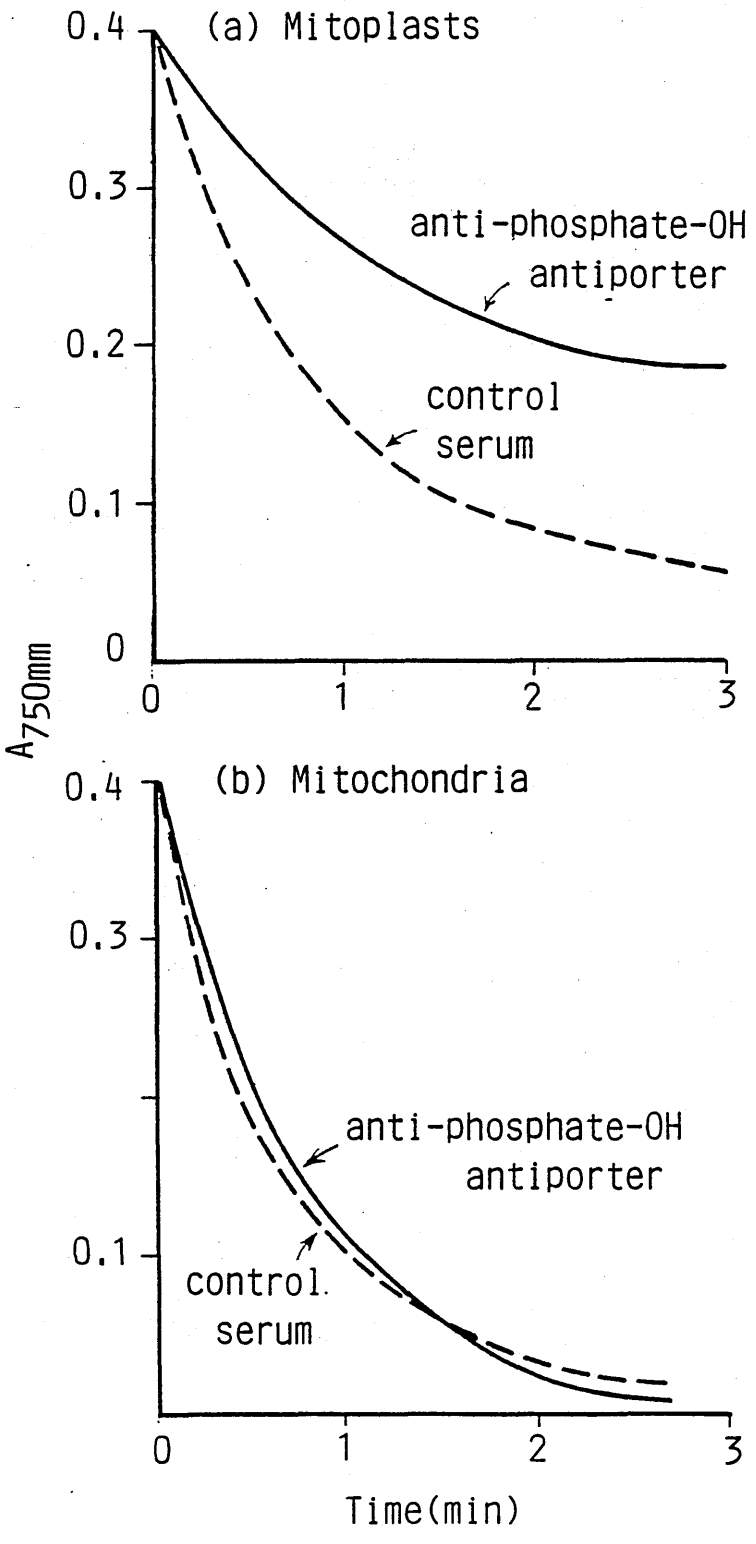

(a) Mitoplasts
0.4
0.3
0.2
0.1
0
anti-phosphate-OH
antiporter
control
serum
0
1
2
3
A750mm
(b) Mitochondria
0.4
0.3
0.1
anti-phosphate-OH
antiporter
control
serum
0
1
2
3
Time(min)

TABLE 3.3.1

COMPARISON OF THE AMINO ACID COMPOSITIONS OF THE RAT LIVER AND BEEF HEART MITOCHONDRIAL PHOSPHATE TRANSPORT PROTEINS

AMINO ACID	MOLES % COMPOSITION (RAT LIVER)	MOLES % COMPOSITION (BEEF HEART)
Cysteine	2.39	0.88
Aspartate (+ Asparagine)	11.94	12.27
Methionine	1.30	1.28
Threonine	8.08	9.12
Serine	7.21	7.40
Glutamate (+ Glutamine)	10.56	8.00
Proline	3.55	2.34
Glycine	12.57	12.42
Alanine	8.65	7.29
Valine	6.75	5.64
Isoleucine	4.24	4.30
Leucine	8.74	8.53
Tyrosine	0.00	3.60
Phenylalanine	3.76	4.64
Histidine	0.87	1.24
Lysine	6.22	8.67
Arginine	3.17	2.31

To measure the content of methionine and cysteine in the protein, these residues were oxidised to cysteic acid and methionine sulphone using performic acid (see section 2.5.3 for further details). The amino acid composition of the phosphate transport protein was subsequently determined by the method described in sections 2.5.4 and 3.3.4.4.

CHAPTER FOUR

CHARACTERIZATION OF ANTISERA RAISED AGAINST THE PHOSPHATE TRANSPORT PROTEIN AND FUMARASE

4.1 INTRODUCTION

In the preceding chapter, a detailed account was given of the purification and identification of the rat liver mitochondrial phosphate transport protein. This protein was also of particular interest from a biosynthetic point of view as it is central to energy metabolism within the cell and is likely to be a transmembraneous inner membrane protein. There is very little information with regard to the biosynthesis and import of mitochondrial transmembrane proteins although the adenine nucleotide translocase protein has been extensively studied in N.crassa cells (Zimmerman and Neupert, 1980). In contrast to the general findings with mitochondrial proteins (Table 1.3.1), these workers discovered that this protein was not made as a larger M_r precursor although there was evidence to suggest that the cytosolic form of the protein existed in a different conformation to the mitochondrial form. As a result of these findings, I was interested in discovering the mode of synthesis of the mitochondrial inner membrane phosphate transport protein to see if this was the general mode of synthesis of mitochondrial transmembraneous proteins.

A second protein of interest with regard to its biosynthesis was fumarase which has a bimodal distribution in many tissues (Akiba et al., 1984). This tricarboxylic acid enzyme is not only found in the mitochondrion but also in the cell cytoplasm where it has a role to play in the urea cycle and tyrosine catabolism. In studying the biosynthesis of this enzyme, an additional problem to be overcome is to determine the mechanism by which this enzyme becomes distributed between two cellular compartments.

A prerequisite for studying the biosynthesis of mitochondrial proteins is the use of a highly specific probe to detect the protein of

interest from a mixture of labelled polypeptides. This probe can be provided by a polyclonal, monospecific antiserum directed against the protein(s) of particular interest. Before an antiserum can be utilised in such a manner, it must be fully characterized. To characterize an antiserum, the antibody titre and precise degree of specificity of interaction with the antigen must be determined. The antibody titre can be measured by the indirect enzyme-linked immunosorbant assay (ELISA) and the monospecificity of the antiserum by the recently developed immune-blotting technique.

In this chapter, the antisera raised against both the phosphate transport protein and fumarase are fully characterized by these methods.

4.2 METHODS

4.2.1 Subcellular Fractionation of Cultured Mammalian Cells

Preparation of a cytosolic fraction from PK-15 cells was performed by the method of Mori et al. (1981). Cells, grown to confluence in a 20oz roller bottle, were washed twice with buffer containing 20mM K^+-Hepes pH 7.4, 0.25M sucrose, 3mM EDTA and 0.1mM PMSF before resuspending in the same buffer at a concentration of 5×10^6 cells/ml. 1ml aliquots (5×10^6 cells) were removed for incubation on ice (2 min) with a range of digitonin concentrations (0.5mg/ml suspension), respectively. After this incubation, the mixture was centrifuged at 14,000g for 1 min and the supernatant fraction retained to measure both fumarase (see section 2.2.2B) and citrate synthetase (see section 2.2.2A) activities. Citrate synthetase activity was measured to test the integrity of mitochondria.

4.3 RESULTS

4.3.1 Determination of the Antibody Titres of Antisera to the Phosphate Transport Protein and Fumarase

The antibody concentrations of the phosphate transport protein and fumarase antisera respectively, were indirectly measured by the non-competitive ELISA assay (Voller et al., 1976). As described in section 2.3.2.4 antigen-antibody complexes can be detected by incubating with a horseradish peroxidase-linked second antibody against the IgG of the species in which the test antibody was elicited. Subsequent incubation of this enzyme-linked complex with a substrate of the enzyme enables direct measurement of the antibody titre by formation of a coloured product. The antibody titre can then be obtained by plotting a graph of absorbance of product formed versus antibody dilution for both a control serum and test antiserum. The point at which there is no significant difference in absorbance between the control and immune antiserum is called the 'titre-point'. Figs. 4.3.1(A) and 4.3.1(B) indicate such determinations for the initial bleedings of rabbit, polyclonal antisera raised against the phosphate transport protein and the enzyme fumarase, respectively. It can clearly be seen that there is no significant difference between the antibody titre values of both antisera, as they lie in the range of 1 in 10,000. This value is in good agreement with the generally accepted value for the initial bleed of any antiserum. Antibody titre was increased by subsequent injection of the rabbit with purified antigens.

4.3.2 Immune-Blotting Analysis of the Phosphate Transport Protein Antiserum

In section 3.3.4.3, this antiserum was shown to partially-inhibit (50%) mitoplast swelling in ammonium phosphate buffer in a specific

manner. This result strongly suggested that the protein isolated from rat liver mitochondria was indeed the phosphate transport protein. To confirm that the rabbit polyclonal antiserum was monospecific, it was tested by using the highly sensitive immune-replica technique of Towbin et al. (1979). Antigen-antibody complexes formed were detected by incubation of the blot with ^{125}I-labelled protein A (2×10^6 cpm/blot) and exposure to autoradiography, as described in section 2.3.2.1(B). Fig. 4.3.2(B) illustrates such an analysis when the antiserum was tested against various quantities of Celite-purified phosphate transport protein. After exposing the autoradiograph for two days at -80^{o}C, a band corresponding to the phosphate transport protein (M_r 34,000) can be seen in each track which increases in intensity as the level of purified protein increases. This result gives an indication of the sensitivity of the technique in that 0.5µg of purified phosphate transport protein can be visualized after the above exposure time. Additionally, the result confirms that the antiserum recognises the antigen to which it was initially raised. However, of interest is the appearance of a doublet at M_r 68,000 which is particularly noticeable at the higher levels of purified protein. A recent report by Tasheva and Dessev (1983), strongly suggested that this M_r 68,000 doublet was of an artifactual nature owing to the presence of 2-mercaptoethanol in Laemmli sample buffer. Confirmation of their findings was provided by an experiment in which Laemmli sample plus 2-mercaptoethanol or DTT in the absence of protein, was run on an SDS-polyacrylamide gel, transferred to nitrocellulose paper and incubated with the phosphate transport protein antiserum (Fig. 4.3.3A, tracks 1 and 2). Clearly, the 68,000 M_r doublet is present in both these samples and arises when sulphydryl group reagents are present in the buffer. This latter point has been confirmed in an experiment where the Celite-purified phosphate transport protein was solubilized by boiling

in Laemmli sample buffer plus 2-mercaptoethanol and then treated with a 5-fold excess of the alkylating reagent, iodoacetamide. Iodoacetamide was used to alkylate free sulphydryl groups on the excess 2-mercaptoethanol. After resolution by SDS-polyacrylamide gel electrophoresis, the protein was transferred onto nitrocellulose paper and subsequently overlayed with phosphate transport protein antiserum for analysis by the immune-replica technique (see section 2.3.2.1B). Although not shown here, this treatment of the protein removed the 68,000 M_r doublet from the corresponding autoradiograph. Clearly, this high M_r doublet arises as an artifact of the system although its actual nature is unknown.

The next step in the characterization of this antiserum, by the immune-replica technique, was to challenge crude mammalian culture cell extracts. This approach can be utilised to confirm that the antiserum is monospecific in its recognition of antigen, a prerequisite for the future biosynthetic studies. In addition, this type of analysis can be performed to establish that the protein is present in the various cell lines and to show that the antiserum directed against the rat liver protein can cross-react with other tissue sources of the protein. Fig. 4.3.3B illustrates the autoradiographic profile of such a blot when crude cytosolic, mitochondrial and whole cell extracts, prepared from PK-15 and BRL cells respectively (see section 2.2.5.3), were treated with the phosphate transport protein antiserum. Numerous high M_r bands can be visualized in all three fractions prepared from each cell line. These additional, non-specific bands vary markedly in M_r. In the mitochondrial fractions of each cell line (tracks 4 and 5), a faint polypeptide band of M_r 34,000 can be visualized when compared with track (1), the Celite-purified, rat liver mitochondrial phosphate transport protein. However, in contradiction to this finding no polypeptide band of this M_r can be seen in the post-nuclear supernatant fractions prepared from these cell

lines (tracks 6 and 7). As expected from its location in the cell, no phosphate transport protein band can be shown to exist in the cytosolic fractions (tracks 2 and 3).

The faint polypeptide band of M_r 34,000 in the mitochondrial fraction prepared from the BRL cells and the lack of this polypeptide in the post-nuclear supernatant fraction, suggests that the level of phosphate transport protein in these cells is low. In the case of the PK-15 cell line, the findings indicate that either the protein is present in low levels within the cell or that it does not cross react very strongly with the antiserum which is raised against the rat liver mitochondrial phosphate transport protein.

Similar results to the above were obtained when rat liver mitochondrial subfractions were blotted and treated with this antiserum.

4.3.3 Effect of Fumarase Antiserum on Enzymic Activity

In order to show that an antiserum is directed against a particular enzyme or protein, one should be able to show an inhibition of that protein's biochemical activity by the antiserum. This was achieved by assaying the activity of the pig heart purified fumarase (EC4.2.1.2) in the presence of anti-fumarase serum, as described in the legend to Fig. 4.3.4. Enzymic activity was calculated from the following equation:

$$\text{Specific activity (Enzyme units/mg protein)} = \frac{\text{A240nm/min} \times 1000}{2.44 \times \text{mg protein/reaction mix}}$$

$$= \text{nmoles/min/mg protein}$$

To observe the effect of the rabbit, polyclonal antiserum on fumarase activity, the percentage of enzymic activity in the presence of

fumarase antiserum as compared to control enzymic activity where no antiserum was added, was plotted against the vol. of antiserum added to the reaction mixture (Fig. 4.3.4). It can clearly be seen from Fig. 4.3.4, that there is a dramatic decrease in enzymic activity on addition of low levels of antiserum (10-20µl), which levels off at about 75% inhibition of enzymic activity. In the presence of pre-immune serum no inhibition of enzymic activity can be found.

4.3.4 Immune-blot Analysis of Anti-fumarase Serum

The immune-replica technique (Towbin et al., 1979), was used to fully characterize the rabbit, polyclonal fumarase antiserum in a similar manner to that employed for the phosphate transport protein antiserum. Initially, the technique was utilised to confirm that the antiserum interacted with the antigen to which it was raised. The effect of incubating a fixed quantity of fumarase (5µg) with serial dilutions of the fumarase antiserum, by the immune-replica assay, is illustrated in Fig. 4.3.5A. There is a very strong interaction of the antiserum with enzyme at low antiserum dilutions (ie. 1 in 20), track (1), which gradually decreases with increasing antiserum dilution. However, even at very high antiserum dilutions (ie. 1 in 500, track 6), a polypeptide band corresponding to fumarase (M_r 47,000) can be detected after autoradiography for two days at -80°C. Apart from demonstrating that the antiserum interacts with the purified pig heart enzyme, this result gives an indication of the sensitivity of fumarase antiserum towards its antigen. Fig. 4.3.5C illustrates the autoradiographic profile obtained when various levels of purified fumarase (0.5 - 10µg) are blotted and treated with a fixed concentration of antiserum (1 in 40 dilution). After a two day exposure at -80°C, a band corresponding to mature fumarase (M_r 47,000) can be visualised which in agreement with the analogous phosphate trans-

port protein blot (Fig. 4.3.2), increases in intensity with raised levels of purified antigen. Fig. 4.3.5B represents the original Coomassie Blue staining profile of Fig. 4.3.5C prior to immune-replica analysis with the fumarase antiserum.

Monospecificity of the fumarase antiserum was demonstrated by showing that the antiserum only reacted with a single component when challenged with crude mammalian tissue culture cell extracts (2.2.5.3), by the immune-replica technique (2.3.2.1B). In this case, the various crude cell extracts were prepared from BRL, PK-15 and NBL-1 cell lines, respectively. Fig. 4.3.6 demonstrates that fumarase can be detected in both the mitochondrial and cytosolic compartments of BRL and PK-15 cells (tracks (2), (3), (5) and (6)). This finding is in agreement with the earlier work of several groups including Nakashima et al. (1976) who have shown that fumarase is distributed between the cell cytoplasm and mitochondrial matrix compartments of rat liver, respectively. Interestingly, the BRL cytosolic form of enzyme appears to be of a higher M_r value (47,000) than the corresponding mitochondrial form (45,000). In contrast, the pig kidney fumarases are of an identical M_r value (47,000). The bimodal distribution of fumarase was confirmed in an experiment in which intact PK-15 cells were treated with a series of concentrations of the detergent-like compound, digitonin (see section 4.2.1). The release of fumarase from PK-15 cells was measured at all concentrations of digitonin used by measuring enzymic activity as described in section 2.2.2B. To act as an indicator of mitochondrial breakage, the activity of the mitochondrial matrix marker, citrate synthetase, was also assayed in each sample (Fig. 4.3.7). At low levels of digitonin where the cell plasma membrane is disrupted preferentially, no measurable citrate synthetase activity could be detected. However, under the same conditions the release of fumarase from the cytosol was shown. This cytosolic activity

corresponds to about 40% of the total cellular activity of fumarase. The existence of a cytosolic pool of enzymic activity confirmed the previous immune-replica data in cultured mammalian cells (see Fig. 4.3.6). At higher levels of digitonin (2 and 5mg/ml cell suspension), citrate synthetase activity could be detected in the supernatant fraction also indicating breakage of mitochondria. A corresponding rise in fumarase activity accompanied mitochondrial disruption, indicating the presence of a second pool of fumarase in this organelle.

Finally, the immune-replica technique was used to demonstrate that the mitochondrial fumarase activity was localized in the matrix compartment of the organelle. Beef heart mitochondria, prepared by the method of Smith (1967), were sonicated for 3X 15S (high power and amplitude 3 settings) on an MSE Ultrasonic disintegrator and the membrane fraction pelleted by centrifugation at 100,000g for 1h. Boxer (1975) previously demonstrated that such mitochondria are largely devoid of outer membrane material. This was shown by assaying the beef heart mitochondria for the outer membrane marker enzyme, monoamine oxidase. When this was performed (Boxer, 1975), only 4% of the total mitochondrial protein represented outer membrane material. As a result of these findings, sonication of beef heart mitochondria in our studies will result in the release of matrix components. Fig. 4.3.8 illustrates the autoradiographic profile of the immune-blot from such samples after treatment with fumarase antiserum. The enzyme (M_r 47,000) could be detected in both the matrix fraction (track 3) and intact mitochondria (track 1) but not in the membrane fraction (track 2). These findings are in agreement with the known mitochondrial function of the enzyme.

4.4 DISCUSSION

4.4.1 Immune-replica Assay

In this study, the specificities of rabbit, polyclonal antisera raised against the rat liver mitochondrial phosphate transport protein and pig heart enzyme fumarase respectively, were largely determined by the immune-replica technique (Towbin et al., 1979), as described in section 2.3.2.1(B). This technique is now routinely used in our laboratory to characterize many different antisera. By transferring SDS-polyacrylamide gel electrophoretically separated proteins on to nitrocellulose paper one has the following advantages:-

i) wet filters are pliable and easy to handle,

ii) the immobilised proteins are readily and equally accessible to antiserum,

iii) transfer analysis generally calls for small amounts of reagents,

iv) processing times can be significantly reduced.

It also has the advantage over the conventional gel methods utilised in the past to determine the specificity of antisera, in that it enables the M_r values of all proteins recognised by the antisera to be calculated directly.

The method of Towbin et al. (1979) was originally adopted in this laboratory to determine the specificity of both rabbit, polyclonal antisera to the rat liver mitochondrial phosphate transport protein and to the pig heart enzyme, fumarase. To saturate additional protein binding sites on the electrophoretic blots, they were soaked in a 3% (w/v) BSA solution (see section 2.3.2.1B), prior to treatment with antiserum. After incubation with antiserum, the immobilised antigen-antibody complexes were decorated with ^{125}I-labelled protein A (prepared as described in

section 2.2.3) and exposed to autoradiography (section 2.2.4.5). In all experiments using BSA as the blocking agent, the backgrounds of the blots after exposure to autoradiography were extremely poor and as a result difficult to interpret. To overcome this problem, the modification of Batteiger et al. (1982) was adopted. It involved the use of the non-ionic detergent Tween 20 (0.5% (v/v)) to block additional protein binding sites on the nitrocellulose paper, instead of BSA. By using a non-ionic detergent to block unoccupied sites on the nitrocellulose paper, several advantages can be gained:

a) The efficiency of electrophoretic transfer can be determined by staining the proteins directly on the blot.

b) Staining for protein after immunological probing enables one to determine if the electrophoretic pattern is conserved during antibody incubation and washes.

c) Comparison of the autoradiogram and the stained blot from which it was derived, allows accurate identification of immunoreactive proteins.

All the immune-blots illustrated in this chapter were treated with Tween 20 and resulted in clearer backgrounds, enabling easier interpretations.

4.4.2 Specificity of the Phosphate Transport Protein Antiserum

Immune-replica analysis of the phosphate transport protein antiserum always demonstrated the presence of a doublet of M_r 68,000, although the M_r value of the purified protein was 34,000. This doublet was even present when the Celite purified rat liver mitochondrial phosphate transport protein was treated to such an analysis. Densitometric scanning

of a Coomassie Blue stained gel containing the Celite purified carrier had previously shown its purity to be greater than 95%.

The possibility that this doublet represented a dimer of the phosphate transport protein was ruled out by the findings in our laboratory of several, completely unrelated antisera reacting with it on immune-replica analysis. In 1983, Tasheva and Dessev reported the existence of a doublet of similar M_r to that mentioned above when resolving a class of proteins bound to DNA by SDS-polyacrylamide gel electrophoresis. They showed that when 2-mercaptoethanol was present in Laemmli sample buffer during SDS-polyacrylamide gel electrophoresis, two additional bands with electrophoretic mobilities corresponding to M_r values of 68,000 and 54,000 respectively, were stainable with silver and to a lesser extent Coomassie Blue. Closer examination of the Coomassie Blue stained polyacrylamide gel used to resolve the phosphate transport protein (Fig. 4.3.2A), confirmed these authors' results. They also demonstrated that even in the absence of protein, when only sample buffer was loaded on to the gel, these two bands could be detected and thus were of a non-protein origin. Fig. 4.3.3(A) illustrated the effect of loading Laemmli sample buffer plus 2-mercaptoethanol or DTT on to a gel and analysing by the immune-replica technique with phosphate transport protein antiserum. The 68,000 M_r doublet and a slightly lower M_r band were strongly labelled in both instances, confirming the non-protein nature of the bands and suggesting that they arise as an artifact of this system. To overcome this problem it has recently been shown in our laboratory that if the protein samples are treated with an excess of the alkylating reagent, iodoacetamide after solubilization in Laemmli sample buffer, the 68,000 M_r doublet can no longer be detected on the auto-radiograph. This finding suggests a role for the free sulphydryl groups on 2-mercaptoethanol in the artifactual nature of this doublet. However,

it does not explain why the antiserum to the phosphate transport protein recognises and binds to the doublet.

When the phosphate transport protein antiserum was tested for specificity against tissue culture cell extracts, numerous additional high M_r bands could be visualized on the autoradiogram. Purification of the IgG fraction from whole antiserum had no effect on the immune-replica pattern of these extracts. The following explanations can be put forward to possibly explain these results:

i) The additional polypeptides of higher M_r which are interacting with the antiserum against the phosphate transport protein (M_r 34,000), may have arisen from the presence in the antigenic preparation of proteolytic fragments derived from larger M_r polypeptides. These proteolytic fragments may comigrate with the phosphate transport protein during SDS-polyacrylamide gel electrophoretic separation and thus will not be differentiated from the phosphate transporter by Coomassie Blue staining. During the preparation of cultured mammalian cell extracts, if there is no problem with proteolysis, the antibodies elicted against the proteolytic contaminants will be able to react with the uncleaved protein giving rise to these polypeptide bands of higher M_r value. A similar finding to this has been reported previously by Suissa and Reid (1983), when studying the specificity of antisera directed against the β-subunit of the yeast F_1-ATPase. They discovered that the antiserum also cross-reacted with the α-subunit of this complex which has been shown to be particularly sensitive to proteolysis. This interpretation of the immune-replica data obtained with the antiserum raised against the phosphate transport protein is unlikely as the preliminary amino acid composition data of the protein illustrates that it contains no tyrosine (see Table 3.3.1). If the phosphate transporter was contaminated with proteolytic degradation fragments of polypeptides of higher M_r value, then one would expect this

to affect the amino acid composition data. It is unlikely that all of the contaminating proteins do not contain any tyrosine residues.

ii) An alternative explanation is that these additional proteins are very antigenic contaminants which are present in the phosphate transporter preparation in low levels and thus are not detectable by Coomassie Blue staining. In agreement with this explanation are the results of earlier findings which showed that the rat liver mitochondrial phosphate transport protein when overlayed with ^{125}I-labelled con A illustrated numerous high and low M_r glycoprotein contaminants (see Fig. 3.3.1B). Although pre-extraction of mitochondria with a 0.5% (v/v) Triton X-100 buffer (see section 3.2.1 method a) appeared to quantitatively remove these glycoproteins, lectin overlay analysis was not performed on subsequent purifications of the phosphate transport protein. As a result, if the preparations used for injecting the rabbit were contaminated with low levels of glycoprotein, antibodies would also be raised against them and they would subsequently be detected by immune-replica analysis. Again, however, this explanation is unlikely because one does not see these additional bands when the purified phosphate transport protein is analysed by the immune-replica technique (see Fig. 4.3.2).

iii) A third explanation of the immune-replica analysis of cultured mammalian cell extracts with the antiserum directed against the phosphate transport protein is based on the hydrophobic properties of the trans-membrane carrier. It is very likely that many of the protein's antigenic determinants are also of a hydrophobic nature. These additional bands on the autoradiograph may, therefore, arise as a result of the constitutive polypeptides exposing similar hydrophobic determinants to those found on the phosphate transport protein and thus being recognised by the antiserum.

This interpretation, however, would have to suggest in yeast, where the antiserum specifically recognises a protein of 29,000 M_r, that no other polypeptides contain similar hydrophobic determinants to those found on the rat liver mitochondrial phosphate transporter (see Fig. 5.3.10a).

iv) Finally, a fourth explanation of the immune-replica findings can be given in which the polypeptide bands of higher M_r values represent either aggregates of the phosphate transport protein or aggregates of the phosphate transport protein with other cellular polypeptides. The aggregation of membrane proteins, even in the presence of SDS, has recently been reported by Finbow et al. (1983). They were carrying out studies on the gap junction protein isolated from a wide variety of tissue sources and illustrated that the protein (M_r 16,000) dimerised when boiled in SDS, to give a polypeptide of M_r 26,000 when resolved by SDS-polyacrylamide gel electrophoresis. To overcome this problem, Finbow et al. (1983) solubilised the gap junction protein in an SDS solution at room temperature. The wide range of M_r values of the additional polypeptides obtained when crude cultured mammalian cell extracts are immune-blotted with the anti-phosphate transport protein serum favours an aggregation of the transporter with other cellular polypeptides. In addition, the purified rat liver phosphate transport protein did not appear to aggregate (see Fig. 4.3.2B). However, once again one has the problem of interpreting the yeast immune-replica data in which the anti-rat liver mitochondrial phosphate transport protein serum specifically recognises a protein of 29,000 M_r (see Fig. 5.3.10a). In this case, it is assumed that for some reason the yeast phosphate transport protein does not have a tendency to aggregate with other cellular proteins.

In summary, although the antiserum directed against the rat liver mitochondrial phosphate transport protein demonstrates the presence of

numerous polypeptides when challenged with crude cell fractions, we believe that this problem is of a technical nature and not due to the lack of purity of the isolated protein.

4.4.3 Specificity of the Rabbit, Polyclonal Antiserum to Pig Heart Fumarase

The immune-replica technique confirmed that the rabbit, polyclonal antiserum raised against pig heart fumarase was monospecific when crude mammalian tissue-culture cell extracts (see section 2.2.5.3) were challenged with the antiserum. However, both the PK-15 and BRL cells appeared to possess a cytosolic form of the enzyme. In the case of the PK-15 cell line, this form of the enzyme was of the same M_r (47,000) as the mitochondrial enzyme, whereas the BRL cytosolic fumarase appeared to be of a slightly higher M_r than its corresponding mitochondrial form.

Cytosolic and mitochondrial pools of fumarase have been reported to exist in many tissues including rat liver, kidney, heart (Akiba et al., 1984) and in yeast (Hiraga et al., 1984). The cytosolic fumarase appears to play a role in the urea cycle and in the tyrosine-oxidizing system. Extensive research in recent years has been directed towards a greater understanding of the physiochemical properties of the two forms of the rat liver enzyme, in particular (Kobayashi et al., 1981). It appeared that the cytoplasmic and mitochondrial forms of fumarase in rat liver were indistinguishable from one another (Table 1.5.1). However, Kobayashi and Tuboi (1983) subsequently discovered that the N-terminal residues of the two enzyme forms differed from each other. The N-termini of the rat liver mitochondrial and cytosolic fumarases were identified as pyroglutamic acid and N-acetylalanine, respectively. In 1984, Hiraga et al. also demonstrated that the two enzyme forms could be separated from each other by Bio-Gel HTP column chromatography. This indicated that there

were some conformational differences between the native proteins of the mitochondrial and cytosolic fumarases.

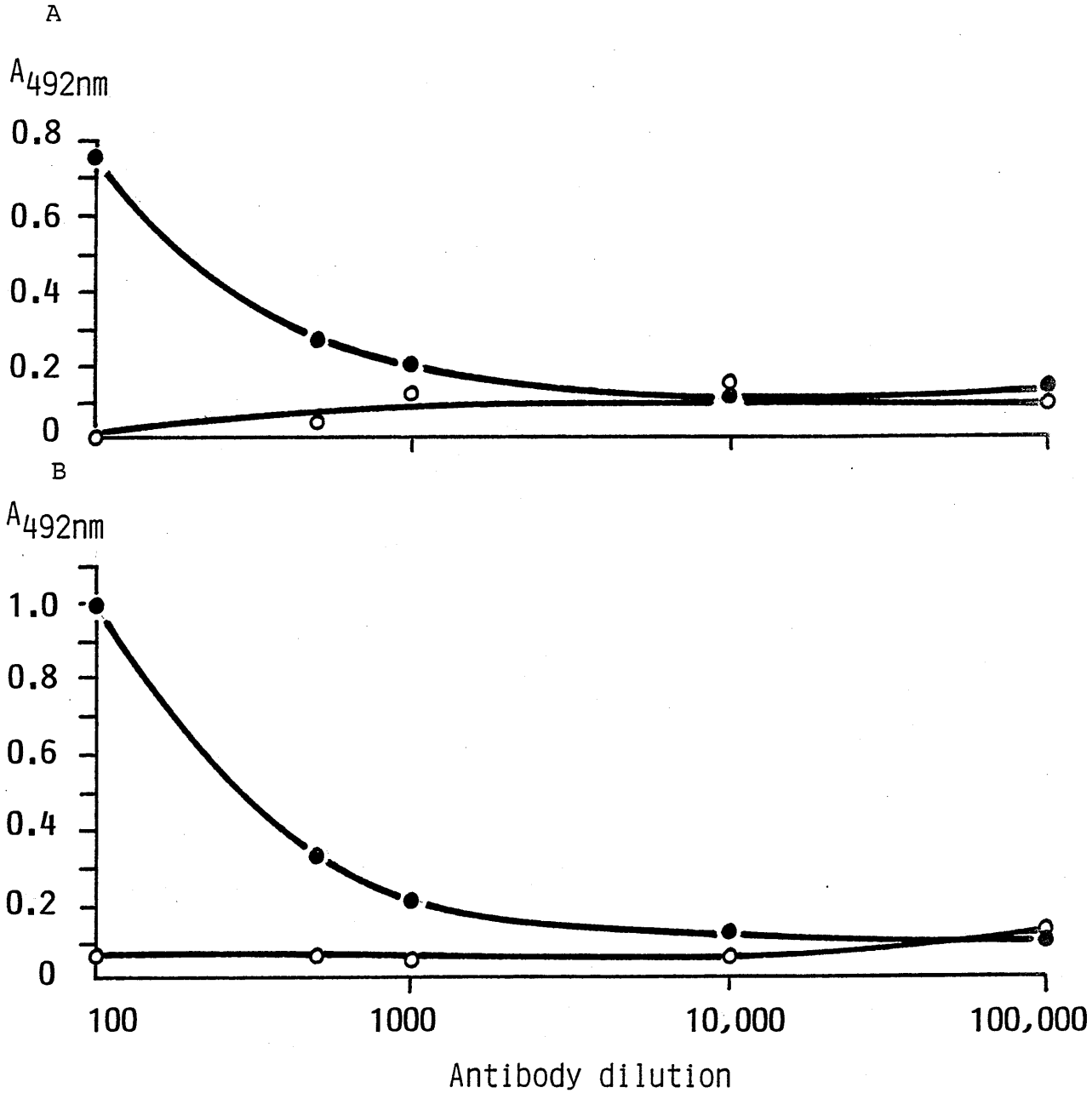
In contrast to the earlier work of these Japanese groups, BRL cytoplasmic fumarase appears to be of a slightly larger M_r than the mitochondrial form, indicating several potential modes of synthesis which will be discussed in chapter five. The results obtained from immune-replica analysis could initially have been interpreted in terms of a contamination of cell cytosol with mitochondrial extracts, since cell disruption was implemented by homogenisation. Confirmation was, however, provided by an experiment in which intact PK-15 cells were treated with increasing concentrations of digitonin (see section 4.2.1). This compound is one of the saponins, a series of steroid glycosides with detergent-like properties that are widespread amongst higher plants. At low concentrations, it can be selectively used to disrupt membranes rich in cholesterol. In this experiment, at low concentrations (0-1mg/5 x 10^6 cells), only the plasma membrane of PK-15 cells would be disrupted. Thus high speed centrifugation (14,000g for 1 min) of cells treated in this manner would spin down all organelles including mitochondria. The resulting supernatant fraction would consequently be rich in cytosolic material. At higher levels of digitonin, the mitochondria which contain very low levels of cholesterol (0.24% of total lipid by weight), will be disrupted. This experimental approach was, therefore, utilised to demonstrate the existence of two pools of the enzyme, fumarase. Intactness of PK-15 mitochondria was measured by monitoring the activity of the matrix-located marker enzyme, citrate synthetase. The results obtained clearly illustrated the presence of a cytosolic pool of fumarase in PK-15 cells when no measurable citrate synthetase activity could be detected. A similar result was obtained when the experiment was performed with BRL cells.

Finally, the immune-replica technique is not only a highly sensitive method for demonstrating the monospecificity of an antiserum but it can also be utilised as an analytical tool, to illustrate the purity of subcellular fractions. Beef heart mitochondrial subfractions were prepared by sonication (see section 4.3.4) and the membrane and soluble fractions isolated by centrifugation. Immune-blotting with fumarase antiserum confirmed that the enzyme was located in the mitochondrial matrix where it functions in the tricarboxylic acid cycle.

Figs. 4.3.1 (A) and (B)

ESTIMATION OF THE TITRES OF THE ANTI-PHOSPHATE TRANSPORT PROTEIN SERUM AND THE ANTI-FUMARASE SERUM

The titres of the polyclonal, rabbit antisera directed against the phosphate transport protein and fumarase, respectively, were estimated by the indirect ELISA assay (see section 2.3.2.4). A fixed concentration of antigen (phosphate transport protein or fumarase) was incubated at room temperature with serial dilutions of antiserum, ranging from 1 in 100 to 1 in 100,000. By plotting the absorbance at 492nm of the product formed from the horse-radish peroxidase linked conjugate (see section 2.3.2.4) against antibody dilution for (o) control serum and (●) phosphate transport protein serum (Fig. 4.3.1A) or fumarase antiserum (Fig. 4.3.1B) respectively, the antibody titres could be calculated.


A
A_{492nm}
0.8
0.6
0.4
0.2
0
B
A_{492nm}
1.0
0.8
0.6
0.4
0.2
0
100
1000
10,000
100,000
Antibody dilution

Figs. 4.3.2 (A) and (B)

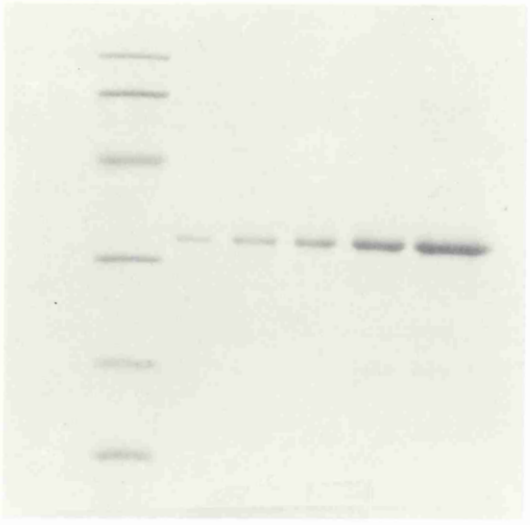
IMMUNE-REPLICA ANALYSIS OF PURIFIED PHOSPHATE TRANSPORT PROTEIN VERSUS ANTISERUM DIRECTED AGAINST THE PROTEIN

Celite-purified rat liver mitochondrial phosphate transport protein (see section 3.2.1 method a) was resolved by SDS-polyacrylamide gel electrophoresis 12.5% (w/v) (see section 2.2.4.1), transferred onto nitrocellulose paper (see section 2.3.2.1A) and analysed by the immune-replica technique (see section 2.3.2.1B).

A) Coomassie Blue staining profile of resolved protein(s) after SDS-polyacrylamide gel electrophoresis; (1) low M_r standards (2) 0.5µg (3) 1µg (4) 2µg (5) 5µg (6) 10µg Celite-purified phosphate transport protein.

B) Autoradiographic profile of above gel after immune-blotting, except that no low M_r standards were run. The autoradiograph was exposed for two days at -80°C (see section 2.2.4.5).

A



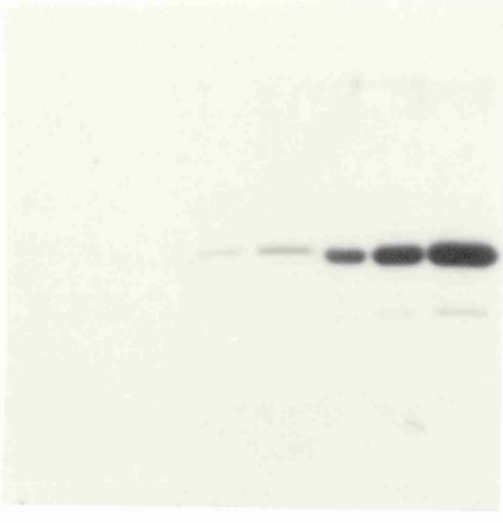
(-)

Phosphate transport protein (M_r 34,000)

(+)

(1) (2) (3) (4) (5) (6)

B



(-)

Doublet (M_r 68,000)

Phosphate transport protein (M_r 34,000)

(+)

(1) (2) (3) (4) (5)

Figs. 4.3.3 (A) and (B)

IMMUNE-REPLICA ANALYSIS OF MAMMALIAN TISSUE-CULTURE CELL EXTRACTS VERSUS PHOSPHATE TRANSPORT PROTEIN ANTISERUM

After separation by SDS-polyacrylamide gel electrophoresis 12.5% (w/v) (see section 2.2.4.1), proteins were transferred onto nitrocellulose paper (see section 2.3.2.1A) before subjection to immune-blotting (see section 2.3.2.1B) and detection by autoradiography (see section 2.2.4.5).

A) To illustrate the artifactual nature of the 68,000 M_r doublet which reacts with the anti-phosphate transport protein, the following samples were utilised; (1) Laemmli sample buffer plus 2-mercaptoethanol (2) Laemmli sample buffer plus DTT (3) Celite eluate.

B) Specificity of this antiserum was determined by challenging the following crude mammalian tissue-culture cell extracts; (1) Celite eluate (2) 40µg PK-15 cytosolic fraction (3) 40µg BRL cytosolic fraction (4) 40µg PK-15 mitochondrial fraction (5) 40µg BRL mitochondrial fraction (6) 40µg PK-15 whole cell extract (7) 40µg BRL whole cell extract.

The autoradiographs were exposed for two days at -80°C.

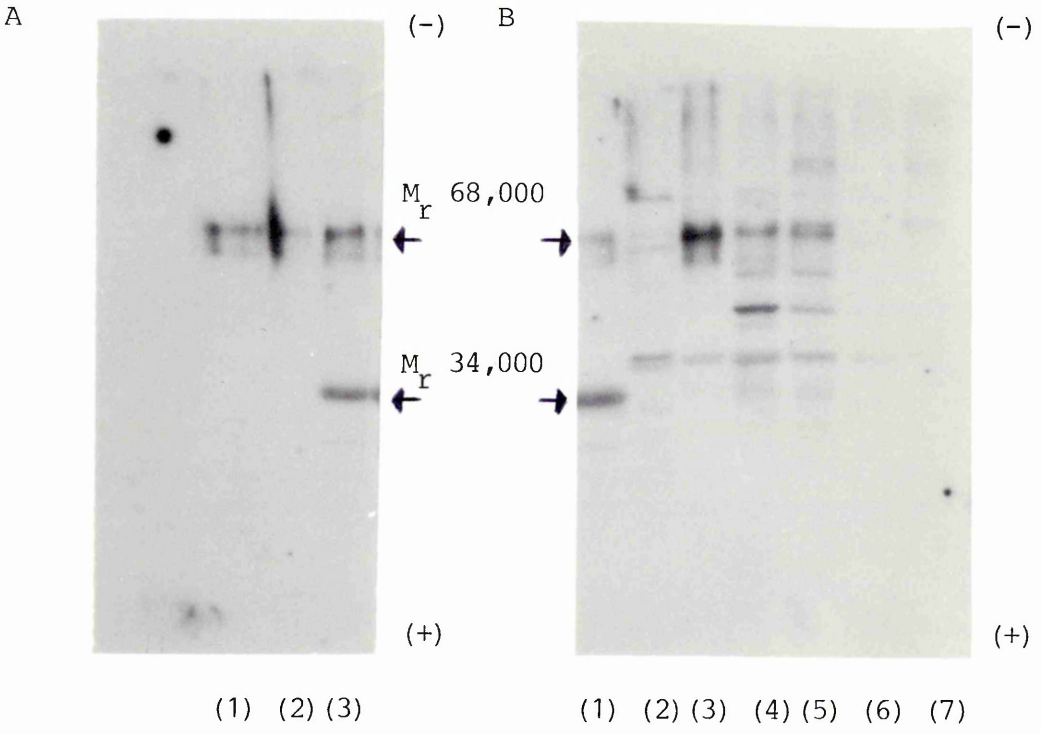
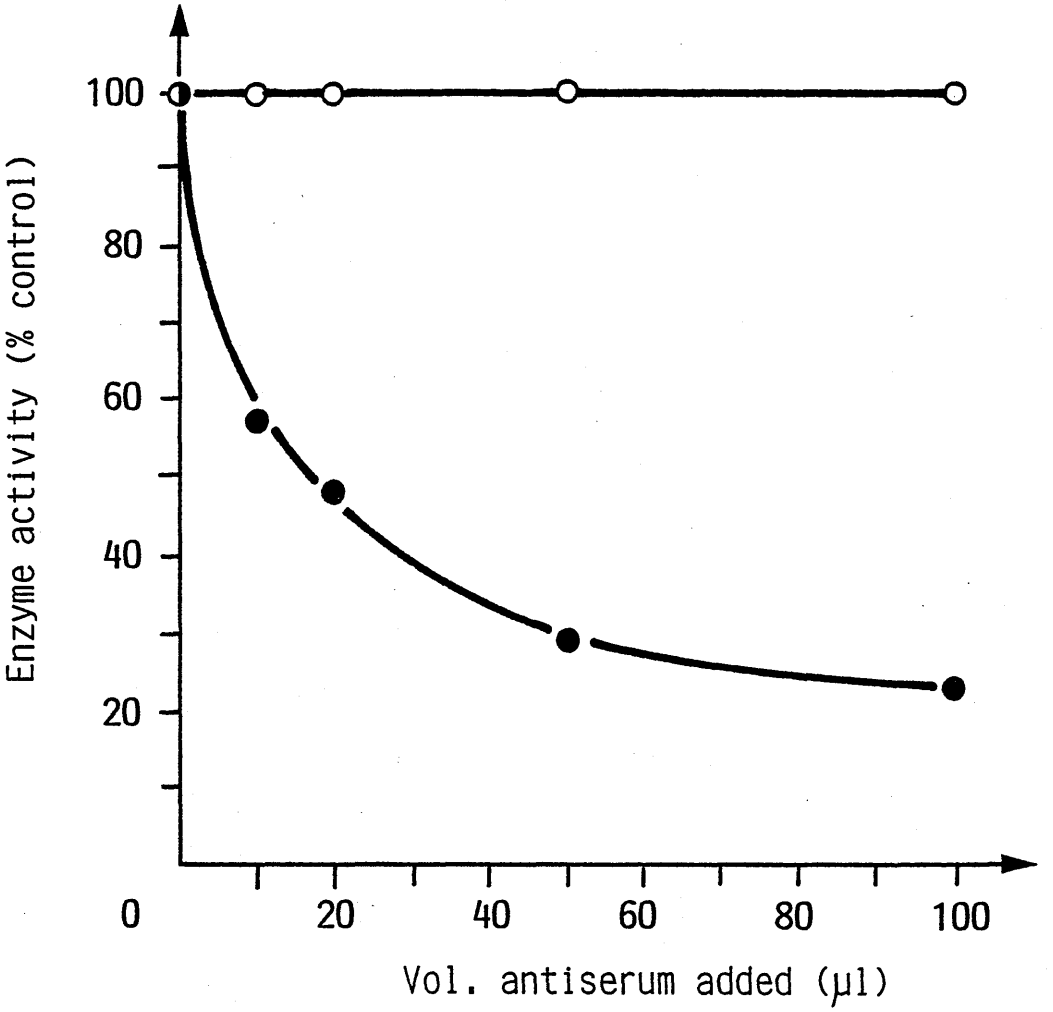

A
(-)
B
(-)
M_r 68,000
M_r 34,000
(+)
(+)
(1) (2) (3)
(1) (2) (3) (4) (5) (6) (7)

Fig. 4.3.4

EFFECT OF POLYCLONAL, RABBIT ANTI-FUMARASE SERUM ON PURIFIED ENZYMIC ACTIVITY

Pig heart fumarase activity (1-5μg) was measured by the method described in section 2.2.2(B), in the presence of increasing levels of (o) control serum and (●) fumarase antiserum, respectively. The enzyme was incubated for 90 min at room temperature with either control serum or fumarase antiserum, prior to its addition to the reaction mixture. A graph was subsequently plotted of the percentage of fumarase activity in the presence of anti-fumarase serum, as compared to control enzymic activity where no antiserum was added, against the vol. of antiserum added.


Enzyme activity (% control)
100
80
60
40
20
0
20
40
60
80
100
Vol. antiserum added (µl)

Figs. 4.3.5 (A), (B) and (C)

IMMUNE-REPLICA ANALYSIS OF PURIFIED PIG HEART FUMARASE VERSUS ANTI-FUMARASE SERUM

Purified fumarase, after resolution by SDS-polyacrylamide gel electrophoresis 10% (w/v) (see section 2.2.4.1) was transferred onto nitrocellulose paper (see section 2.3.2.1A) and treated to immune-replica analysis (see section 2.3.2.1B) with anti-fumarase serum. Visualization of the reactive bands was achieved by autoradiography (see section 2.2.4.5).

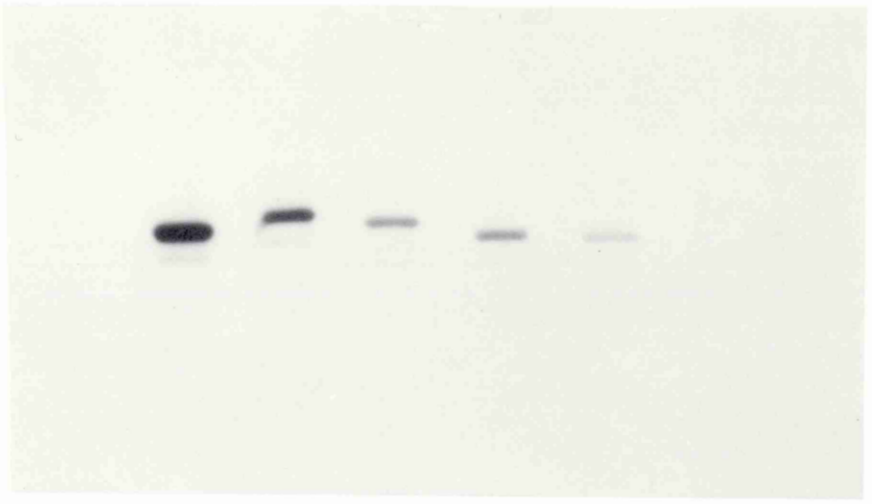
A) Autoradiographic profile of the titration of a fixed level of fumarase (5µg) with a series of dilutions of fumarase antiserum; (1) 1 in 20 (2) 1 in 40 (3) 1 in 80 (4) 1 in 100 (5) 1 in 200 (6) 1 in 500.

B) Autoradiographic profile of a range of levels of purified fumarase versus fixed antiserum dilution (1 in 40); (1) 10µg (2) 5µg (3) 2µg (4) 1µg (5) 0.5µg purified fumarase.

C) Coomassie Blue staining profile of B including low M_r standards (track 6) (see section 2.2.4.3 for details of standards).

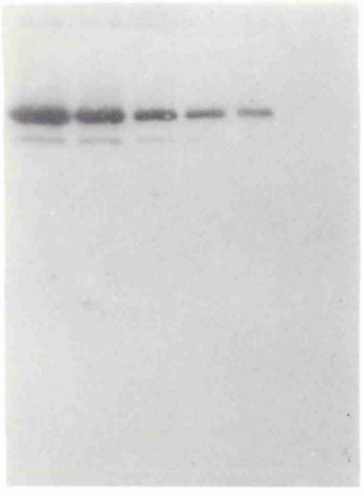
Both autoradiographs were exposed for 24hrs at -80°C.

A



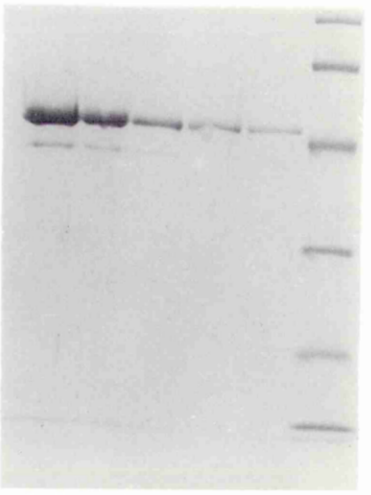
(1) (2) (3) (4) (5) (6)

B



(1) (2) (3) (4) (5)

C



(1) (2) (3) (4) (5) (6)

Fig. 4.3.6

IMMUNE-REPLICA ANALYSIS OF CULTURED MAMMALIAN CELL EXTRACTS VERSUS ANTI-FUMARASE SERUM

Crude cultured mammalian cell extracts, prepared as described in section 2.2.5.3, were initially subjected to SDS-polyacrylamide gel electrophoresis 10% (w/v) (see section 2.2.4.1) followed by immune-blotting analysis (see sections 2.3.2.1A and B). Specificity of the antiserum was determined by exposing the blot to autoradiography (see section 2.2.4.5).

The autoradiographic profile of the following samples is illustrated: (1) BRL cell extract (2) BRL mitochondria (3) BRL cytosol (4) 0.2µg of purified fumarase (5) PK-15 cell extract (6) PK-15 mitochondria (7) PK-15 cytosol (8) 0.2µg purified fumarase (9) NBL-1 cell extract. In all cases, 40µg of cell extract was loaded on to the gel. Tracks 1-3 were exposed for six days whereas tracks 4-9 for two days at -80°C.

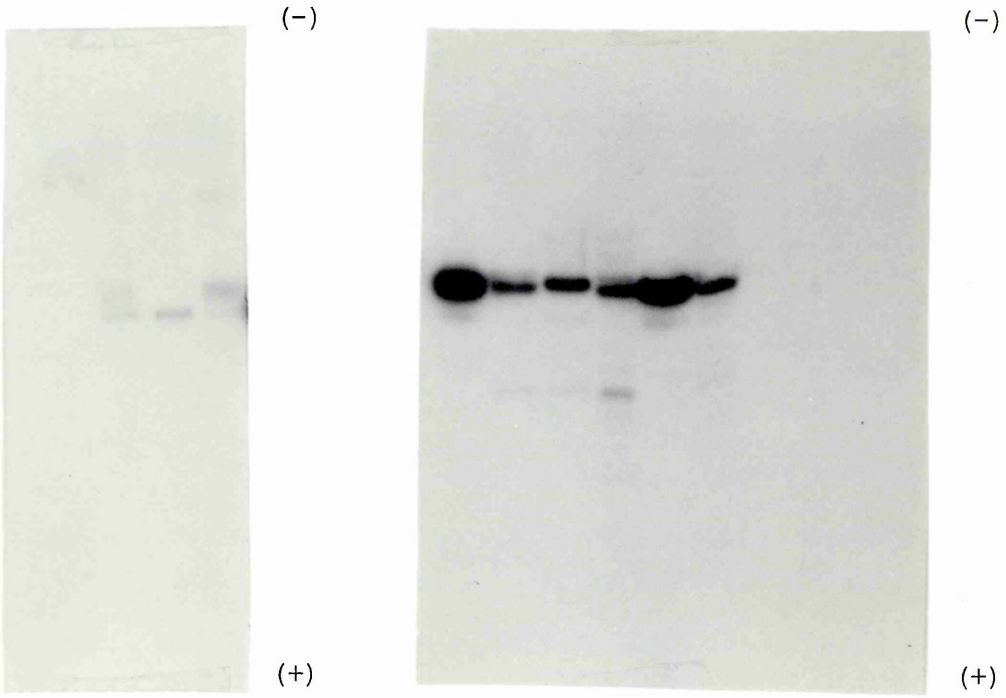

(−)
(+)
(−)
(+)
(1) (2) (3)
(4) (5) (6) (7) (8) (9)

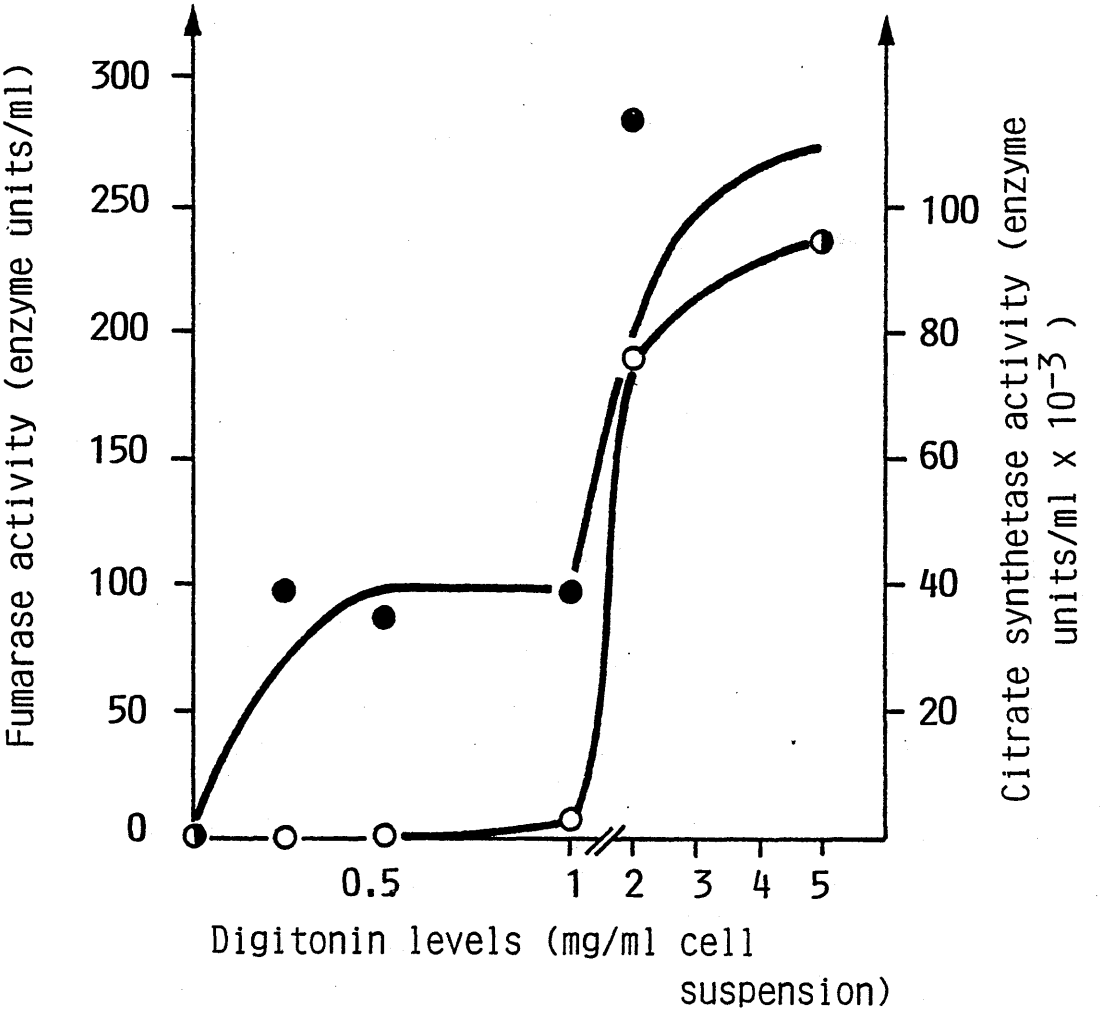
Fig. 4.3.7

ESTIMATION OF FUMARASE AND CITRATE SYNTHETASE ACTIVITIES IN DIGITONIN TREATED PK-15 CELLS

PK-15 cells were treated with a range of digitonin concentrations (0-5mg/ml cell suspension), as described in section 4.2.1, to prepare a fraction which represented the cell cytosol. The bimodal distribution of fumarase was demonstrated by measuring the release of enzymic activity (see section 2.2.2B) at each digitonin concentration. As a control, the mitochondrial matrix marker enzyme, citrate synthetase was also assayed (see section 2.2.2A) in each fraction to test the integrity of the mitochondria.

● - fumarase activity (enzyme units/ml cell extract)

o - citrate synthetase activity (enzyme units/ml cell extract)


Fumarase activity (enzyme units/ml)
300
250
200
150
100
50
0
0.5
1
2
3
4
5
100
80
60
40
20
Citrate synthetase activity (enzyme units/ml x 10^{-3})
Digitonin levels (mg/ml cell suspension)

Figs. 4.3.8 (A) and (B)

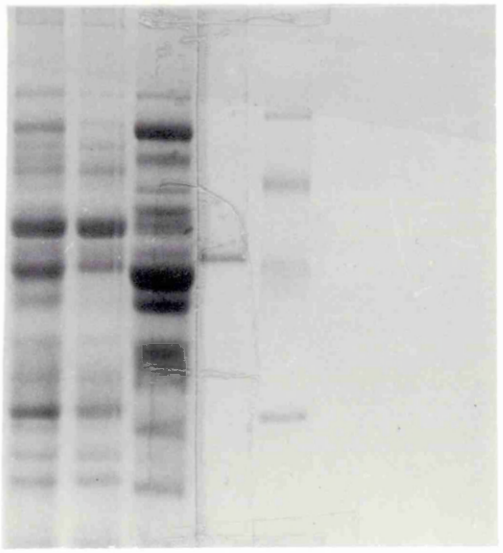
IMMUNE-REPLICA ANALYSIS OF BEEF HEART MITOCHONDRIAL SUBFRACTIONS VERSUS ANTI-FUMARASE SERUM

Beef heart mitochondrial subfraction proteins, prepared as described in section 4.3.5, were separated by SDS-polyacrylamide gel electrophoresis 10% (w/v) (see section 2.2.4.1) and then analysed by the immune-blotting technique (see sections 2.3.2.1 A and B) with fumarase antiserum (1 in 40 dilution).

A) Coomassie Blue staining profile of; (1) intact beef heart mitochondria (2) mitochondrial membrane fraction (3) mitochondrial matrix (4) 2µg purified fumarase (5) low M_r standards.
40µg of mitochondrial subfractions were loaded onto each track.

B) Autoradiographic profile of above gel except that track (4) only contained 0.2µg of purified fumarase and track (5) contained 30,000 cpm of ^{125}I-labelled low M_r standards. The autoradiograph was exposed for 24h at -80°C.

A



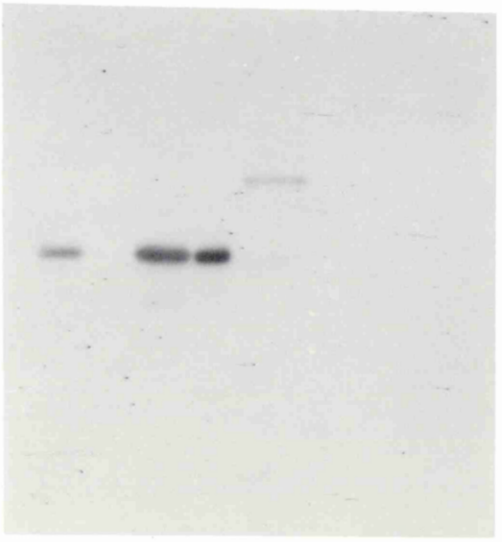
(−)

← M_r 47,000

(+)

(1) (2) (3) (4) (5)

B



(−)

← M_r 47,000

(+)

(1) (2) (3) (4) (5)

CHAPTER FIVE

BIOSYNTHETIC STUDIES ON FUMARASE AND THE PHOSPHATE TRANSPORT PROTEIN

5.1 INTRODUCTION

A detailed account of the present understanding of the mechanism(s) of biosynthesis of nuclear-encoded mitochondrial proteins was given in section 1.3. The vast majority of knowledge gained in this field has come from biosynthetic studies carried out in the lower eukaryotes S.cerevisiae and N.crassa. In vitro translational systems and in vivo studies employing pulse-labelled cells have shown that the overall mechanism of import, from the synthesis of the precursor in the cell cytoplasm to its subsequent uptake and translocation into the correct mitochondrial compartment, occurs as summarized in table 1.3.2.

Attention in higher eukaryotic systems has largely focused on the synthesis and import of nuclear-encoded mitochondrial proteins utilising in vitro translational systems. Some work has, however, been performed in vivo including that of Mori et al. (1981) who studied the synthesis of the urea cycle enzymes, ornithine transcarbamylase and carbamoyl phosphate synthetase in isolated rat liver hepatocytes. Primary rat liver cell cultures were used in these studies rather than an established rat liver cell line because the urea cycle enzymes are lost on prolonged incubation of hepatocytes in culture. They were able to confirm the previous results in yeast and N.crassa in that these two proteins were made as larger M_r precursors. Little research has been performed in vivo employing cultured mammalian cells, although the work that has been carried out includes that of Mihara et al. (1982) who studied the mechanism of synthesis of L-glutamate dehydrogenase and D-β-hydroxybutyrate dehydrogenase. Again, these workers were able to demonstrate that these two proteins were initially synthesised as larger M_r precursors.

As a result of the lack of in vivo data employing established cell lines to study the biosynthesis of mitochondrial proteins in higher

eukaryotic cells, I decided to concentrate on the synthesis of nuclear-encoded mitochondrial proteins in cultured mammalian cells. In addition, the in vivo data in higher eukaryotes has concentrated on the mechanism of synthesis and import of soluble mitochondrial proteins and has not investigated the biosynthesis of integral membrane proteins. By utilising pulse-labelled cultured mammalian cells, I hoped to gain a clearer insight into the mechanism of synthesis of mitochondrial proteins destined for two different locations within the organelle, in order to support the earlier findings in yeast and N.crassa.

In particular, the enzyme fumarase was of interest as it is not only found in the mitochondrial matrix, where it functions in the tri-carboxylic acid cycle, but also because it is located in the cell cytoplasm of most tissues (see section 1.3.1). The bimodal distribution of this enzyme presents an interesting problem in that several potential modes of synthesis can be envisaged to occur which would result in this type of distribution (discussed in detail in section 5.4.2). Polyclonal, monospecific rabbit antisera raised against this protein, which was fully characterized by the methods described in the previous chapter, was used as the specific probe to follow the biosynthesis of the enzyme in pulse-labelled cultured mammalian cells.

I was also interested in studying the biosynthesis of the mitochondrial integral, inner membrane phosphate transport protein in a similar system to the above. This carrier protein is likely to be transmembraneous as has been suggested for the extensively studied adenine nucleotide translocase of the mitochondrial inner membrane. Neupert and co-workers (1979, 1980) have demonstrated both in vitro and in vivo that the N.crassa adenine nucleotide translocator is synthesised in the cytoplasm as a protein of similar M_r to the mature polypeptide. I was, therefore, interested in determining whether the phosphate transport protein was synthesised in a

similar fashion. However, as reported in section 4.3.2, immune-replica analysis of the polyclonal, rabbit antiserum directed against the rat liver phosphate transport protein, showed that the antiserum cross-reacted with several polypeptides when challenged with crude mammalian tissue-culture cell extracts. As a result of this finding, biosynthetic studies were not pursued in cultured mammalian cells, although as will be shown in section 5.3.4, the antiserum did cross-react specifically with yeast extracts prepared from the rho$^-$ mutant strain D273-10B-1. A detailed account of the synthetic studies performed in the yeast cells will be given in section 5.4.3.

5.2 METHODS

5.2.1 L-[^{35}S]-Methionine Labelling of Cultured Mammalian Cells

The procedure listed below was generally used for incorporation of L-[^{35}S]-methionine into cellular protein in BRL, PK-15 and NBL-1 cell lines, respectively. Variations on its theme will be discussed in the appropriate section.

Initially, cells (3 x 10^5 cells/ml) were grown in plastic Petri dishes in normal growth medium (see section 2.4.1) by incubating overnight in a humified incubator at 37^{o}C containing a 5% CO_2/95% air atmosphere. The original cell suspension was prepared by the method described in section 2.4.2 and cell numbers determined by using a haemocytometer. Normal growth medium was then discarded, cell monolayers washed once with low methionine medium (1 in 20 dilution of normal growth medium with (-) methionine medium) prior to incubation for 2h at 37^{o}C in this medium. At this stage 5-200μCi [^{35}S]-methionine (1100 Ci/mmol) was added to the dish to initiate radiolabelli of cell protein. After appropriate incubation times (see legends to Figs. and Tables), the medium was removed, cells washed 3X with PBS (see section

2.3.2.2) to remove soluble counts and lysed with either 0.3-1.0ml of a 1% (v/v) Triton X-100 buffer (see section 2.3.2.2) or 1ml of 2% (w/v) SDS, 10mM Tris-HCl pH 8.8 buffer. At this point three different strategies were adopted to process the cell extract further, depending on the nature of the experiment;

i) if cells had been labelled for immune-precipitation studies, the Triton X-100 extract was processed according to the procedure described in section 2.3.2.2;

ii) if cells were labelled for optimizing growth conditions (see section 5.3.1), the extract was treated in the following manner;
a 25µl aliquot (total vol. 300µl) was spotted onto Whatman 3MM filter discs and washed extensively overnight in 10% (w/v) trichloroacetic acid (TCA) at 4°C, TCA was removed, the discs washed once for 20 min with ethanol and then air-dried. Finally, the dried discs were placed in scintillation vials and counted by adding 4.5ml of scintillation fluid, as previously described (see section 2.3.2.2);

iii) if cells had been labelled with [^{35}S]-methionine in the presence of inhibitors of protein synthesis (see section 5.3.2), after solubilization in 2% (w/v) SDS, 10mM Tris-HCl pH 8.8 buffer, they were treated with 4 vol. of acetone to precipitate protein and subsequently prepared for SDS-polyacrylamide gel electrophoresis (see section 2.2.4.2).

5.2.2 Accumulation of Mitochondrial Precursor Proteins in Yeast Cells

The yeast rho$^-$ mutant strain D273-10B-1 was routinely grown at 21°C on a rich medium containing 1% (w/v) yeast extract, 2% (w/v) Bacto-peptone and 0.1M potassium phosphate buffer pH 6.0.

To accumulate large amounts of cytoplasmically-made mitochondrial precursor proteins, the mutant strain D273-10B-1 was grown at 21°C on rich

medium supplemented with 2% (w/v) galactose. On reaching the mid-log phase of growth, the culture was divided into six equal aliquots and CCCP added to five of them at final concentrations of 1,2,5,10 and 20μM, respectively. A sixth fraction to which no CCCP had been added, acted as a control culture. The cells were grown for a further 7.5h at 21°C, after which time they were cooled and harvested at 4°C by centrifugation at 1500g for 10 min. The pellets were washed twice in 0.1M potassium phosphate buffer containing 10μM CCCP, the uncoupler being added to prevent processing of the precursor proteins. After washing, the cells were resuspended in 8ml of 0.1M potassium phosphate buffer pH 6.0 to which was added 2.0ml 100% (w/v) TCA, to precipitate the protein. Subsequently, the protein was collected by centrifugation at 1500g for 10 min and the pellets were resuspended in 50mM Tris-HCl pH 7.0 containing 10% (w/v) SDS and 2mM EDTA. If necessary, the pH was adjusted to 7.0 with 1M Tris-base prior to boiling the samples at 100°C for 15 min. Insoluble material was removed by centrifugation at 1500g for 10 min. Occasionally, this extraction procedure was repeated to ensure that protein solubilization was complete. The solubilized protein was precipitated by the addition of 4 vol. of acetone before resuspending in Laemmli sample buffer minus 2-mercaptoethanol and pyronin y, to enable protein estimations to be carried out (see section 2.2.1). After the amount of protein had been determined, the samples were prepared for SDS-polyacrylamide gels (see section 2.2.4.2) and subsequent analysis by the immune-replica technique.

Finally, yeast mitochondria were prepared from the wild-type strain D273-10B (α; ATCC 25657) by the method of Daum et al. (1982) and analysed by SDS-polyacrylamide gel electrophoresis (see section 2.2.4.1) and immune-blotting (2.3.2.1).

5.3 RESULTS

5.3.1 Optimization of Growth Conditions

For studies on the biosynthesis of nuclear-encoded mitochondrial proteins in cultured mammalian cells, the growth conditions had to be optimized initially to ensure maximal rate of incorporation of [^{35}S]-methionine into total cellular proteins. BRL cells were utilised with regard to these studies although the optimal conditions obtained were directly applicable to PK-15 and NBL-1 cells. Detailed descriptions of the labelling procedures are given in the respective legends to Figs. and Tables.

The initial radiolabelling experiments were performed in (-) methionine (-) serum growth medium, as this medium does not contain any free non-radioactive methionine. The cells are, however, likely to contain internal pools of non-radioactive methionine which will reduce the specific activity of [^{35}S]-methionine within the cells. It was necessary to optimize the incubation time in this medium prior to the initiation of incorporation of [^{35}S]-methionine into total cellular protein, so that the levels of the endogenous cellular methionine pools would be depleted to enhance the rate of incorporation of [^{35}S]-methionine. The cells would be expected to contain intracellular pools of methionine since they can synthesise this amino acid from cysteine or it can be generated from protein turnover. Several dishes were cultured in which the cells were pre-incubated in this labelling medium for various times before the addition of isotope and subsequent incorporation for 30 min at 37^{o}C (Fig. 5.3.1). The rate of incorporation of [^{35}S]-methionine increased linearly up to 2h pre-incubation, thereafter decreasing slowly. The reduction in the rate of incorporation of radioisotope upon prolonged incubation in this medium is likely to be due to a deterioration in the condition of the cells in the absence of externally-added methionine. Whilst the level of incorporation of [^{35}S]-methionine into BRL cellular protein was sufficient for use in the future immune-

precipitation studies, the above experiment did not demonstrate whether the rate of protein synthesis was affected by the omission of non-radioactive methionine from the external medium. As a result, it was necessary to investigate the effects of varying the concentration of non-radioactive methionine in the external growth medium, on the extent of incorporation of [^{35}S]-methionine into cellular protein.

Table 5.3.1 illustrates the effect of varying the composition of growth medium on the extent of incorporation of [^{35}S]-methionine into BRL cellular protein, during a 30 min labelling period. This experiment was performed in order to determine the optimal growth medium in which the specific activity of the intracellular radioactive pools was greatly enhanced without limiting drastically the rate of incorporation of [^{35}S]-methionine into cell protein. No significant difference in the incorporation of radioisotope into cellular protein was found when either (-) methionine or low methionine containing serum was used. The levels of free methionine in these media are very similar and, therefore, as a result one would expect to obtain the same extent of incorporation of [^{35}S]-methionine. When the cells were labelled in either of these media minus serum, however, the levels of incorporation of [^{35}S]-methionine into cellular protein were also very similar. The following explanations can be given to explain this finding since the methionine concentrations in these media vary by about 1000-fold with respect to each other (see table 5.3.1);

either (i) methionine is rate-limiting in the (-) methionine (-) serum medium such that the rate of incorporation of radioisotope is dramatically reduced,

or (ii) there is a high endogenous intracellular pool of methionine in the cells which tends to reduce the effective specific activity of added

[^{35}S]-methionine to a similar degree in these respective media.

When cells were labelled in (+) methionine growth medium, a marked decrease in the extent of incorporation of [^{35}S]-methionine was found. The free methionine concentration in this medium was greatly increased (about 7-fold) with respect to (-) methionine and low methionine media plus serum. As a result, the endogenous methionine pools would be expected to be larger, reducing the effective concentration of [^{35}S]-methionine and thus lowering the rate of incorporation of the isotope. Additionally, this finding confirms that the levels of methionine in the (-) methionine and low methionine media plus serum are not rate-limiting, since there is a comparable increase in the extent of incorporation of [^{35}S]-methionine into BRL cellular protein with a reduction in the free methionine concentrations of these media. The presence or absence of serum in the growth medium did not appear to have a significant effect on the incorporation of the radioisotope into total cell protein.

Fig. 5.3.2 illustrates the extent of incorporation of [^{35}S]-methionine into BRL cellular protein at different times up to 24h. The cells were labelled in both low methionine plus serum medium and (-) methionine plus serum medium, respectively. It can clearly be seen that there is a linear increase in the incorporation of [^{35}S]-methionine up to about 12h in both media, at which point there is no further net incorporation of [^{35}S]-methionine. The parallel increase in the rate of incorporation of radioactive isotope in the two media confirmed the previous data (table 5.3.1). It should be noted that by 12h, 80-100% of the label was incorporated into protein.

In summary, the biosynthetic labelling studies, as a result of the findings in this section, were performed in low methionine plus serum medium.

5.3.2 Optimization of Conditions for Accumulating Precursor Polypeptides

In section 1.3.5.1 a detailed account was given of the energy requirements involved in the import of cytoplasmically-synthesised mitochondrial proteins. It was shown that a membrane potential must exist across the mitochondrial inner membrane for successful transfer of proteins destined for the inner membrane or matrix. Depletion of the membrane potential by the use of uncouplers of oxidative phosphorylation has been utilised to accumulate the extramitochondrial precursor forms of many proteins destined for location within this organelle.

Initially, conditions had to be established whereby import would be blocked by uncoupler without dramatically inhibiting cellular protein synthesis. To follow the biological activity of uncoupler in vivo employing BRL cells, the effect of uncoupler on the synthesis of proteins encoded by the mitochondrial genome was studied. Mitochondrially-encoded protein synthesis can be measured by labelling cells with [^{35}S]-methionine in the presence of cycloheximide (1mg/1ml), an inhibitor of cytoplasmic protein synthesis in eukaryotic cells.

After extraction of radio-labelled proteins, as described in section 5.2.1 (iii), SDS-polyacrylamide gel electrophoresis 10% (w/v) was performed to resolve the proteins (see section 2.2.4.1), which were subsequently detected by fluorography (see section 2.2.4.6). In the presence of cycloheximide alone (Figs. 5.3.3a, b; tracks 5 and 6 respectively), four to six polypeptide bands could be visualized which ranged in M_r from 42,000 to 23,000. Early studies on the synthesis of proteins encoded by the mitochondrial genome (reviewed by Mason and Schatz, 1974) in which yeast cells were labelled with [^{3}H]-leucine in the presence of cycloheximide, also demonstrated that six polypeptides could be resolved by SDS-polyacrylamide gel electrophoresis. The identity of some of these proteins was provided by immune-precipitation of specific components from mitochondrial

extracts prepared from cells labelled in the above manner. Mason and Schatz (1973) utilising this type of approach were able to show that the three largest subunits of the cytochrome c oxidase complex are synthesised by the mitochondrial translation system. In conjunction with the yeast findings described above and the M_r values of the proteins reported from the recent DNA sequence data of the human mitochondrial genome (Anderson et al., 1981), the BRL cell bands were assigned a function (see table 5.3.2).

The effects of the uncouplers CCCP and FCCP on mitochondrial protein synthesis, at final concentrations of 20μM and 10μM respectively, are illustrated in Fig. 5.3.3a, tracks 1 and 2. It can clearly be seen that at these levels CCCP had little or no effect on the synthesis of mitochondrially-encoded proteins whereas 10μM FCCP completely inhibited protein synthesis. This result suggests that FCCP is the more efficient uncoupler of oxidative phosphorylation, under the conditions employed in this experiment. Subsequently, it was decided to test the effects of a series of concentrations of FCCP on mitochondrial protein synthesis, in order to find the optimum level of this uncoupler to utilise in future biosynthetic studies (Fig. 5.3.3b, tracks 1 and 2). No inhibitory effect was found when 1μM FCCP was used (track 1), whereas 10μM FCCP (track 2) completely abolished mitochondrial protein synthesis, in agreement with the previous result. In addition, although not illustrated here, 3μM FCCP was tested and shown not to inhibit protein synthesis. As a control, to confirm that the uncoupler was inhibiting the synthesis of mitochondrially-encoded proteins, BRL cells were labelled with [^{35}S]-methionine in the presence of cycloheximide (1mg/ml) and chloramphenicol (300μg/ml) (Fig. 5.3.3a, track 6). No polypeptide bands could be visualized after treating cultured BRL cells in this manner. This finding supported the view that FCCP was exerting its biological function, under the conditions employed, by inhibiting synthesis of mitochondrially-encoded polypeptides.

As a result of these observations, the effect of the uncoupler, FCCP (10μM) on the incorporation of [^{35}S]-methionine into total cell protein was investigated, over a 4h incubation time-course (Fig. 5.3.4). After 30 min, inhibition of cellular protein synthesis was about 50-70% and remained at this level up to 4h incubation, in the presence of this uncoupler.

A second potential method for studying extramitochondrial precursor polypeptides is to inhibit the activity of the matrix-located processing protease (see section 1.3.5.2). Raymond and Shore (1981) demonstrated that treatment of rat liver explants with the protease inhibitor, p-aminobenzamidine (1mM), interfered with the normal processing of the precursor to carbamyl phosphate synthetase although no precursor accumulation in the cytosol took place. In addition, they showed that p-aminobenzamidine had no effect on protein synthesis. When BRL cells were labelled with [^{35}S]-methionine in the presence of cycloheximide (1mg/ml) and p-aminobenzamidine (0.1mM and 1mM respectively), mitochondrial protein synthesis was markedly inhibited at the higher concentration (Fig. 5.3.3a, track 4; Fig. 5.3.3b, tracks 3 and 4). Additionally, p-aminobenzamidine (1mM) dramatically interfered with total cellular protein synthesis. As a result of these findings, p-aminobenzamidine was no longer utilized to study mitochondrial protein precursors.

Another reported inhibitor of the rat liver matrix-located processing protease, leupeptin was investigated in a similar manner to the above experiment. Although leupeptin, at a final concentration of 2mM, did not inhibit BRL mitochondrial protein synthesis, it was later discovered (Conboy et al., 1982) that this protease activity does not play an obligatory role in the maturation of mitochondrial precursor proteins of higher M_r value (discussed in detail in section 1.3.5.3).

In summary, precursor accumulation experiments were carried out by labelling cultured mammalian cells with [^{35}S]-methionine, in the presence

of 10μM FCCP. It was assumed also that the overall rate of synthesis of individual proteins was inhibited to the same extent by the presence of uncoupler in that there was no preferential inhibitory effects on particular protein(s).

5.3.3 Mechanism of Synthesis of Fumarase in Mammalian Culture Cells

In the preceding chapter (see section 4.3.3), the greatest degree of cross-reactivity of antiserum raised against pig heart fumarase with mammalian culture cell extracts, was observed in PK-15 and NBL-1 cells. Preliminary biosynthetic labelling experiments were performed with these cell lines, in order to establish the mechanism of synthesis of fumarase, which has been shown to be bimodally distributed in most tissues (Akiba et al., 1984). The M_r of the mitochondrial and cytosolic fumarases have been determined in rat liver and both shown to be about 49,000. In order to determine the nature of the precursor form of this enzyme, import into mitochondria can be blocked by utilizing the uncoupler, FCCP (see section 5.3.2). FCCP acts by dissipating the electrochemical potential across the mitochondrial inner membrane, a requirement for efficient uptake of most cytoplasmically-synthesised precursor polypeptides.

Initially, however, PK-15 and NBL-1 cells were labelled overnight with 100μCi [^{35}S]-methionine, in the absence of uncoupler, after which time a soluble cell extract was prepared (see section 2.3.2.2) for analysis by immune-precipitation (see section 2.3.2.3) and detection by fluorography (see section 2.2.4.6). This experiment was performed in order to illustrate that the newly-synthesised enzyme could be recognised by the antiserum. The anti-fumarase serum specifically precipitated a single polypeptide of M_r 47,000 from both cell lines (Fig. 5.3.5, tracks 2, 3, 6 and 7). The M_r value obtained is in agreement with the value of N-[^{3}H]-ethylmaleimide labelled fumarase (track 1) which was co-electrophoresed to aid the identification of immune-precipitated protein. In addition, the findings support the

immune-replica data, which illustrates that the mitochondrial and cytosolic forms of the enzyme are of the same M_r (see Fig. 4.3.7). To confirm that the polypeptide band of M_r 47,000 is specifically recognised by fumarase antiserum, a control immune-precipitation was performed with pre-immune serum (tracks 4 and 5).

Subsequently, precursor experiments were performed in PK-15 cells by labelling with [^{35}S]-methionine for 4h in the absence or presence of FCCP. In the absence of uncoupler and in agreement with the previous data (Fig. 5.3.5), a polypeptide band of M_r 47,000 can be visualized which corresponds to both the mature mitochondrial and cytosolic forms of enzyme (Fig. 5.3.6, track 5). When the cells were labelled with [^{35}S]-methionine in the presence of 10μM FCCP, however, not only could this polypeptide band be seen but also a protein of higher M_r (track 3). The M_r of this precursor form (51,000) was estimated to be about 4,000 larger than the mature protein. It is assumed that the large M_r protein is the precursor to mitochondrial fumarase whereas the polypeptide of similar M_r to the mature enzyme represents cytosolic fumarase.

Finally, the above experiment was essentially repeated in the BRL cell line. Although the antiserum raised against the pig heart enzyme cross-reacted weakly with BRL fumarase, it had previously been shown that the cytosolic and mitochondrial fumarases were of a different M_r (47,000 and 45,000 respectively). In the absence of FCCP, two polypeptides could be visualized on the fluorograph with M_r values of 47,000 and 45,000 respectively (Fig. 5.3.7, track 2). This result, therefore, confirmed the immune-replica data and illustrated that these two different M_r fumarase forms existed <u>in vivo</u>. In contrast to PK-15 cells, addition of 10μM FCCP not only blocked import of mitochondrial fumarase but also appeared to inhibit the synthesis of both fumarase forms (track 4). As a result, the level of uncoupler was reduced to 5μM and the cells labelled with

[^{35}S]-methionine for 4h. A single protein component of M_r 47,000 was resolved by SDS-polyacrylamide gel electrophoresis, under these labelling conditions (track 3). The protein band is likely to be composed of cytosolic fumarase as well as the high M_r precursor form of mitochondrially-located fumarase.

The potential mechanisms of synthesis of this enzyme arising from these findings are discussed in detail in section 5.4.2.

5.3.4 Precursor Accumulation Studies in Yeast Cells

In 1982(a), Reid and Schatz reported that the yeast rho$^-$ mutant strain D273-10B-1 could be utilized to accumulate large amounts of precursors to mitochondrial polypeptides. Incubation of this yeast strain with uncoupling concentrations of CCCP for 7.5h in which cell growth was allowed to continue, enabled the precursors to be detected directly by the immune-replica technique.

Fig. 5.3.8 illustrates the effect of CCCP on the growth of the rho$^-$ mutant of S.cerevisiae. The turbidity of the cell suspension was measured after 7.5h of growth in the absence or presence of increasing levels of uncoupler and the resulting absorbance at 540nm plotted against uncoupler concentration. It can be seen that with increasing uncoupler levels, growth is gradually inhibited and reaches a maximum of 50% with 20μM CCCP, in agreement with the findings of Reid and Schatz (1982a). The turbidity of the initial innoculum of each culture is also shown (A_{540nm} 0.15). Cell protein extracts were prepared from each culture, by the method described in section 5.2.2 and the proteins initially resolved by SDS-polyacrylamide gel electrophoresis (see section 2.2.4.1). After transferring the proteins to nitrocellulose paper, they were examined by the immune-replica technique with anti-fumarase and anti-phosphate transport protein sera, respectively.

It was assumed in these experiments that there was a constant amount of protein in each yeast cell, under the various inhibitory conditions employed.

Fig. 5.3.9(a) demonstrates the autoradiographic profile of these samples after treatment with anti-fumarase serum. In the absence of uncoupler (track 1), a polypeptide of M_r 41,000 can be visualized and it is assumed that this band corresponds to the mature fumarase in yeast. On addition of the uncoupler, CCCP (1-20μM) this band disappears gradually and a polypeptide of higher M_r (46,000) appears in a parallel fashion (tracks 2 and 6). The autoradiograph was subsequently scanned with an Ultroscan 2202 laser densitometer to determine the percentage areas of the mature and precursor polypeptides. The percentage areas of mature protein obtained were then plotted against uncoupler concentration (Fig. 5.3.9b). The amount of mature fumarase found in cells grown in the absence of CCCP is expressed as 100%. In the presence of 1-5μM CCCP, the proportion of mature enzyme decreases rapidly, until it reaches a minimum level at 5μM CCCP. This point is indicative of an optimization of precursor accumulating conditions where there is a maximum inhibition of processing of precursor without dramatically affecting protein synthesis. At higher concentrations of CCCP (10 and 20μM), protein synthesis is reduced and as a consequence cell growth is inhibited more markedly (up to 50%). Although the amount of precursor per g of total cell protein will be the same, the reduction in cell growth results in an increase in the proportion of mature polypeptide assuming that the mature protein is stable during the period of growth (Fig. 5.3.9b). The above result was confirmed when a similar analysis of these yeast extracts was performed with anti-pyruvate dehydrogenase complex serum. The E_2 subunit of this enzyme was shown to be made as a larger M_r precursor and the relative proportion of mature E_2, in the presence of increasing uncoupler, closely resembled that obtained for

fumarase. It has recently been shown (Hiraga et al., 1984) that yeast cells like most mammalian cells contain both cytosolic and mitochondrial fumarase activity, although the M_r values of these species are not known. In addition the distribution of activity between cytosol and mitochondria appears to vary markedly with growth conditions. It was not possible from the above blots to establish in detail, the mechanism of synthesis of these two forms.

On examination of yeast extracts with phosphate transport protein antiserum, a specific band of M_r 29,000 could be detected at each concentration of uncoupler (Fig. 5.3.10a). However, in contrast to the enzyme fumarase, no higher M_r precursor appeared with increasing uncoupler concentration. When the percentage area of mature phosphate transport protein was calculated and plotted against uncoupler concentration (Fig. 5.3.9b), there was little or no decrease in the proportion of the mature polypeptides. This result suggests that the phosphate transport protein is synthesised in the presence of uncoupler, as a protein of similar M_r to the mature transporter. If a larger M_r precursor was being synthesised in the presence of uncoupler, but was relatively unstable, one would have expected to obtain a similar result to that for fumarase and the E_2 subunit of pyruvate dehydrogenase complex.

Finally, one of the problems to arise from the findings with the yeast blots was to confirm the identity of the cross-reacting polypeptides, which in both instances differed in M_r value from the corresponding mammalian proteins. In the case of the phosphate transport protein, the identity of the polypeptide band of M_r 29,000 was partially provided by an experiment in which mitochondria were isolated from yeast cells (see section 5.3.3) and analysed by the immune-replica technique (see section 2.3.2.1). The antiserum raised against the rat liver mitochondrial transporter, cross-reacted very strongly with a polypeptide of M_r 29,000, confirming that

this protein was of a mitochondrial origin.

5.4 DISCUSSION

5.4.1 Optimization of Conditions for Studying Mitochondrial Precursors

The existence of precursor polypeptides to nuclear-encoded mitochondrial proteins has been demonstrated in several laboratories adopting one of the following experimental approaches;

i) *in vitro* translational systems employing either cell-free homogenates or rabbit reticulocyte lysates programmed with specific mRNA. In the absence of mitochondria, only the cytoplasmic, precursor forms are synthesised;

ii) *in vivo* pulse-labelling experiments where intact cells are labelled with [^{35}S]-methionine for very short times. In this case, both precursor and mature polypeptides are detected, unless cells are fractionated into cytosol and mitochondria, respectively.

One of the problems with these experimental approaches is that very small quantities of precursor are synthesised, enabling their detection to be quite difficult at times. As a result of this, a third approach which generally enhances precursor accumulation *in vivo* has been utilised, whereby the import mechanism is interfered with directly. This type of approach assumes that there is not a feedback mechanism in operation whereby accumulation of precursor molecules in the cytosol represses the expression of nuclear-encoded mitochondrial proteins. In addition, this experimental approach relies on (a) the accumulated precursor being stable on prolonged incubation in the presence of an inhibitor of import and (b) the antiserum raised against the mature enzyme recognising the precursor form of the

protein.

Import of proteins destined for the mitochondrial inner membrane or matrix requires the presence of a membrane potential across the inner membrane Although the actual role played by the membrane potential in import is not fully understood, its importance has been demonstrated by utilising mitochondria isolated from N.crassa (Schleyer et al., 1982), rat (Schleyer et al., 1982; Kolansky et al., 1982) and yeast (Gasser et al., 1982a) in the in vitro import assay, described in section 1.3.5.1. By using specific inhibitors of the respiratory chain, of the F_1F_0-ATPase and ionophores, it was possible to maintain a high concentration of matrix ATP while depleting the electrochemical gradient across the inner membrane. Under these conditions, mitochondria were no longer able to efficiently import and process precursor proteins. A detailed account of these experiments can be found in section 1.3.5.1.

Several groups have utilised this approach in vivo, whereby import and processing were blocked by interfering with the membrane potential, but translation allowed to continue (Nelson and Schatz, 1979; Reid and Schatz, 1982a). The most commonly used compound to block import by this type of mechanism is the uncoupler, CCCP. Uncouplers act by making the mitochondrial inner membrane permeable to protons and thus dissipate the electrochemical potential, which is essential for the transfer of proteins across the inner mitochondrial membrane. In yeast, at least, if translocation into mitochondria is blocked but translation allowed to continue, the precursor pools are large. However, it should be noted also that there is considerable variation in the stability of precursor molecules accumulated in the presence of uncoupler. Pre-cytochrome c_1 (Reid and Schatz, 1982a) is rapidly degraded whereas the precursor to the β-subunit of the yeast F_1-ATPase is not only stable, but also retains the ability to be translocated into the mitochondria for at least 30 min after synthesis

(Reid and Schatz, 1982a).

Prior to performing the biosynthetic studies in cultured mammalian cells, conditions had to be established where addition of uncoupler would block import of proteins without dramatically interfering with protein synthesis. The rationale behind these experiments was that by inhibiting the generation of an electrochemical potential across the mitochondrial inner membrane, intra-mitochondrial ATP synthesis would also be inhibited. Since mitochondrially-encoded proteins require ATP for their synthesis, at optimum levels of uncoupler, one would expect to see an inhibition in the expression of these gene products. By labelling cells in the presence of cycloheximide, a potent inhibitor of eukaryotic protein synthesis and consequently cytoplasmic protein synthesis in cultured mammalian cells, it was possible to follow the expression of the mitochondrial genome products, in an isolated manner.

In the presence of cycloheximide alone, six polypeptide bands were resolved by SDS-polyacrylamide gel electrophoresis 10% (w/v) and detected by fluorography. DNA sequence data from three mammalian mitochondrial genomes (Anderson et al., 1981, Bibb et al., 1981; Anderson et al., 1982) has predicted that 13 different reading frames exist, each capable of synthesising a functional protein. The M_r values of these 13 different reading frames, as predicted from the human mitochondrial DNA sequence (Anderson et al., 1981), range from 66,000 to 7,500. In contrast to the findings reported in this study, Attardi and Ching (1979) using SDS-urea-polyacrylamide gel electrophoresis 15% (w/v) were able to resolve a significantly larger number of these polypeptide bands. The following reasons can be put forward to explain the discrepancy in the number of bands resolved on a 10% (w/v) SDS-polyacrylamide gel;

i) the M_r values of four of the mammalian mitochondrial DNA products

are less than 20,000, which on the above gel system are likely to migrate to the dye front;

ii) variability in the number of bands may also be due in part to differences in the rate of synthesis and/or metabolic stability of different polypeptides;

When BRL cells were labelled with [^{35}S]-methionine in the presence of cycloheximide and CCCP, at levels of this uncoupler utilised to accumulate precursors in yeast (Reid and Schatz, 1982a), no inhibition in the synthesis of mitochondrially-encoded polypeptides was found. However, the fluoro-derivative FCCP was shown to be an effective inhibitor of mitochondrial translation at a concentration of 10μM. Additionally, it was illustrated that total cell protein synthesis was inhibited by about 50%, in agreement with the findings of several other groups (Reid and Schatz, 1982a; Fenton et al., 1984).

Another potential method for accumulating extramitochondrial precursor proteins is to inhibit the activity of the matrix-located processing protease, which plays a major role in the maturation of larger M_r precursor forms. It is assumed in this type of inhibition that as long as no feedback mechanism is in operation, events prior to processing will occur normally. In 1981, Raymond and Shore investigated the effect of the addition of the protease inhibitor, *p*-aminobenzamide on the processing of the precursor to the mitochondrial matrix enzyme, carbamyl phosphate synthetase. The experiments were performed in rat liver explants and it was shown that the normal processing of the precursor was inhibited, in that no mature enzyme could be found in the mitochondria. In addition, however, no precursor could be detected in the mitochondrion and no accumulation of precursor in the cytoplasm was found when compared to cells labelled for 30 min in the absence of *p*-aminobenzamidine. It was suggested from these results that

the precursor to carbamyl phosphate synthetase was rapidly degraded, in a non-specific manner, upon reaching the blocked processing protease in the mitochondrial matrix. Since the stability of different precursor molecules has been shown to vary markedly under accumulating conditions (Reid and Schatz, 1982a), this type of mechanism of inhibition was pursued.

Initially, as with the uncoupler FCCP, the effect of *p*-aminobenzamidine on both mitochondrial and total cellular protein synthesis was investigated. In this case, one would not expect to see any direct effects on protein synthesis. It was shown, however, that at a final concentration of 1mM and prolonged incubation time that *p*-aminobenzamidine interfered dramatically with the expression of both mitochondrially and nuclearly-encoded proteins. Since *p*-aminobenzamidine has a similar structure to the amino acid arginine, it is possible that these effects on protein synthesis are due to arginine and *p*-aminobenzamidine competing for the same site on the tRNA synthetase, specific for arginine.

The biosynthetic studies performed to determine whether fumarase was initially made as a large M_r precursor or not, were carried out in cultured mammalian cells in the presence of the uncoupler FCCP.

5.4.2 Mechanism of Synthesis of Fumarase in Cultured Mammalian Cells

In 1976, Nakashima et al. discovered that the tricarboxylic acid cycle enzyme, fumarase, is not solely located in the mitochondrial matrix but can also be found in the cell cytoplasm of rat liver. Subsequently a detailed study of the structural and physical properties of the two rat liver fumarase activities was achieved by Kobayashi et al. (1981) and is summarized in table 1.5.1. The mitochondrial and cytosolic fumarases could not be separated from one another by their physicochemical, catalytic and immunological properties. The M_r values of the two enzymes were shown

to be identical by SDS-polyacrylamide gel electrophoresis (M_r 49,000). Recently, Akiba et al. (1984) discovered that fumarase is bimodally distributed between the mitochondrial matrix and cell cytoplasm of most tissues. In addition to fumarase, malate dehydrogenase and aspartate aminotransferase are compartmentalized in the cell cytosol and mitochondria. However, in contrast to fumarase, the two enzymic forms of these proteins are distinguishable from each other in terms of their physiochemical and immunological properties. The bimodal distribution of these proteins raises the question as to their mechanism of biosynthesis.

Extensive studies on the synthesis of the aspartate aminotransferases (Sonderegger et al., 1982), using an in vitro translation system and non-cross-reacting antibodies, demonstrated that the cytosolic form was made as a protein of same M_r as the mature enzyme. The mitochondrial matrix isoenzyme was, however, synthesised as a precursor of higher M_r. The synthesis of a high M_r precursor to the matrix-located aspartate amino-transferase is expected, since the majority of matrix proteins have been shown to be synthesised in this manner (see Table 1.3.1). The differences in the properties of these two forms along with the biosynthetic findings suggest that the mitochondrial and cytosolic aspartate aminotransferase isoenzymes are encoded by two separate genes. Similarly, the malate dehydrogenase isoenzymes have been shown to be encoded by separate genes (Van Heyningen et al., 1975). Mihara et al. (1982) illustrated that mitochondrial malate dehydrogenase was synthesised as a protein of higher M_r when translation was performed in a wheat germ cell-free system programmed with rat liver RNA.

In this chapter, preliminary data to determine the mechanism of synthesis of fumarase was obtained by the in vivo labelling of cultured PK-15 and BRL cells with [^{35}S]-methionine. Information as to the nature of the biosynthetic intermediates of the mitochondrial and cytosolic enzyme

forms was obtained by blocking import of proteins into mitochondria. As discussed in the preceding section (see 5.4.2), this can be achieved by the use of uncouplers of oxidative phosphorylation which act by dissipating the membrane potential across the mitochondrial inner membrane, a pre-requisite for import. When PK-15 cells were labelled with [^{35}S]-methionine in the absence of uncoupler, a single specific protein was immune-precipitated which had an M_r value of 47,000 corresponding to both the mature mitochondrial and cytosolic fumarases. On addition of 10μM FCCP, two specific polypeptide bands were immune-precipitated with anti-fumarase serum (see Fig. 5.3.6). A protein of 47,000 M_r and one of 51,000 M_r could be detected under these conditions. The polypeptide of 47,000 M_r, synthesised in the presence of 10μM FCCP, can be assumed to be the cytosolic form of fumarase, since its mode of synthesis should not be unduly affected by uncoupler. On the other hand, the high M_r polypeptide produced by blocking import into mitochondria, is likely to be the cytoplasmic precursor form of the mitochondrially-located fumarase. Upon import into mitochondria, this precursor would be expected to be cleaved at its amino-terminal end by the matrix-located protease (McAda and Douglas 1982) to generate mature mitochondrial fumarase (M_r 47,000).

The above data is in agreement with the recently published findings of Ono et al. (1985) who studied the biosynthesis of fumarase in vitro employing a rabbit reticulocyte lysate programmed with total rat liver RNA. They were able to demonstrate the existence of two different putative precursor polypeptides to rat liver fumarase in this system. One of the polypeptides was synthesised as a precursor with a larger M_r (50,000) than the mature subunit of fumarase (M_r 45,000) whereas the other protein had the same M_r as the mature enzyme. Further evidence as to the identity of these proteins was provided by showing that the addition of 10μg of purified cytosolic fumarase competitively inhibited their

immune-precipitation. In addition, Ono et al. (1985) were able to perform in vitro import studies in which the [^{35}S]-labelled cell-free translation products were incubated with isolated rat liver mitochondria at 30°C. Under these conditions, they illustrated that only the high M_r precursor fumarase could bind to and subsequently be imported into mitochondria. From their findings, Ono et al.. (1985) have suggested that the large M_r precursor represents the cytosolic polypeptide of mitochondrial fumarase and the protein of similar M_r is the mature, cytoplasmic fumarase.

In marked contrast to the report of Ono et al. (1985) and the findings with PK-15 cells, when the BRL cell line was preliminary investigated with regard to the biosynthesis of fumarase, a different result was found. Two polypeptides of differing M_r could be detected on the fluorograph (see Fig. 5.3.7), in the absence of uncoupler. The demonstration that two different M_r forms of fumarase existed in BRL cells, under normal conditions, was a novel discovery but did support the earlier immune-replica data (see Fig. 4.3.6). It was shown in those experiments that the BRL mitochondrial and cytosolic fumarases differed in M_r, the mitochondrial form being of a lower M_r than cytoplasmic fumarase. On addition of FCCP (5μM), a single specific polypeptide component (M_r 47,000) could be resolved by SDS-polyacrylamide gel electrophoresis 10% (w/v). This polypeptide had the same M_r as the cytoplasmic enzyme. One interpretation of this result is that the cytoplasmically-located fumarase in BRL cells is the precursor form of the mitochondrial enzyme which arises due to the slow uptake of the fumarase precursor. Alternatively, the cytoplasmic precursor to mitochondrially-located fumarase is of a similar M_r to the cytosolically-located enzyme and is, therefore, hard to resolve on above gel system.

Although both PK-15 and BRL total cell protein synthesis is only inhibited by about 50% in the presence of 10μM FCCP, there were conflicting findings when immune-precipitation performed with anti-fumarase serum. It was

originally assumed that the effects of FCCP on protein synthesis were general and did not affect specific proteins. In BRL cells, however, no detectable fumarase could be resolved following SDS-polyacrylamide gel electrophoresis whereas in PK-15 cells two proteins specifically recognised by antiserum. These findings suggest that the synthesis of BRL cell fumarases is more susceptible to the addition of uncoupler than the PK-15 cellular fumarases.

As a result of the in vivo biosynthetic studies in PK-15 and BRL cells, how does fumarase become distributed between the two intracellular compartments, cytosol and mitochondria? Two potential modes of synthesis can be considered;

i) the mitochondrial and cytoplasmic fumarase activities are encoded by two separate nuclear genes. In the PK-15 cell line the nuclear gene for the mitochondrially-located enzyme contains additional information to encode the amino-terminal signal sequence. On the other hand, in BRL cells the genes for mitochondrial and cytosolic fumarases have the same coding capacity but the mitochondrial gene encodes an amino-terminal sequence that can be recognised and cleaved by the matrix-located protease (McAda and Douglas 1982).

As stated earlier in this section, the existence of separate genes for the two forms of malate dehydrogenase has been shown (Van Heyningen et al. 1975) and a similar mechanism has been suggested for the aspartate aminotransferases (Sonderegger et al., 1982). In both instances the two isoenzyme forms have been shown to differ markedly from one another with respect to their physiochemical and immunological properties. However, in the case of rat liver fumarases, Kobayashi et al. (1981) clearly demonstrated that these properties were very similar and the proteins in fact had the same M_r. It was also recently reported (Hiraga et al.,

1984) that rabbit IgG raised against the mitochondrial fumarase of rat liver could equivalently neutralize the fumarase activities in the cytosolic and mitochondrial extracts. Further evidence to rule out this mechanism of synthesis of fumarase has come from somatic-cell hybridization studies (Tolley and Craig, 1975; McKusick and Ruddle, 1976). Analysis of the segregation of human fumarase in human-mouse hybrids demonstrated the existence of one gene responsible for the synthesis of both mitochondrial and cytosolic fumarase. From the last few statements, it would appear that this type of mechanism of synthesis of bimodally distributed fumarase is unlikely and a more plausible explanation is as follows:

ii) the mitochondrial and cytosolic fumarases are encoded by a single gene which can generate two distinct mRNA species by the mechanism known as 'alternative splicing'. Many eukaryotic genes have been shown to be comprised of mosaic structures in which intervening sequences(introns) are interspersed between protein coding regions (exons). Intervening sequences appear in the primary transcript product but are removed by splicing during mRNA maturation. The generation of two mRNA species from a single gene has been shown for the synthesis of immunoglobulin heavy chain polypeptides (reviewed by Wall, 1980). The earliest immunoglobulin to appear is of the IgM subclass and this molecule can exist either in a membrane-bound or secretory form. Amino-terminal protein sequences of the two forms are identical although their carboxyl terminal sequences are quite different. The mu (μ) mRNAs appear to be generated from a single heavy chain transcription unit by alternative patterns of RNA splicing. It has been proposed that developmentally regulated polyadenylation determine which mRNA will be produced.

Another example of 'alternative splicing' occurs with the mouse amylase protein which is synthesised in the liver and salivary gland of

mouse and is encoded by a single gene. The mRNAs present in each tissue are identical in their coding region but differ in the first part of the 5' nontranslated leader (Young et al., 1981). There are two separate exons for the 5' nontranslated leaders which vary in length. One of the leader exons is spliced on to the coding region exon depending on the tissue.

The generation of two fumarase activities by this type of mechanism has been proposed by Ono et al. (1985) from their findings on the synthesis of rat liver fumarase. It has recently been illustrated (Kobayshi et al., 1983), that the carboxy termini of the rat liver fumarases are identical although their amino-termini differ in sequence (Sato et al., 1984). From these findings and those in PK-15 cells it can be assumed that there are two separate exons for the amino-terminal regions of the two enzyme forms. It can be envisaged that these exons were derived from a common ancestral gene by gene duplication. They have subsequently evolved independently such that one codes for the amino-terminus of cytosolic fumarase and the other for mitochondrial fumarase. 'Alternative splicing' brings the amino-terminal sequences together with the constant coding region containing the carboxyl-terminal sequence. In addition, the mitochondrial amino-terminal exon must contain a sequence which is recognised by the matrix protease, so that the mature mitochondrial enzyme is of the same M_r as the cytosolic form.

In the case of BRL cells, it can be assumed that the two amino-terminal region exons have evolved in such a manner that the mitochondrial exon again contains information to enable this region to be recognised by the matrix-located protease. The fact that in this cell line the cytoplasmically located fumarase is of a higher M_r than the mitochondrial protein, would be due to its amino-terminal exon being of a similar size to that of the mitochondrial amino-terminal exon, without containing the protease

recognition site (see Fig. 5.4.1).

In BRL cells, a third mechanism of synthesis can be proposed whereby only one mRNA species is generated from one gene for fumarase. In this situation, the bimodal distribution of the enzyme may arise due to the slow uptake of the precursor into mitochondria, such that a steady-state equilibrium of the enzyme is established between the cytosol and mitochondria.

5.4.3 Precursor Studies in Yeast Cells

A more direct approach to study the biosynthesis of fumarase in vivo is to follow the experimental procedure, described by Reid and Schatz (1982a). They reported that the respiration-deficient yeast rho^- mutant strain D273-10B-1 could be utilised to accumulate large amounts of precursors to mitochondrial polypeptides. The rho^- mutants have been shown to lack cytochromes aa_3, b and c, as well as a functional ATPase complex (reviewed by Mason and Schatz, 1974). This arises because these mutants have lost large segments or, in some cases, all of their mitochondrial DNA and, as a result, they all seem to have lost a functional mitochondrial protein synthesising system. The proteins still present in these defective mitochondria are, therefore, made by the cytoplasmic protein synthesising system. These proteins include the enzymes of the tricarboxylic acid cycle, cytochromes c and b_2 and the mitochondrial F_1-ATPase subunits. In addition, the rho^- mutant mitochondria possess an apparently normal outer membrane and a distinct inner membrane which does not differ greatly from that in wild-type mitochondria. In 1979 Nelson and Schatz demonstrated that rho^- mutant strain of S.cerevisiae could import cytoplasmically-synthesised polypeptides into their mitochondria, even although their mitochondria lacked a functional ATPase complex as well as a respiratory chain. Since an electrochemical potential must exist across mitochondrial

inner membrane for import to occur (Gasser et al., 1982a), this finding suggested that rho$^-$ mutants generate a modest potential, perhaps via the adenine nucleotide translocator (Klingenberg and Rottenberg, 1977).

Reid and Schatz (1982a) chose the rho$^-$ mutant strain in preference to wild-type yeast because exploratory experiments indicated that its mitochondrial import system was more sensitive to CCCP than that of wild-type yeast cells. The yeast cells were grown on a fermentable carbon source, galactose, since they do not have an operational respiratory chain. As a result, ATP is generated by substrate level phosphorylation reactions in the glycolytic pathway. This ATP can be partially used to maintain a membrane potential across the mitochondrial inner membrane, so that cytoplasmically-synthesised polypeptides can be efficiently imported into the organelle. Reid and Schatz (1982a) showed that long term growth of cells, in the presence of CCCP, was only inhibited by about 50%, a result confirmed in this study (see Fig. 5.3.8). Therefore, by adding CCCP to an early exponential phase culture of respiration-deficient rho$^-$ yeast cells and allowing growth to continue for 7.5h precursors should accumulate outside the mitochondria. Reid and Schatz (1982a) demonstrated that the precursors to the β-subunit of F_1-ATPase and cytochrome c_1, could be detected directly by the immune-replica technique, following extraction of yeast proteins, as described in section 5.2.2. This method of analysis of mitochondrial precursor polypeptides has the obvious advantage over conventional methods, described in sections 5.3.3 and 5.4.3, in that it rules out the need for immune-precipitation and thus is less time consuming.

A similar series of experiments was performed in this study, employing antisera raised against pig heart fumarase and rat liver mitochondrial phosphate transport protein. A pre-requisite for these studies is that the antisera raised against the mammalian proteins will cross-react with

the yeast proteins. Preliminary experiments with fumarase antiserum, indicate that a larger M_r precursor polypeptide is generated with increasing uncoupler concentrations and there is a simultaneous decrease in the proportion of mature polypeptide (Fig. 5.3.9a). The precursor has an M_r value (46,000) which is about 5,000 greater than the mature polypeptide (M_r 41,000). One of the problems with this result is that the specifically cross-reacting polypeptides have M_r values which are lower than the pig heart counterpart. Although the chromatographic properties of Baker's yeast mitochondrial and cytosolic fumarases have been studied (Hiraga et al., 1984), no estimation of the M_r values of the two yeast fumarases has been published.

If it is assumed that the mature yeast fumarase has an M_r value of 41,000 and that the mitochondrial and cytosolic forms are of the same M_r, then the simplest interpretation of immune-replica data is as follows; the precursor polypeptide that is being synthesised in the presence of the uncoupler CCCP is the cytoplasmic pre-form of the mitochondrial enzyme. However, because of the nature of the experiment, it is not possible to establish whether cytosolic form of the enzyme is being synthesised as a larger M_r precursor or as a polypeptide of the same M_r as the mature sized enzyme. In these experiments one is not solely looking at newly-synthesised proteins but also at pre-existing polypeptides. As a result, you would expect to see mature fumarase at all levels of CCCP, unless there is a very rapid turnover rate for the protein. The immune-replica data partially confirms the biosynthetic data obtained from PK-15 cells (discussed in section 5.4.3), in that a precursor polypeptide of higher M_r value can be detected when yeast cells are incubated in the presence of CCCP.

Interestingly, the immune-replica data obtained with the phosphate transport protein antiserum, in an analogous experiment to the above, reveals a strongly cross-reacting polypeptide of M_r 29,000 (Fig. 5.3.10a).

On addition of uncoupler (1-20μM), there is no sign of a higher M_r precursor form to this protein and the relative proportion of mature phosphate transport protein varies little with increasing CCCP (Fig. 5.3.9b). This latter result suggests that in the presence of uncoupler, precursor to this protein is being synthesised which has the same M_r value as the mature polypeptide. Although this data has to be treated with caution because the mammalian antiserum is cross-reacting with a yeast protein of lower M_r, the result conforms to the findings of Neupert and coworkers (1979, 1980). They studied the biosynthesis of the N.crassa ADP/ATP translocator both in vitro and in vivo and showed that the precursor polypeptide was of the same M_r as the mature protein. Since both transporters are likely to be highly hydrophobic, transmembrane proteins, it may be expected that their biosynthetic pathways follow parallel routes. The preliminary findings with the yeast rho^- mutant strain, indicates that this may be the case and that this may also be the general mode of synthesis of mitochondrial, inner transmembraneous proteins.

Confirmation as to the identity of the cross-reacting yeast protein was suggested by performing an immune-replica analysis of purified yeast mitochondria with the rat liver mitochondrial phosphate transport protein antiserum. In this case, a very strong polypeptide band of M_r 29,000 was recognised by the antiserum, confirming at least the mitochondrial location of this protein (see Fig. 5.3.10b).

A major dilemma to arise from the yeast immune-replica data is that the antiserum raised against the mammalian protein appears to be very specific in its recognition properties, in conflict with the previous data (see section 4.3.2). When crude mammalian culture cell extracts were challenged with this antiserum, numerous additional polypeptides could be visualized on the autoradiograph. Why does the antiserum react so specifically with yeast phosphate transport protein? One possibility

is that the yeast phosphate transport protein does not have the same tendency as its rat liver counterpart to aggregate with itself or other proteins when boiled in SDS. As a result, there would be a reduction in the degree of non-specific binding to these aggregates, which was previously proposed to be a potential source of these additional polypeptide bands (see section 4.4.2).

Fig. 5.3.1

THE EFFECT OF PRE-INCUBATION TIME IN (-) METHIONINE (-) SERUM MEDIUM ON THE INCORPORATION OF [^{35}S]-METHIONINE INTO BRL CELL PROTEIN

BRL cells (3 x 10^5 cells/ml) were initially incubated overnight at 37^{o}C in 5.5cm (diameter) plastic Petri dishes containing 3ml of normal growth medium (see section 2.4.1). The cells were subsequently transferred into (-) methionine (-) serum medium and pre-incubated for 0,1,2,4,5 and 24h, respectively. After the appropriate pre-incubation time in the above medium, incorporation of the radioactive amino acid was initiated by the addition of 20µCi [^{35}S]-methionine and continued for 30 min at 37^{o}C. To measure the extent of incorporation of radioisotope into cellular protein following termination of the labelling reaction, the procedure described in section 5.2.1 (ii), was closely followed.

Dishes were labelled in duplicate and a single aliquot counted from each sample.

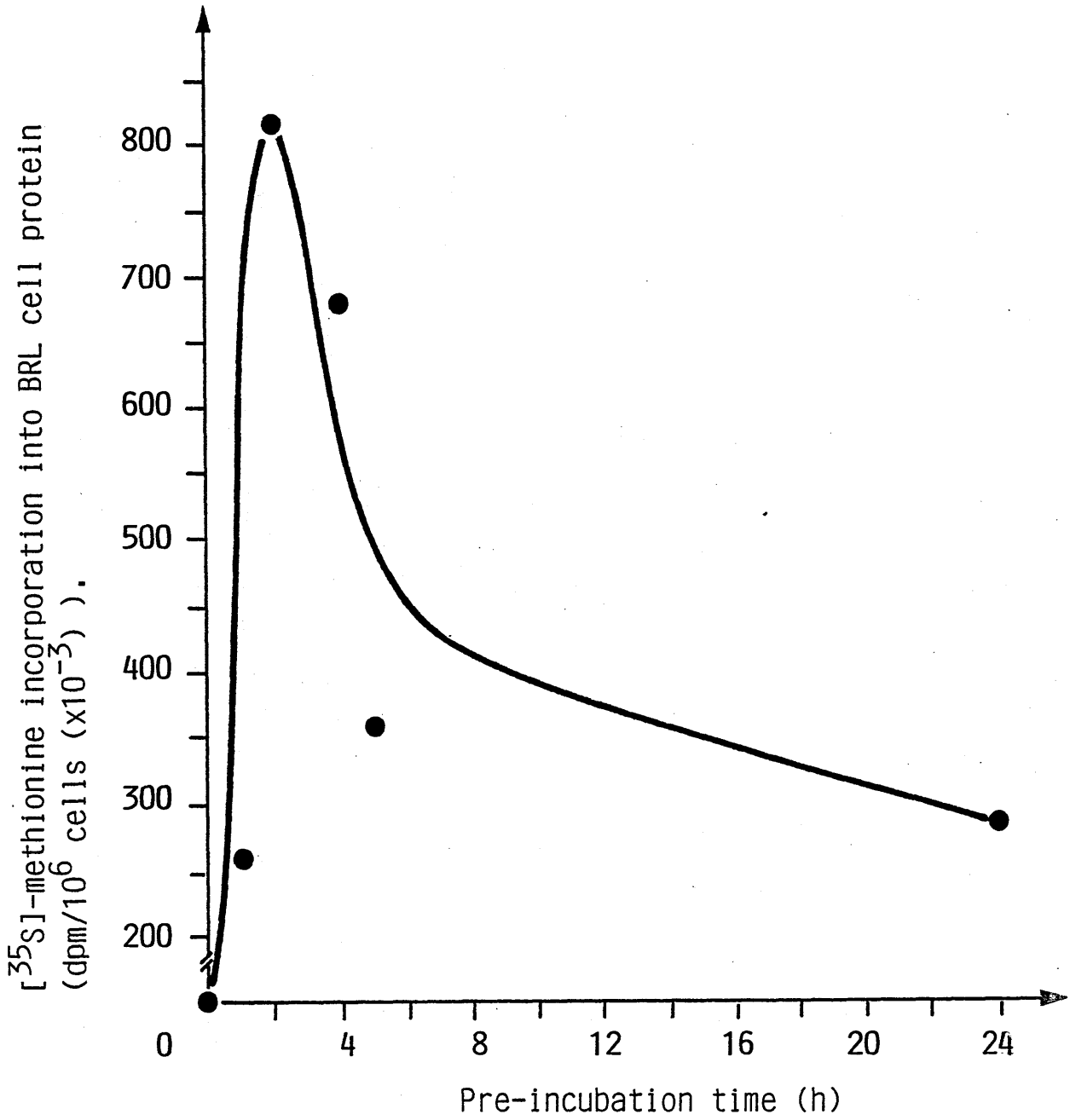

[35S]-methionine incorporation into BRL cell protein
(dpm/10^6 cells (x10^-3)).
800
700
600
500
400
300
200
0
4
8
12
16
20
24
Pre-incubation time (h)

Table 5.3.1

THE EFFECT OF THE COMPOSITION OF LABELLING MEDIUM ON THE INCORPORATION OF [^{35}S]-METHIONINE INTO TOTAL BRL CELL PROTEIN

Cells (3×10^5 cells(ml) were incubated overnight in 3.5cm (diameter) plastic Petri dishes at 37°C (see section 2.4.1), before being transferred into 1ml of various media (listed in table opposite). After a further 1.5h pre-incubation at 37°C, radiolabelling was initiated by the addition of 20µCi [^{35}S]-methionine and continued for 30 min. Incorporation of radioisotope was then terminated, a cell extract prepared and the extent of incorporation of [^{35}S]-methionine into BRL cell protein determined by the procedures described in section 5.2.1 (ii).

Dishes were labelled in duplicate and two equal aliquots (25µl) taken from each sample. [^{35}S]-methionine incorporation into BRL cell protein was subsequently calculated from four different samples.

Growth Medium	Methionine conc. in labelling medium (μM)	Average incorporation of [^{35}S]-methionine into Cellular Protein (dpm/10^5 cells)
(-) methionine (-) serum	0.018	189,290 ± 5,069
(-) methionine (+) serum	1.5	198,545 ± 5,441
low methionine (-) serum	0.5	162,540 ± 5,076
low methionine (+) serum	2.0	171,666 ± 5,620
(+) methionine (-) serum	10	25,465 ± 2,284
(+) methionine (+) serum	11.5	26,725 ± 2,394

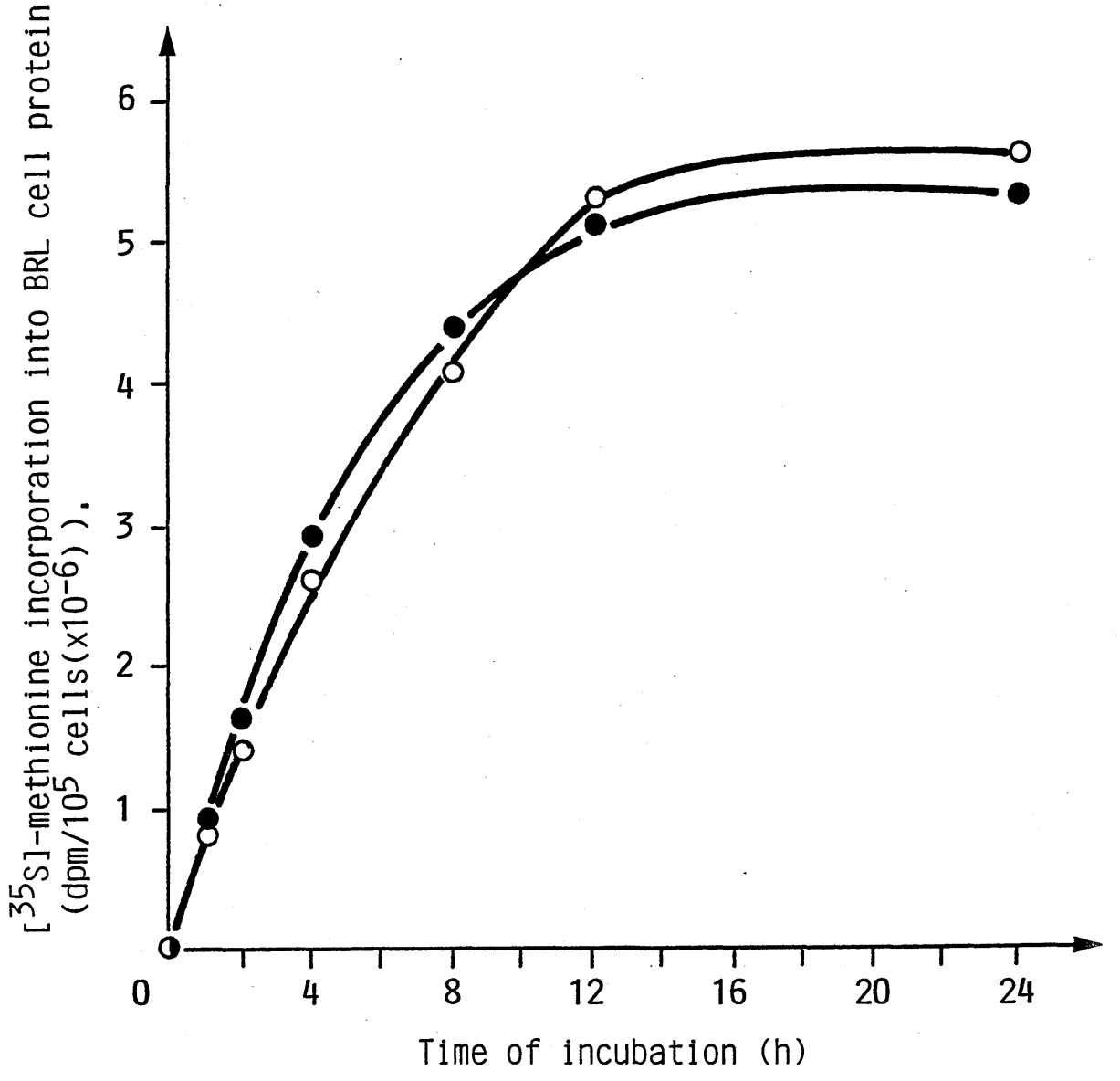
Fig. 5.3.2

TIME-COURSE TO MEASURE THE EXTENT OF INCORPORATION OF [^{35}S]-METHIONINE INTO BRL CELLULAR PROTEIN IN (-) METHIONINE AND LOW METHIONINE MEDIA

After an overnight incubation at 37^{o}C in 3.5cm (diameter) plastic Petri dishes containing 1ml normal growth medium (see section 2.4.1), BRL cells (3 x 10^{5} cells/ml) were transferred to (-) methionine or low methionine media, respectively and pre-incubated for 1.5h at 37^{o}C. 10μCi [^{35}S]-methionine was added to each dish (in duplicate) and incubated for 0,1,2,4,8,12 and 24h, respectively. At the indicated time points, radiolabelling was terminated by washing the dishes 3X with PBS (see section 2.3.2.2). Cell extracts were subsequently prepared (see section 5.2.1 ii) and the extent of incorporation of [^{35}S]-methionine into cellular protein measured as described in section 5.2.1.

●——● (-) methionine medium

o——o low methionine medium


[35S]-methionine incorporation into BRL cell protein
(dpm/10^5 cells(x10^-6)).
6
5
4
3
2
1
0
4
8
12
16
20
24
Time of incubation (h)

Figs. 5.3.3 (A) and (B)

A STUDY OF THE EFFECTS OF VARIOUS INHIBITORY COMPOUNDS ON THE SYNTHESIS OF MITOCHONDRIALLY-ENCODED PROTEINS

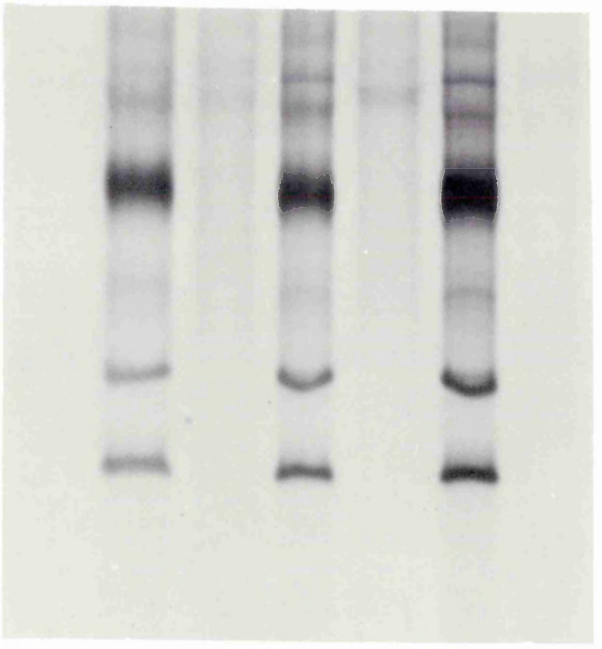
BRL cells (3×10^5 cells/ml) were initially incubated overnight at 37°C in 8.5cm (diameter) plastic Petri dishes containing 10ml of normal growth medium (see section 2.4.1). This medium was discarded and the cells placed in low methionine medium (see section 5.2.1) for 2h prior to the addition of various inhibitors of protein synthesis (see below). After a further pre-incubation of 30 min at 37°C, [^{35}S]-methionine was added to each dish (250µCi/dish) and the labelling reaction continued for 4h at 37°C. A cell extract was subsequently prepared, according to the method described in section 5.2.1 (iii), before resolving the proteins by SDS-polyacrylamide gel electrophoresis 10% (w/v) (see section 2.2.4.1) and detecting the radiolabelled polypeptides by fluorography (see section 2.2.4.6). In this case, polyacrylamide gels were cast between 14 x 8cm glass plates, in a perspex box designed to accommodate four pairs of plates:

A) (1) Cycloheximide (1mg/ml) + 20µM CCCP (2) Cycloheximide (1mg/ml) + 10µM FCCP (3) Cycloheximide (1mg/ml) + leupeptin (2mM) (4) Cycloheximide (1mg/ml) + p-aminobenzamidine (1mM) (5) Cycloheximide (1mg/ml) (6) Cycloheximide (1mg/ml) + chloramphenicol (300µg/ml).

B) (1) Cycloheximide (1mg/ml) + 1µM FCCP (2) Cycloheximide (1mg/ml) + 10µM FCCP (3) Cycloheximide (1mg/ml) + 0.1mM p-aminobenzamidine (4) Cycloheximide (1mg/ml) + 1mM p-aminobenzamidine (5) Cycloheximide + alcohol 1% (v/v) (6) Cycloheximide (1mg/ml).

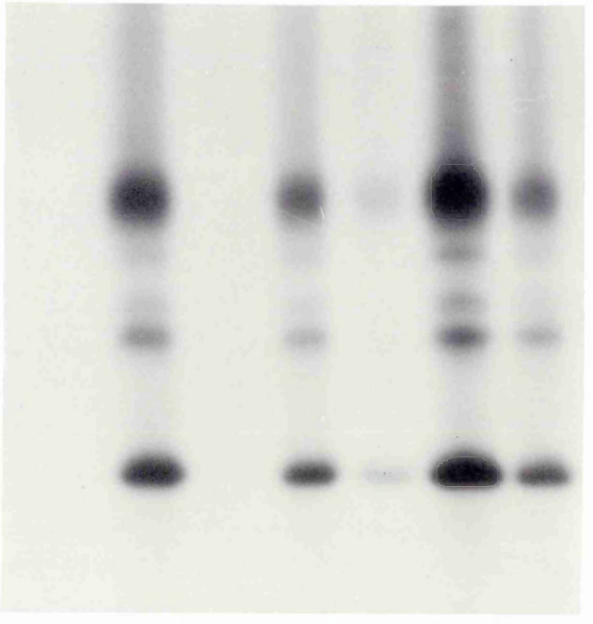
The fluorographs were exposed for one week at -80°C.

A



(1) (2) (3) (4) (5) (6)

B



(1) (2) (3) (4) (5) (6)

Table 5.3.2

FUNCTIONAL ASSIGNMENT OF MITOCHONDRIALLY-ENCODED BRL CELL PROTEINS

BRL proteins encoded by the separate mitochondrial genome were labelled with [^{35}S]-methionine in the presence of an inhibitor of eukaryotic cytoplasmic protein synthesis, cycloheximide (see Figs. 5.3.3 A and B). The M_r values of the [^{35}S]-methionine labelled polypeptides were estimated by the method described in section 2.2.4.3. The proteins were identified by comparing their M_r values with the values obtained from human mitochondrial DNA sequence and from the studies performed by Mason and Schatz (reviewed in 1974). See section 5.3.1 for further details of their experiments.

Polypeptide Band	M_r from BRL cells	Functional Assignment
I	42,000	Cyt b
II	36,000	URF I
III	30,000	CO III
IV	28,000	CO II
V	27,000	ATPase 6
VI	23,000	URF 6

Fig. 5.3.4

TIME-COURSE OF THE EFFECT OF 10μM FCCP ON THE INCORPORATION OF [^{35}S]-METHIONINE INTO TOTAL BRL CELL PROTEIN

Cells were initially incubated overnight at 37^{o}C in 5.5cm (diameter) plastic Petri dishes containing 3ml of a single cell suspension (3 x 10^{5} cells/ml) in normal growth medium (see section 2.4.1). This medium was subsequently discarded, the cells placed in low methionine medium (see section 5.2.1) and incubated for 1.5h at 37^{o}C. 5 min, prior to the addition of 10μCi [^{35}S]-methionine, FCCP (10μM) was added to duplicate dishes which were then labelled for 0.5,1,1.5,2 and 4h respectively at 37^{o}C. As a control, a parallel series of dishes were labelled with [^{35}S]-methionine, in the absence of uncoupler. Radiolabelling was terminated at the indicated times, a cell extract prepared and the extent of incorporation of [^{35}S]-methionine into total cellular protein determined by the methods described in section 5.2.1 (ii).

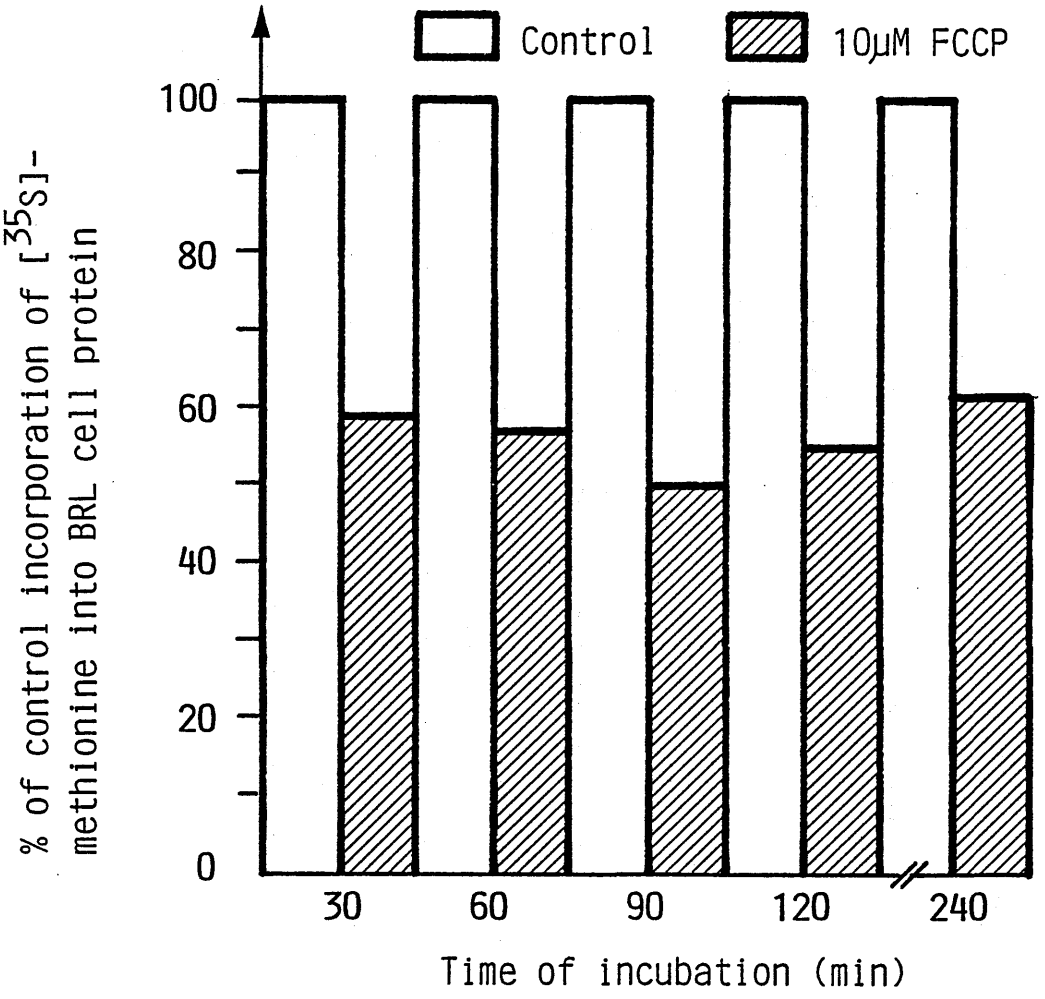

Control
10µM FCCP
100
80
60
40
20
0
30
60
90
120
240
% of control incorporation of [35S]-methionine into BRL cell protein
Time of incubation (min)

Fig. 5.3.5

IMMUNE-PRECIPITATION OF FUMARASE FROM PK-15 AND NBL-1 CELLS LABELLED OVERNIGHT WITH [^{35}S]-METHIONINE

Cells (3 x 10^6 cells/10ml) were grown overnight at 37^{o}C by incubating them in 8.5cm plastic Petri dishes containing normal growth medium (see section 2.4.1). In order to enable the immune-precipitation of mature fumarase, the cells were subsequently placed in low methionine medium containing serum (see section 5.2.1), pre-incubated for 1.5h at 37^{o}C then radiolabelled overnight by the addition of 100µCi [^{35}S]-methionine. Incorporation of the radioisotope was terminated by washing the cell monolayer 3X with PBS (see section 2.3.2.3) and a soluble cell extract prepared (see section 5.2.1 i) for subsequent immune-precipitation studies (see section 2.3.2.3).

The immune-precipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis 10% (w/v) (see section 2.2.4.1) and visualized by fluorography (see section 2.2.4.6); track (1) N-[^{3}H]-ethylmaleimide-labelled fumarase (2) and (3) mature fumarase from NBL-1 cells (4) and (5) pre-immune serum (6) and (7) mature fumarase from PK-15 cells.

The fluorographs were exposed for one week at -80^{o}C.

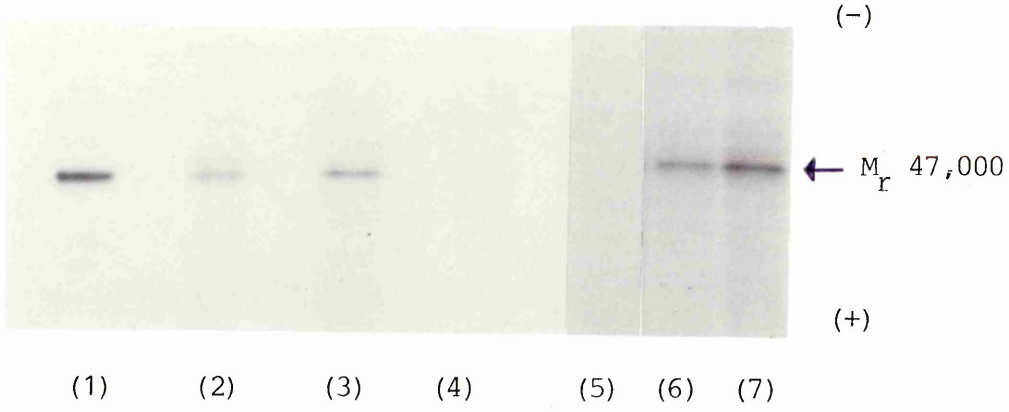

(−)
M_r 47,000
(+)
(1)
(2)
(3)
(4)
(5)
(6)
(7)

Fig. 5.3.6

BIOSYNTHESIS OF FUMARASE IN PK-15 CELLS LABELLED WITH [^{35}S]-METHIONINE IN THE PRESENCE OF AN INHIBITOR OF MITOCHONDRIAL IMPORT

PK-15 cells (3 x 10^6 cells/10ml) were initially grown overnight at 37^{o}C as described in the legend to Fig. 5.3.5. The cells were subsequently placed in low methionine medium containing serum (see section 5.2.1) for 1.5h at 37^{o}C, before the addition of FCCP (10μM). Radiolabelling of cellular proteins was initiated 5 min after the addition of uncoupler, by placing 200μCi [^{35}S]-methionine in the dish and continued for 4h. A separate dish was labelled in the absence of FCCP (10μM), to act as a control.

Soluble cell extracts, immune-precipitations, SDS-polyacrylamide gel electrophoresis and fluorography were subsequently performed as described in legend to Fig. 5.3.5; track (1) [^{125}I]-labelled low M_r standards (2) N-[^{3}H]-ethylmaleimide labelled fumarase (3) 4h label plus 10μM FCCP (4) pre-immune serum immune-precipitation (5) 4h label in the absence of uncoupler.

Tracks (1) to (4) were exposed to fluorography for three weeks at -80^{o}C; track (5) was exposed for three days at -80^{o}C.

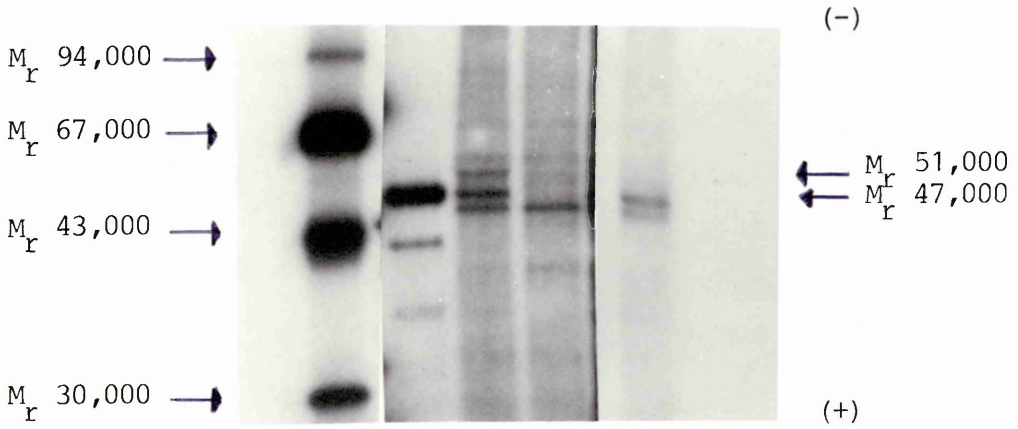

(-)
M_r 94,000
M_r 67,000
M_r 43,000
M_r 30,000
M_r 51,000
M_r 47,000
(+)
(1) (2) (3) (4) (5)

Fig. 5.3.7

BIOSYNTHESIS OF FUMARASE IN BRL CELLS LABELLED WITH [^{35}S]-METHIONINE IN THE PRESENCE OF AN INHIBITOR OF MITOCHONDRIAL IMPORT

BRL cells (3 x 10^6 cells/ml) were labelled with [^{35}S]-methionine in the absence or presence of FCCP (5 and 10μM), as described in the legend to Fig. 5.3.6 and following immune-precipitation (see section 2.3.2.3) were analysed by SDS-polyacrylamide gel electrophoresis (see section 2.2.4.1) and fluorography (see section 2.2.4.6).

Track (1) pre-immune serum (2) 4h label minus uncoupler (3) 4h label plus 5μM FCCP (4) 4h label plus 10μM FCCP (5) N-[^{3}H]-ethylmaleimide labelled fumarase (6) [^{125}I]-labelled low M_r standards.

The fluorograph was exposed for one week at -80°C.

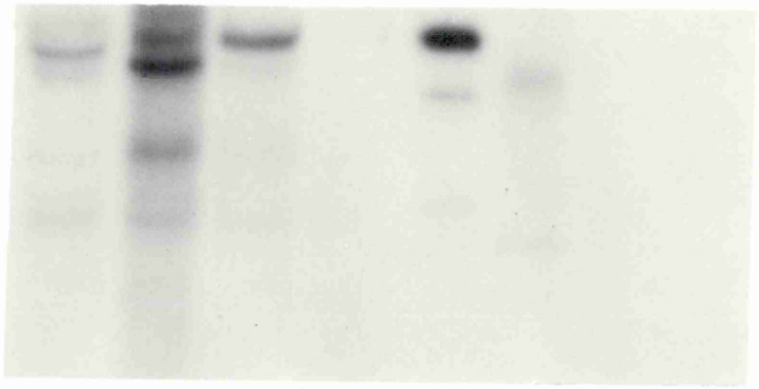

(-)
M_r 47,000
M_r 45,000
(+)
(1)
(2)
(3)
(4)
(5)
(6)

Fig. 5.3.8

EFFECT OF THE UNCOUPLER CCCP ON THE GROWTH OF THE RHO$^-$ MUTANT STRAIN OF S.CEREVISIAE

The yeast rho$^-$ mutant strain D273-10B-1 was grown at 21^{o}C on rich medium supplemented with galactose 2% (w/v) (see section 5.2.2). On reaching the mid-log phase of growth, the culture was divided into six equal aliquots and CCCP was added to five of them at final concentrations of 1,2,5,10 and 20μM, respectively. Growth was continued for a further 7.5h at 21^{o}C. The turbidity of each sub-culture was measured spectrophotometrically at a wavelength of 540nm, after this period of growth. A graph was subsequently plotted of A540nm versus concentration of CCCP.

●——● Growth curve in the presence of increasing CCCP concentration

o——o Turbidity of initial innoculum

Effect of the uncoupler CCCP on the growth of yeast rho$^-$ mutant

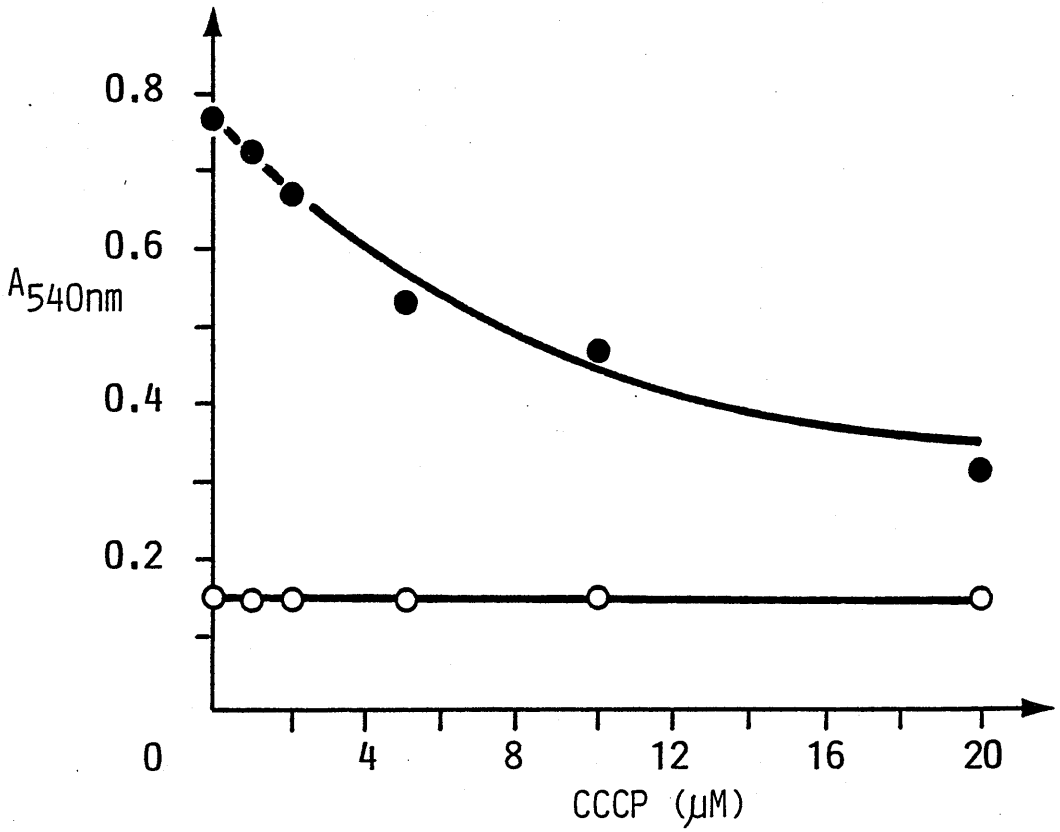


Fig. 5.3.9 (A) and (B)

A) IMMUNE-REPLICA ANALYSIS OF THE YEAST RHO$^-$ MUTANT STRAIN TREATED WITH INCREASING CONCENTRATIONS OF CCCP (FUMARASE ANTISERUM)

The rho$^-$ strain D273-10B-1 was grown in the absence or presence of CCCP as described in the legend to Fig. 5.3.8 and harvested 7.5h after addition of CCCP. TCA was added to 20% (w/v), the cells were broken and proteins were extracted with SDS as described in section 5.2.2. Aliquots of the cell extracts containing 40µg of protein each were subjected to SDS-polyacrylamide gel electrophoresis 10% (w/v) (see section 2.2.4.1) and subsequently transferred to nitrocellulose sheets for immune-replica analysis (see section 2.3.1) with fumarase antiserum. Lane (1) 0µM CCCP (2) 1µM CCCP (3) 2µM CCCP (4) 5µM CCCP (5) 10µM CCCP (6) 20µM CCCP (7) 0.2µg pig heart fumarase (8) [^{125}I]-labelled low M_r standards.

The autoradiogram was exposed for five days at -80°C.

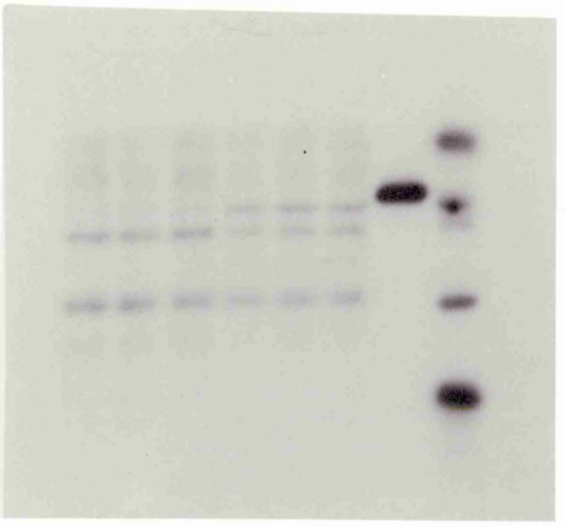
B) QUANTITATION OF PRECURSOR ACCUMULATION IN YEAST CELLS AFTER GROWTH IN THE PRESENCE OF CCCP

Determined the loss of mature fumarase and phosphate transport protein in rho$^-$ cells grown at various levels of CCCP by scanning the respective autoradiographs (see Figs. 5.3.9A and 5.3.10A) with an Ultroscan 2202 laser densitometer. The amount of mature protein found in cells grown in the absence of CCCP is expressed as 100%.

●——● Fumarase

o——o Phosphate transport protein

A


(-)
M_r 45,000
M_r 41,000
(+)
(1) (2) (3) (4) (5) (6) (7) (8)

B

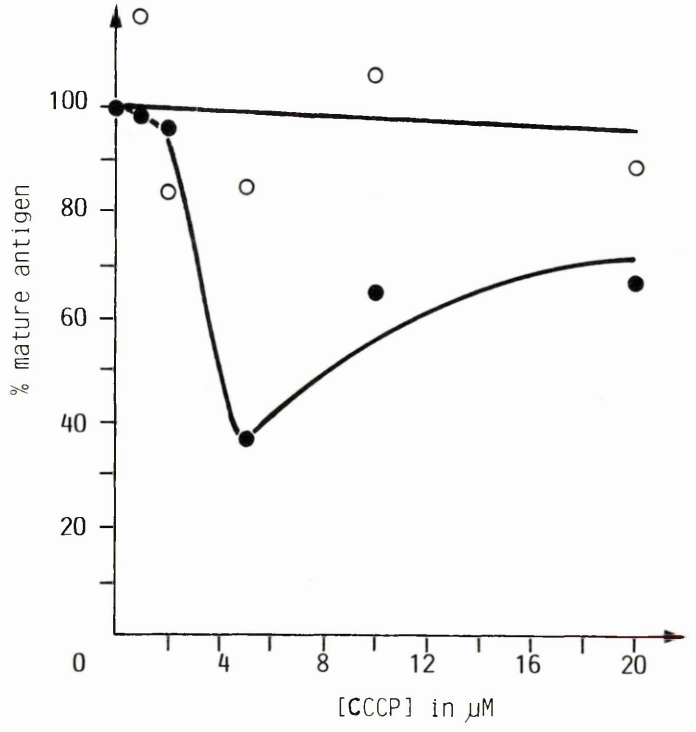

% mature antigen
100
80
60
40
20
0
4
8
12
16
20
[CCCP] in µM

Fig. 5.3.10 (A) and (B)

A) IMMUNE-REPLICA ANALYSIS OF THE YEAST RHO$^-$ MUTANT STRAIN TREATED WITH INCREASING CONCENTRATIONS OF CCCP (PHOSPHATE TRANSPORT PROTEIN ANTISERUM)

A similar procedure to that described in the legend to Fig. 5.3.9(A) was utilised to perform the above experiment, the only exception being that an antiserum directed against the rat liver mitochondrial phosphate transport protein was used for the immune-replica analysis.
Lanes (1) to (6) 0-2μM CCCP (7) Celite-purified rat liver mitochondrial phosphate transport protein (10μl) (8) [^{125}I]-labelled low M_r standards.

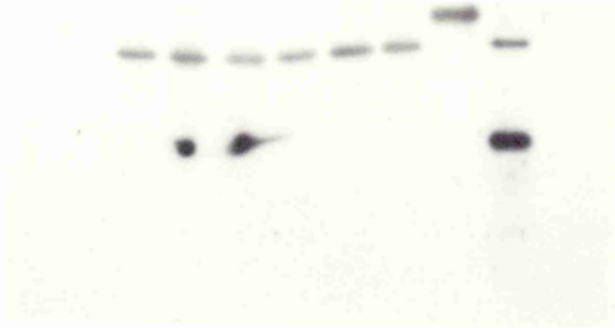
The autoradiogram was exposed for five days at -80°C.

B) IMMUNE-REPLICA ANALYSIS OF A YEAST MITOCHONDRIAL EXTRACT WITH PHOSPHATE TRANSPORT PROTEIN ANTISERUM

Yeast mitochondria were prepared from the wild-type strain D273-10B-1 (α, ATCC 25657) by the method of Daum et al. (1982) and analysed by SDS-polyacrylamide gel electrophoresis 10% (w/v) (see section 2.2.4.1) and immune-blotting with anti-phosphate transport protein serum (see section 2.3.1).
Lane (1) Celite-purified rat liver mitochondrial phosphate transport protein (10μl) (2) 80μg (3) 60μg (4) 40μg mitochondrial extract, respectively.

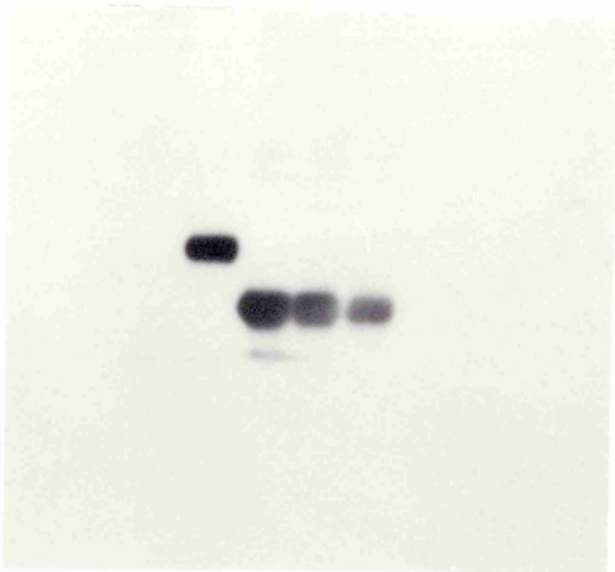
Autoradiography was performed for five days at -80°C.

A



(1) (2) (3) (4) (5) (6) (7) (8)

B



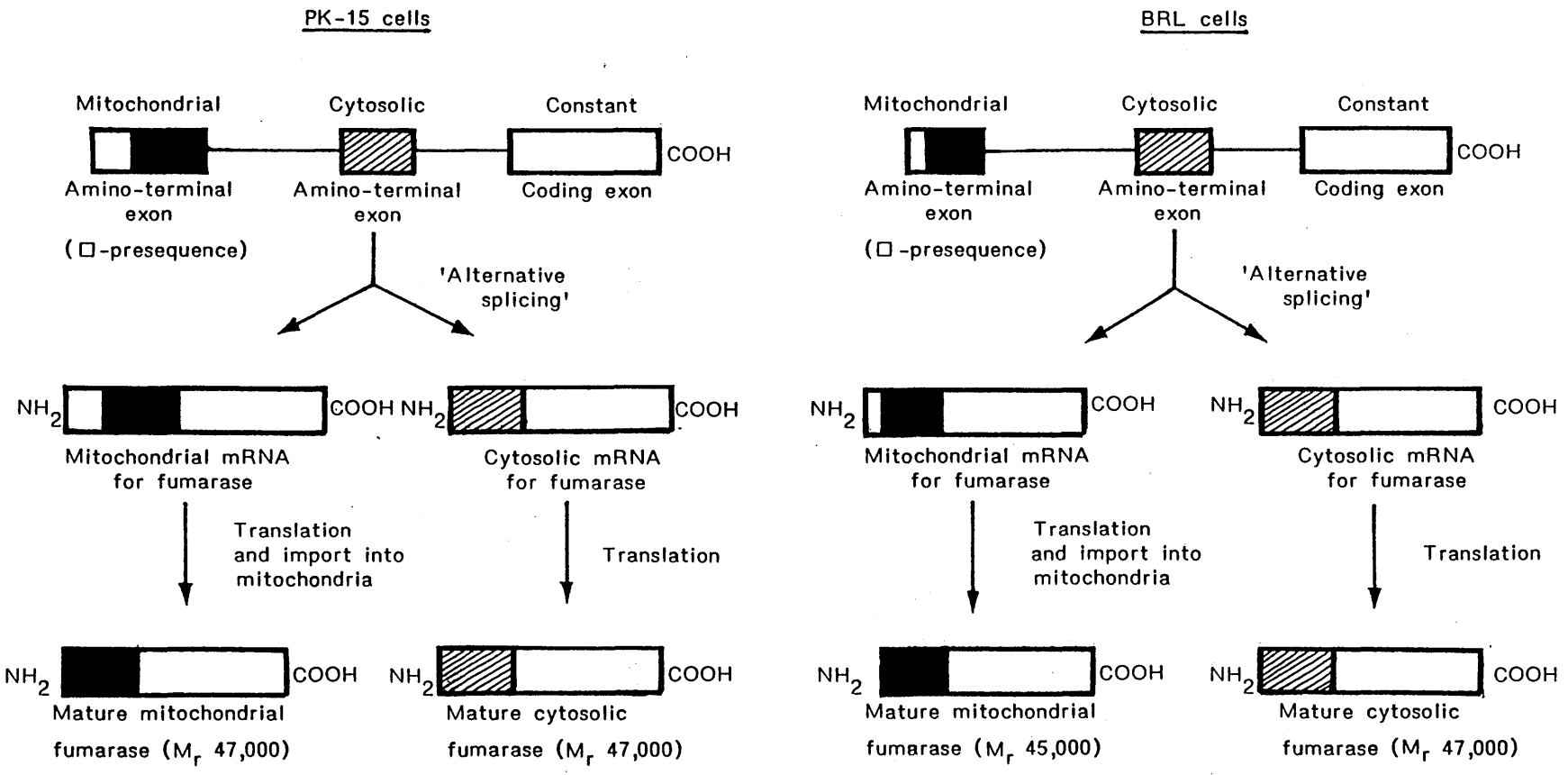
(1) (2) (3) (4)

Fig. 5.4.1

GENERATION OF TWO mRNA SPECIES ENCODING THE MITOCHONDRIAL AND CYTOSOLIC FORMS OF FUMARASE

The findings from the biosynthetic studies performed on the bimodally distributed fumarase in PK-15 and BRL cells, can be explained by the following model. This model proposes that in both cell lines the mitochondrial and cytosolic fumarases are generated from one gene by the mechanism of 'Alternative Splicing'. This result in the synthesis of two separate mRNAs encoding the mitochondrial and cytosolic forms of fumarase (see section 5.4.2 for further details).

Generation of two mRNA species encoding the mitochondrial and cytosolic forms of fumarase



CHAPTER SIX

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

The biogenesis of mitochondria requires an input from two genetic systems; the mitochondrial and nucleo-cytoplasmic systems. In the case of some of the mitochondrial inner membrane respiratory complexes, their synthesis is dependent on both genetic systems (see section 1.1). One such complex is cytochrome c oxidase which consists of at least nine non-identical subunits. The three largest subunits (I, II, III) are coded by mitochondrial DNA, whereas the six small subunits (IV-VII, VIIa, VIII) are coded by nuclear DNA. The interdependence of the two systems has recently been demonstrated by Dowhan et al. (1985) who showed that the assembly of cytochrome c oxidase in yeast was dependent on the synthesis of cytoplasmically-made subunit IV. They constructed a S.cerevisiae mutant which lacked subunit IV, by using a gene interruption technique. This mutant was devoid of cytochrome c oxidase activity although it still contained the other cytochrome c oxidase subunits but they were not assembled into a stable complex.

The majority of mitochondrial proteins are nuclear-encoded and as a result a central feature of mitochondrial assembly is the import of nuclear-encoded mitochondrial proteins from their site of synthesis on free cytoplasmic polysomes. The key steps in this import process have been identified by isotope-tracer studies with intact cells and by incubation of isolated mitochondria or mitochondrial subfractions with radiolabelled protein precursors in vitro. Since the preliminary studies of Maccecchini et al. (1979) where it was shown that cytoplasmic precursor polypeptides of higher M_r were synthesised to F_1-ATPase α, β and γ subunits in an in vitro translational system, the field of mitochondrial import has advanced greatly. In general, the import of cytoplasmically-synthesised mitochondrial proteins destined for location within the

intermembrane space, inner membrane or matrix of the organelle involves the following steps;

i) specific interaction of the precursors with the mitochondrial outer membrane via protease sensitive sites (Riezman et al., 1983; Gasser and Schatz, 1983);

ii) translocation of polypeptides across one or both mitochondrial membranes is energy-dependent. The most likely source of energy involves the existence of an electrochemical potential across the mitochondrial inner membrane (Gasser et al., 1982a);

iii) processing the large M_r precursors by a matrix-localised protease (Böhni et al., 1980; McAda and Douglas, 1982).

There are several exceptions to this mechanism of import as is summarised in table 1.3.2. In addition, to date, the import of proteins distributed in the mitochondrial outer membrane does not appear to involve any one of the above three steps.

Extensive research has been performed on the synthesis of proteins located in the mitochondrial matrix (Lewin et al., 1980, Raymond and Shore, 1981; Böhni et al., 1983). In all cases, with the possible exception of one (Hampsey et al., 1983), these proteins are synthesised as larger M_r precursors which, upon translocation into the mitochondrial matrix, are cleaved at their amino-termini by the matrix-located protease (Böhni et al., 1980) to generate the mature protein. In agreement with these findings are the preliminary results reported in the last chapter on the mechanism of synthesis of fumarase. The mitochondrial location of this enzyme is the matrix where it functions in the tricarboxylic acid cycle. A larger M_r precursor (2000-4000 greater) was shown to be synthesised to fumarase in the PK-15 and BRL mammalian cell lines (Figs.

5.3.6 and 5.3.7) and in the yeast rho$^-$ mutant strain D273-10-B-1 (Fig. 5.3.9a). This was achieved by blocking the import of cytoplasmically-synthesised precursor into mitochondria using uncouplers of oxidative phosphorylation. In yeast, however, the identity of the enzyme has not been firmly established since the antiserum raised against the pig heart enzyme cross-reacted with a protein of lower M_r than its mammalian counterpart. It should also be noted that the mechanism of synthesis of this enzyme is complicated by the fact that fumarase has been shown to be located in the cell cytoplasm (Akiba et al., 1984) of most tissues and in yeast (Hiraga et al., 1984). For a detailed account of the proposed mechanism of synthesis of this bimodally distributed enzyme, see section 5.4.2.

Additionally, I was interested in studying the biosynthesis of the mitochondrial inner membrane phosphate transport protein as it is thought to be an integral, transmembrane protein. To date, only one other transmembraneous polypeptide has been studied in detail, this being the N.crassa ADP/ATP translocator of the inner membrane (Zimmerman and Neupert, 1980). The mechanism of synthesis of this protein is slightly different to that employed for the synthesis of mitochondrial matrix proteins, in that the cytoplasmic precursor form is of the same M_r as the mature protein. However, the precursor form of the protein does differ from the mitochondrially located polypeptide with respect to its conformational properties, in that it exists in the cytoplasm as a soluble aggregate of M_r between 150,000 and 500,000 and binds to hydroxylapatite (Zimmerman and Neupert, 1980). The import mechanism does, on the other hand, appear to include an interaction with protease sensitive sites on the mitochondrial outer membrane and requires the existence of an inner membrane electrochemical potential. As reported in chapter four, the antiserum raised against the rat liver phosphate transport protein cross-reacted with

several polypeptides of varying M_r value when challenged with mammalian cell culture extracts (see Fig. 4.3.4). However, the antiserum did appear to cross-react specifically with yeast cell extracts. When the yeast rho$^-$ mutant strain D273-10-B-1 was grown in the presence of increasing levels of CCCP (0-20μM), a specific polypeptide of 29,000 M_r was illustrated to cross-react with the antiserum (see Fig. 5.3.10a). However, no large M_r precursor could be detected with increasing uncoupler concentration. If the identity of this protein can be established then the preliminary findings will agree with those obtained for the ADP/ATP translocase. It could then be postulated that this is the general mode of synthesis of mitochondrial inner transmembraneous proteins.

6.2 Update on Mitochondrial Biogenesis

As stated earlier (see section 1.3.5.1), the transport of precursors into the mitochondrion requires an electrochemical potential across the mitochondrial inner membrane and is followed by the removal of the amino-terminal precursor extension by a chelator-sensitive protease in the matrix (Böhni et al., 1980, 1983, Miura et al., 1982, McAda and Douglas, 1982). Precursors whose prepiece has been removed in vitro by the addition of the solubilised matrix protease can no longer be imported into the mitochondria (Gasser et al., 1982b). This observation suggests that the transient prepiece has some function in the import pathway and in recent years a great deal of attention has been directed towards gaining a more detailed understanding of the role of the transient prepiece.

Initial interest has been directed towards studying the structures of the amino-terminal presequences of higher M_r precursor polypeptides in an attempt to find common structural elements which may be involved in targeting proteins to the mitochondrion. This has been achieved by

utilising recombinant DNA technology to determine the nucleotide sequences of the genes coding for imported mitochondrial proteins and thus enabling a prediction of the likely protein sequence. This experimental approach was adopted because the methods utilised to study the biosynthesis of nuclear-encoded mitochondrial proteins result in the synthesis of very small amounts of precursor. When secretory proteins were examined with respect to this property there was little obvious homology among their signal sequences although closer examination of primary structure (Garnier et al., 1980), revealed a central region of hydrophobic residues with a minimum length of nine residues. Several genes have recently been sequenced which code for mitochondrial proteins including the proteolipid subunit of N.crassa ATPase (Viebrock et al., 1982), yeast cytochrome c peroxidase (Kaput et al., 1982), yeast cytochrome c_1 (Sadler et al., 1984) and yeast cytochrome c oxidase subunit IV (Maarse et al., 1984). In all cases, the amino-terminal presequences were shown to render the protein more basic and in general more hydrophilic. Interestingly, the presequence of cytochrome c peroxidase precursor, which is processed in a two-step manner (see section 1.3.5.2), contains a 23 residue long apolar segment (Kaput et al., 1982). It is proposed that this segment integrates the protein into the mitochondrial inner membrane, such that the 18 amino-terminal residues of precytochrome c peroxidase face the matrix and the remainder of the pre and mature sequence are exposed to the intermembrane space.

To further characterize the role played by the amino-terminal prepiece, Hurt et al. (1984a) studied the import of subunit IV of yeast cytochrome c oxidase into mitochondria after its gene had been altered either by deletion or fusion to another gene. The in vitro transcription-translation system first described by Stueber et al. (1984) was used to synthesise the various protein constructs derived from the wild-type subunit IV gene.

Hurt et al. (1984a) were able to demonstrate by this type of approach that the amino-terminal region of the subunit IV precursor directs the polypeptide into mitochondria. When the amino-terminal region encoding the first 53 amino acids of the precursor was fused to the gene coding for the cytosolic protein, dihydrofolate reductase, the resulting fusion protein was transported into the mitochondrial matrix. The location of the fusion protein was confirmed by subfractionating the mitochondria into its various compartments and testing for the presence of radiolabelled protein. A subsequent report by Hurt et al. (1984b) found that only the region encoding the first 22 amino acids of the 25 amino acid transient prepiece of cytochrome c oxidase subunit IV was required for directing dihydrofolate reductase to the mitochondrial matrix. Additional evidence to confirm the role played by the amino-terminal presequence in the import of proteins into the mitochondrion was provided by Douglas et al. (1984). They fused the gene coding for the amino-terminus of the yeast F_1-ATPase β-subunit to the E.coli β-galactosidase gene and were able to demonstrate that this hybrid molecule is targeted to the mitochondria. The actual location of the protein was not fully defined, although it was shown to be protected from added protease under conditions in which an outer membrane enzymatic marker was digested. The results described above suggest the following roles for the amino-terminal extension of mitochondrial precursor polypeptides of higher M_r:

i) the prepiece renders some hydrophobic polypeptides more soluble (Viebrock et al., 1982).

ii) the prepiece contains the information for targeting the attached 'mature' polypeptide to its correct intramitochondrial location (Hurt et al., 1984a).

So far the emphasis has been placed on studying the signal which directs proteins of higher M_r to the mitochondrion. Recombinant DNA technology has, however, recently been utilised by Hase et al. (1984) in order to identify the sequences which target the major 70,000 M_r protein of the yeast mitochondrial outer membrane and which permanently anchor it to the lipid bilayer of the outer membrane. As stated earlier in this chapter (see section 6.1), proteins destined for location within the mitochondrial outer membrane are not made as larger M_r precursors. By manipulating the cloned gene, Hase et al. (1984) deleted 13 different regions throughout the polypeptide. All the information for targeting and anchoring the 70,000 M_r protein was contained within the amino-terminal 41 amino acids. When this entire region was deleted, the protein was recovered in the cytosol fraction. However, several restricted deletions within this amino-terminal region appeared to affect targeting and anchoring differentially; most of the altered protein remained in the cytosol but a small fraction was misrouted into the mitochondrial matrix space. They have suggested from these findings that targeting is mediated by a region which includes the 11 amino-terminal amino acids whereas the permanent membrane anchor is provided by a typical transmembrane sequence between residues 9 and 38.

Finally, although a basic understanding of the mechanisms of synthesis and import of nuclear-encoded mitochondrial proteins has been attained in the last few years, little is knownabout how the import pathway is regulated and most of its molecular components remain to be identified or isolated. One approach to solving these problems has recently been adopted by Yaffe and Schatz (1984) and involves the use of temperature-sensitive mutants. The isolation of mutants in mitochondrial protein import was based on the following assumptions:

i) import is an essential cellular process (Gbelska et al., 1983);

ii) a block in this process would result in the accumulation of precursors of some mitochondrial proteins (Reid and Schatz, 1982a).

Yaffe and Schatz (1984) isolated two mutants (mas 1 and mas 2) which were shown to accumulate the precursor to the β-subunit of the mitochondrial F_1-ATPase at the non-permissive temperature (37°C). In addition, the mutation resulted in the arrest of cellular growth at 37°C. Import of the F_1-ATPase β-subunit at 37°C was greatly reduced in both mutant strains when compared with wild-type cells. Although the temperature-sensitive step in import in both mas 1 and mas 2 was not firmly established, it was shown to occur before the arrival of the precursors in the mitochondrial matrix. This type of experimental approach should facilitate the molecular analysis of mitochondrial protein import as the mutant can be used to isolate known proteins of the import machinery, to clone the genes coding for these proteins and to overproduce these components. The field of mitochondrial protein import has now taken on a new and exciting direction which should enable a more detailed understanding of the overall process.

6.3 FUTURE WORK

6.3.A Fumarase

A detailed account was given in chapter five on the preliminary findings relating to the mechanism of synthesis of the bimodally distributed fumarase in mammalian culture cells. In order to confirm these results, future research should initially be directed towards establishing the relationship between the precursor and mature fumarases. There are several potential methods of achieving this including:

i) pulse-chase experiments in which PK-15 and BRL cells are initially labelled with [^{35}S]-methionine in the presence of uncoupler, as described

previously (see legend to Fig. 5.3.6). Following this labelling period, the cells are transferred into normal growth medium (see section 2.4.1) containing cold methionine and chased for various selected times. In this type of experiment it should be possible to see the precursor form of fumarase being converted into the mature protein, as the period of chase increases. In addition to confirming the relationship between the precursor and mature enzyme, this type of experimental approach also gives an indication of the stability of the precursor,

ii) competition experiments in which the immune-precipitation reaction of a cell extract prepared from cells labelled in the presence of uncoupler with fumarase antiserum, is performed in the presence of unlabelled mature fumarase. In this case, no polypeptides should be visualized on the fluorograph indicating that both the precursor and mature proteins are competed out by the unlabelled enzyme and thus are related to each other.

The next step would be to confirm that the large M_r precursor synthesised in PK-15 cells blocked in import is the precursor form of mitochondrial fumarase whereas the mature sized protein is the cytosolically located enzyme. This can be achieved by radiolabelling cells in the presence of uncoupler, preparing a cell-free extract from the cells and subsequently incubating it with isolated mitochondria. Immune-precipitation from the resulting mitochondrial and post mitochondrial supernatant fractions should illustrate a decrease in the proportion of precursor in the post-mitochondrial supernatant fraction with a corresponding increase in the amount of mature radioactive fumarase in the mitochondria. In addition, the amount of mature fumarase in the supernatant fraction should not vary under these conditions.

6.3.B Phosphate Transport Protein

As previously discussed (see section 5.4.3), the antiserum raised against the rat liver mitochondrial phosphate transport protein could only be shown to cross-react specifically with a protein of 29,000 M_r in yeast extracts prepared from intact cells grown in the presence of CCCP or from mitochondria. Although the cross-reacting protein has been shown to be of mitochondrial origin in this study, its identity has not been conclusively proven in yeast. The first priority would be to purify the phosphate transport protein from yeast cells, possibly by a similar procedure to that adopted for the rat liver protein (see section 3.2.1 method a). A second approach which may be used to tentatively identify the protein is to utilise its known sulphydryl group sensitivity. Yeast mitochondria could be incubated with N-[^{3}H]-ethylmaleimide before being subjected to immune-precipitation with anti-phosphate transport protein serum. The specifically precipitated protein would then be visualized by fluorography. If the protein can be identified it may be feasible to study its mechanism of synthesis in more detail and to confirm the yeast rho^- mutant immune replica data which suggested that the protein was initially synthesised as a precursor of the same M_r as the mature polypeptic If the phosphate transport protein can be shown to be synthesised in the cytoplasm as a protein of similar M_r to the mature protein, by incubating the purified phosphate transporter in an <u>in vitro</u> import system, it should be possible to study its mechanism of import in more detail.

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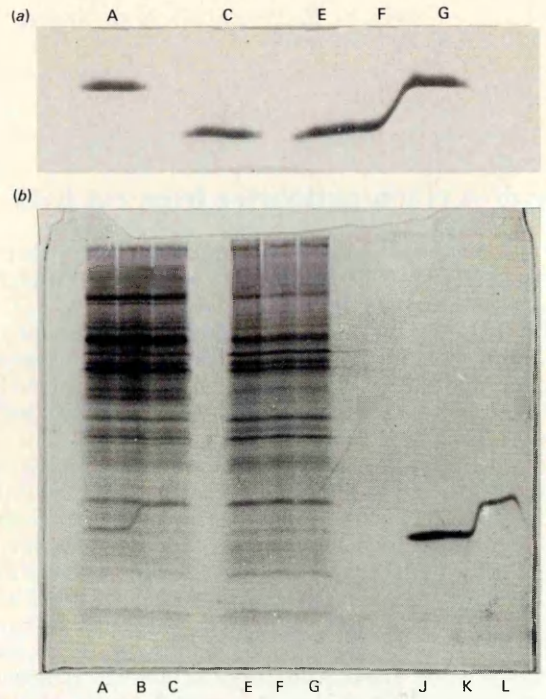

Fig. 1. *SDS/polyacrylamide-gel electrophoresis of (a) calmodulin pretreated with either 0.8 mM-Ca^{2+} ions or 5 mM-EGTA, and (b) crude synaptic membranes from bovine brain*

(*a*) Tracks contain the following: A, calmodulin, EGTA; B, blank; C, calmodulin, Ca^{2+}; D, blank; E, F, calmodulin, Ca^{2+}; G, calmodulin, EGTA. (*b*) Membrane samples were dissolved in sample buffer containing either 0.8 mM-$CaCl_2$ or 5 mM-EGTA before electrophoresis. Tracks: A, B, washed membranes, Ca^{2+}; C, washed membranes, EGTA; D, blank; E, F, Triton-washed membranes, Ca^{2+}; G, Triton-washed membranes, EGTA; H, I, blanks; J, K, calmodulin, Ca^{2+}; L, calmodulin, EGTA.

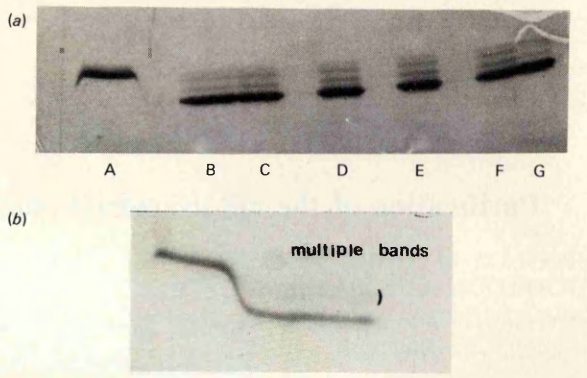

Fig. 2. *SDS/polyacrylamide-gel electrophoresis of (a) calmodulin pretreated with Ca^{2+}, and (b) calmodulin (10 μg) with either EGTA (5 mM) or Ca^{2+} (0.8 mM)*

(*a*) Tracks contain calmodulin (10 μg) with: A, 5 mM-EGTA; B, no Ca^{2+}; C–G, 5, 10, 15, 20, 600 μM-Ca^{2+} respectively.

al., 1979), or not show (Burgess *et al.*, 1980), this differential mobility, and thus the uniqueness of this sigmoid pattern to calmodulin is not fully established.

Calmodulin has been observed bound to post-synaptic membranes (Wood *et al.*, 1980) and brain microsomes (Sobue *et al.*, 1982). Total release of calmodulin from membranes was reported to require washing of membranes with detergent (Kakiuchi *et al.*, 1978) and it has been shown (Sharma & Wang, 1981) that Triton X-100 bound to calmodulin in a Ca^{2+}-dependent manner and inhibited calmodulin activation of phosphodiesterase. However, Grab *et al.* (1979) have reported calmodulin in post-synaptic densities which had been prepared by washing membranes with 0.5% Triton X-100.

Synaptic membranes and Triton-washed (0.05%) membranes were prepared from bovine brain (Chang *et al.*, 1981) and electrophoresed as described for calmodulin. Native membranes showed horizontal protein bands through adjacent tracks, the one exception being a 'sigmoid' pattern for one protein band which co-migrated with pure bovine brain calmodulin (Fig. 1*b*). Other proteins migrating with calmodulin, either Ca^{2+}-free or -bound, although having mobilities unaffected by Ca^{2+}, masked the positions of calmodulin, unless adjacent tracks were subjected to electrophoresis. Using this procedure, however, the sigmoid pattern of calmodulin allowed confident identification of the protein. In Triton-washed membranes, no sigmoid pattern was observed (Fig. 1*b*), showing that Triton-washing indeed released calmodulin from membranes.

Multiple bands of calmodulin have been reported on SDS/polyacrylamide-gel electrophoresis by Burgess *et al.* (1980), who concluded that these bands represented calmodulin that had lost one or more of its Ca^{2+} binding sites due to denaturation, and by Sharrard *et al.* (1983), who suggested that the intermediate bands may be due to proteolysis.

Three intermediate bands can be seen at high protein loading (Fig. 2*a*) with all buffers made with deionized, distilled water (>15 MΩ/cm), the intensity of these bands being increased at intermediate Ca^{2+} concentrations. Continuity of the intermediate bands with the Ca^{2+}-free calmodulin band (EGTA track) suggested that these bands represented Ca^{2+}-bound calmodulin species (Fig. 2*b*). Thus it seems probable that these intermediate bands represent calmodulin that had lost either different amounts of Ca^{2+} or Ca^{2+} from different sites on denaturation. The high protein concentration being greatly in excess of the micromolar dissociation constants for calcium binding would allow most endogenous Ca^{2+} to be protein-bound. However, the discrete multiple bands suggest that, in the absence of EGTA, SDS must greatly decrease Ca^{2+} dissociation from calmodulin.

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Purification of the mitochondrial phosphate–hydroxyl ion antiporter from rat liver

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Inorganic phosphate must be transported continuously into the mitochondrial matrix to maintain the steady-state oxidative phosphorylation of ADP. Specific uptake of this anion occurs via at least two distinct transport systems: an electroneutral inorganic phosphate–dicarboxylic acid exchange carrier and an inorganic phosphate–hydroxyl antiporter which catalyses the influx of about 90% of the mitochondrial phosphate.

An interesting property of these phosphate transporters, which has facilitated their identification, is that they are inhibited by a low concentration of sulphydryl group reagents. The phosphate carrier protein is inhibited by *N*-ethylmaleimide, mersalyl and *p*-chloromercuribenzoic acid (Fonyo & Besman, 1968; Tyler, 1969; Coty & Pederson, 1974), whereas the phosphate–dicarboxylate exchange protein is blocked by mersalyl and *p*-chloromercuribenzoic acid but is insensitive to *N*-ethylmaleimide (Meyer & Tager, 1969; Meijer *et al.*, 1970; Quagliariello & Palmieri, 1972; Coty & Pederson, 1974).

In rat liver mitochondria, the phosphate carrier protein has been purified by modifying the method of Kolbe *et al.* (1981). This involves selective extraction of the phosphate carrier in a Triton X-100 buffer (20mM-LiCl, 20mM-H_3PO_4, 0.5mM-dithiothreitol, 0.1mM-EDTA and 8% (w/v) Triton X-100, pH7.0), and hydroxylapatite chromatography before adsorption on Celite. Fig. 1 compares the Coomassie Blue staining pattern of the phosphate carrier purified from rat-liver and beef heart mitochondria, on a 14–20% sodium dodecyl sulphate/polyacrylamide gradient gel. The beef heart protein has been purified also as described by Kolbe *et al.* (1981). There is a major difference between the protein product(s) at the hydroxylapatite stage in the two preparations. In the rat liver sample, only one major band is observed, which migrates with a M_r of 34000, whereas in the beef heart preparation there are two major bands, which migrate with M_r of 34000 and 31000. The 31000 M_r band is probably the adenine nucleotide translocase as shown by Wohlrab (1980). The translocase protein can be removed by adsorption on Celite, as was shown by Kolbe *et al.* (1981), and is illustrated here in Fig. 1. In this laboratory, another method for separating the translocase from the phosphate carrier protein has also been utilized. This involves the differential elution of the proteins from a Procion Red A column with NaCl. The phosphate carrier protein can be eluted with 0.25M-NaCl, whereas the translocase requires 3M-NaCl for elution from the column.

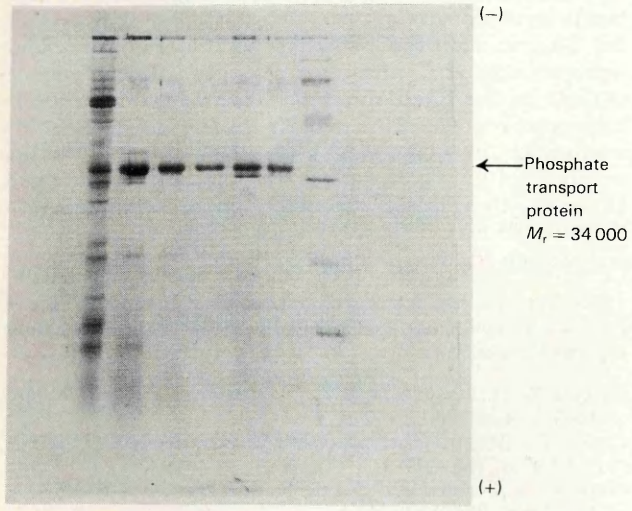

Fig. 1. *Sodium dodecyl sulphate 14–20% (w/v)/polyacrylamide gradient gel analysis of purified phosphate hydroxyl antiporter from rat-liver and beef-heart mitochondria*

Coomassie Blue profile of different stages in the purification. From rat-liver mitochondria: (*a*) 8% (w/v) Triton X-100 extract, (*b*) hydroxylapatite eluate, (*c*) Celite-adsorbed fraction, (*d*) 0.25M-NaCl eluate from Procion Red A column. From beef-heart mitochondria: (*e*) hydroxylapatite eluate, (*f*) Celite-adsorbed fraction, (*g*) marker proteins 94000–14000 M_r.

The results discussed above, contrast markedly with the recent findings of Kolbe *et al.* (1982), who purified the phosphate carrier protein from bovine and pig heart mitochondria. The hydroxylapatite eluates, when resolved on 14–20% (w/v) sodium dodecyl sulphate/polyacrylamide gradient gels, demonstrated four to five Coomassie blue staining bands, which varied in M_r from 34500 to 30000. They explained their findings in terms of a model, which suggests that the phosphate carrier protein is proteolytically degraded during its isolation from mitochondria. Multiple banding was not observed in this laboratory. Densitometic scanning of the gel demonstrated that the protein was greater than 90% pure.

An important additional step in purifying the phosphate carrier proteins from rat liver, by the method of Kolbe *et al.* (1981), was the pre-extraction of the mitochondria with 0.5% (w/v) Triton X-100 buffer. Early preparations, although demonstrating only one major band, when overlayed with ^{125}I-labelled concanavalin A showed the presence of numerous low and high M_r glycoproteins. This initial extraction in 0.5% (w/v) Triton X-100 buffer solubilized about 80% of the mitochondrial protein and removed these contaminants.

The sulphydryl group sensitivity of the phosphate carrier protein was employed in the identification of the protein. Labelling studies with [^{3}H]*N*-ethylmaleimide showed that only the 34000 M_r band was labelled in both the hydroxylapatite and Celite fractions, when purified from rat liver mitochondria. This result is consistent with the known sulphydryl group sensitivity of the phosphate carrier protein. Further confirmation is provided by preliminary experiments with an antiserum directed against the 34000 M_r protein. Partial inhibition of mitoplast swelling in isotonic ammonium phosphate buffer (Chappell & Crofts, 1966) was achieved when mitoplasts were incubated with the specific antiserum.

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Organization and distribution of carbohydrate on mitochondrial membranes

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All eukaryotic membranes, including those of intracellular organelles, contain small amounts of covalently associated carbohydrate, mainly in the form of glycoprotein (Hughes, 1976). In mitochondria, it is clearly important to establish the presence, nature and topographical distribution of these glyco-conjugates, since resolution of these points will provide valuable information on the origins, mode of synthesis and assembly of outer and inner membranes.

Earlier reports on the presence of carbohydrate (De Bernard *et al.*, 1971; Glew *et al.*, 1973) and glycosyl transferase activities in this organelle (Gateau *et al.*, 1978) have not been developed to the stage where we can eliminate the possibility that these represent contaminants of non-mitochondrial origin. Moreover, no well-characterized glycoproteins have been isolated from the organelle, although a protein, M_r 33000, involved in Ca^{2+} uptake, appears to contain associated carbohydrate (Panfili *et al.*, 1976). There has also be controversy over possible glycosylation of the mitochondrial F1-ATPase (Andreu *et al.*, 1978; Nalin *et al.*, 1979).

In our laboratory, we can detect the presence of several (10–15) concanavalin A-reactive glycoproteins in highly purified mitochondria and derived subfractions. The broad specificity of this lectin enables over 70% of mitochondrially associated glycoprotein to be isolated by affinity chromatography on concanavalin A–Sepharose 4B.

Interestingly, using WGA, specific for sialic acid and GlcNAc, only two major glycosylated species with apparent M_r of 92000 and 105000 are found to interact with this lectin in rat liver mitochondria. We have concluded that these components are located in mitochondrial membranes based on the following observations: (a) rat liver mitochondria or mitoplasts (outer membrane removed) are readily agglutinated by WGA, a reaction which is inhibited by GlcNAc; (b) WGA can be employed to isolate sealed 'inside-out' vesicles containing no accessible carbohydrate from a mixed population of inner membrane fragments (D'Souza & Lindsay, 1981); and (c) fluorescein–WGA binds specifically to isolated mitochondria.

The external location of sialic acid on the 92000 M_r component has been confirmed by neuraminidase treatment of intact mitochondria or mitoplasts. This band exhibits a decreased mobility on SDS/polyacrylamide-gels on removal of sialic acid as revealed by fluorography after incubation with ^{125}I-labelled WGA. In contrast, the higher M_r species is unaffected by neuraminidase and is absent from mitoplasts, indicating a location in the outer membrane.

The unique nature of the major WGA-reactive glycoprotein has been confirmed by surface-specific modification of accessible galactosyl residues employing the galactose oxidase/tritiated borohydride procedure of Gahmberg (1976). Fig. 1 shows an SDS/polyacrylamide-gel profile of ^{3}H-labelled glycoproteins in intact mitochondria as detected by fluorography. The most striking feature is the presence of two prominent bands with M_r of 92000 and 105000 (track c). Prior incubation with neuraminidase, which exposes sub-terminal galactose, leads to a marked enhancement in Na^3HBH_4 labelling (3–5 fold) over control values (track d). The effect of this pretreatment is confined exclusively to the lower M_r component which becomes extensively tritiated (track b), again exhibiting a slightly decreased mobility. However, this novel sialoglycoprotein is not susceptible to periodate oxidation as evidenced by its subsequent lack of

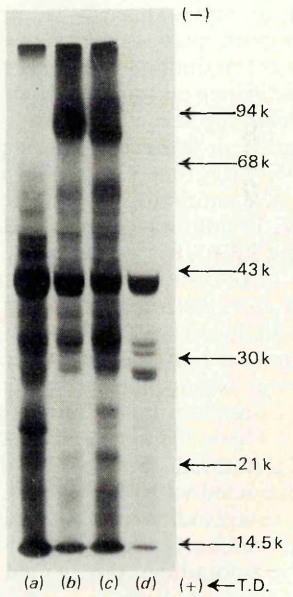

Fig. 1. *External labelling of rat liver mitochondrial glycoproteins*

Sucrose-density purified mitochondria are labelled with 1 mCi Na^3HBH_4 (5–20 Ci/mmol) after pretreatment for 30 min at 4°C with neuraminidase and galactose oxidase (track b), galactose oxidase only (track c) or no pretreatment (track d) according to the procedure of Gahmberg (1976). For comparison, total mitochondrial sialoglycoprotein is oxidized with periodate (Blumenfeld *et al.*, 1972) before tritiation with borohydride (track a). All samples are visualized by fluorography after resolution of SDS/polyacrylamide-gels. T.D., tracker dye front; k, kilodaltons.

Abbreviations used: GlcNAc, *N*-acetylglucosamine; SDS, sodium dodecyl sulphate; WGA, wheat germ agglutinin.

reactivity with tritiated borohydride (track a). This insensitivity to periodate treatment indicates the presence of a C-8 substituted neuraminic acid (O–$COCH_3$ or O–$COCH_2OH$). Removal of the outer membrane does not affect the 92000 M_r species while the 105000 M_r species is again released.

The intriguing properties and distribution of this novel WGA-reactive sialoglycoprotein are under further investigation, since it may span the bilayer of both inner and outer membranes.

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Prostaglandin synthase activity in tissues from guinea pigs fed fatty acid- and aspirin-supplemented diets

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PUFA supplementation of the diet of guinea pigs increased the prostaglandin synthase activity in the tissues (McGrath, 1980). Aspirin-like drugs have been found to inhibit prostaglandin synthase activity (Vane, 1971).

The present work examines the relationship between dietary PUFA and prostaglandin synthase activity in five tissues (adipose tissue, brain, kidney medulla, liver, lung) and the effect of simultaneously administered aspirin thereon. Groups of animals of the same age (5 months) isolated in cages were maintained on diets for 4 or 8 weeks. They were fed *ad libitum* on standard laboratory diet (group A), laboratory diet supplemented with 12.5% (w/w) sunflower oil (group B), or laboratory diet supplemented with 12.5% (w/w) olive oil (group C). Half of each group were killed and half were maintained on the diets for a further 4 weeks receiving, in addition, aspirin (20mg/kg per day). The animals were killed, tissues removed, chopped, homogenized in 4vol. of 100mM-Tris/HCl buffer, pH8.2, at 4°C, and centrifuged at 12000*g* for 15min at 4°C. The supernatants were centrifuged at 200000*g* for 30min at 4°C to yield a microsomal fraction (Flower *et al.*, 1973). The microsomal fraction was resuspended in buffer. Prostaglandin synthase activity was determined in incubation at 37°C for 15min with [1-^{14}C]arachidonic acid (2.2 μM), adrenaline (5mM), reduced glutathione (5mM) in Tris/HCl, pH8.2. The products were extracted with ethyl acetate and dried under nitrogen. The prostaglandins were separated by t.l.c. using chloroform/methanol/acetic acid (95:5:5) (Srivastara, 1978), located by autoradiography, eluted and determined by liquid scintillation counting.

Although the diets were isocaloric, intake by the different groups of animals varied in *ad libitum* feeding. Animals fed standard (group A) and sunflower oil (group B) diets have similar growth rates as determined by gain in body weight; slightly higher growth rates were observed in animals fed olive oil (group C).

Total lipid content determined by Folch extraction (Folch *et al.*, 1957) showed similar patterns in four tissues in that sunflower oil and olive oil supplementation progressively increased the lipid weight per g of wet tissue (group A < group B < group C). In the fifth tissue (adipose tissue) the lowest lipid content was found in animals fed sunflower oil (group B < group A < group C).

Analysis of extracted lipid using the lipoxygenase method (Waltking, 1972) showed the PUFA content of all tissues, expressed as mg of PUFA per g of wet tissue, to follow the series: group B > group C > group A. Prostaglandin synthase activity was lowest in tissues from animals fed standard diet, and highest in those from animals fed sunflower oil diets (group A < group C < group B) (Fig. 1).

Aspirin administered for 4 weeks to guinea pigs fed specified diets did not effect the animals growth rates, total lipid content or PUFA content. In addition aspirin administration resulted in decreased prostaglandin synthase activity in all tissues (Fig. 1).

The sensitivity of prostaglandin synthase to aspirin (Sigma, London) expressed as IC_{50} varied with the tissue: adipose tissue 760 μM, brain 133 μM, kidney 300 μM, liver 60 μM and lung 52 μM. These values were not affected by the diet nor by aspirin administration.

Changes in the PUFA content of tissues resulting from supplementation with sunflower and olive oil are consistent with the PUFA content of these oils. Increases in total lipid

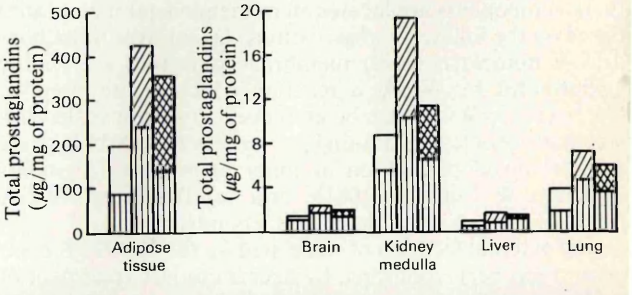

Fig. 1. *Microsomal prostaglandin synthase activity in tissues from guinea pigs fed modified diet (μg/mg of protein)*

Guinea pigs fed standard (group A), sunflower oil (group B) and olive oil (group C) diets *ad libitum* for 4 weeks or for 8 weeks with aspirin during weeks 5–8. Total prostaglandins determined by extraction, t.l.c. separation and liquid scintillation counting of products of incubation. □ Group A without aspirin; ▨ group B without aspirin; ■ group C without aspirin; ▥ groups A, B and C with aspirin.

Abbreviations used: PUFA, polyunsaturated fatty acids; t.l.c., thin-layer chromatography; IC_{50}, concentration of inhibitor resulting in 50% inhibition of prostaglandin synthase.