Studies on three mitochondrial proteins: the phosphate and adenine nucleotide inner membrane carriers and the pyruvate dehydrogenase complex

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This thesis is submitted for the degree of

Doctor of Philosophy

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October, 1991

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For my parents

and Jennifer

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CHAPTER 1 (INTRODUCTION)

ABBREVIATIONS

In addition to the accepted abbreviations (Instructions to Authors, *Biochem. J.* [1985] **225** 1-26), the following have been employed:-

ANPP	4-azido-2-nitrophenylphosphate
ANT	adenine nucleotide translocase
ATR	atractyloside
BKA	bongkrekic acid
BCOADC	branched chain 2-oxo acid dehydrogenase complex
BSA	bovine serum albumin
CAT	carboxyatractyloside
CCCP	carbonyl cyanide *m*- chlorophenylhydrazone
CD	circular dichroism
DCCD	*N,N'*- dicyclohexylcarbodiimide
DSC	differential scanning (micro)calorimetry
EDTA	ethylenediamine tetraacetate
EGTA	ethylene glycol-*bis* (β– aminoethyl ether) *N,N,N',N'*- tetraacetate
EMA	eosin-5-maleimide
FPLC	fast protein liquid chromatography
GdnHCl	guanidine-HCl
GIP	general insertion protein
HEPES	*N*- 2-hydroxyethylpiperazine-*N'*- 2-ethane-sulphonic acid
HPLC	high pressure liquid chromatography
hsp	heat shock protein
HTP	hydroxylapatite
Iodogen™	(1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril)
KP_i	potassium phosphate buffer (KH_2PO_4/K_2HPO_4)
LDAO	lauryl dimethyl amine oxide
leupeptin	acetyl-L-leucyl-L-leucyl-L-arginal
MOPS	3-(*N*- morpholino) propane-sulphonic acid
M_r	relative molecular mass

NaP_i	sodium phosphate buffer (Na_2HPO_4/NaH_2PO_4)
NEM	*N*- ethylmaleimide
NRS	non-immune rabbit serum
OGDC	2-oxoglutarate dehydrogenase complex
PAGE	polyacrylamide gel electrophoresis
PBP	phosphate-binding protein
pCMB	*p*- chloromercuribenzoate
PDC	pyruvate dehydrogenase complex
PFK	phosphofructokinase
P_i	orthophosphate
PMSF	phenylmethylsulphonyl fluoride
PP_i	pyrophosphate
PTP	phosphate transport protein
PTS	phosphate transport system
SDS	sodium dodecyl sulphate
SMP	submitochondrial particles
SRP	signal receptor particle
TEMED	*NNN'N'*- tetramethylethylethylene-diamine
UCP	uncoupling protein

The following represent growth media for yeast, supplemented as denoted:

YPD	2% (w/v) glucose
YPDG	0.1% (w/v) glucose, 3% (v/v) glycerol
YPEG	2% (v/v) glycerol, 3% (v/v) ethanol
YPG	2% (w/v) galactose
YPL	2% (w/v) sodium lactate, pH 6.0
YPS	2% (w/v) sodium succinate, pH 6.0

ACKNOWLEDGEMENTS

I would initially like to thank Professors M.D.Houslay and the late R.M.S.Smellie for providing the facilities of the Biochemistry Department at the University of Glasgow for the duration of the research project which has resulted in this thesis. I would also like to thank my supervisor, Dr.J.Gordon Lindsay for his leadership, guidance and invaluable advice throughout my time in the laboratory, and his continual promptings during the write-up. My gratitude is also extended to SERC for funding this project, paying the rent, and providing enough for the (very) occasional liquid refreshment. Further to this, I am grateful to Dr.J.G.Lindsay and Mr.T.Mathieson for continuing to permit me the use of the word processors in the Biochemistry Department after I had started working elsewhere.

This research would not have been possible without the invaluable cooperation and technical assistance of my co-workers, both past and present, in lab.C35, especially the following: Dr. Jeffrey Hodgson, Dr. James Neagle, Jacqueline Rice, Fiona McCallum, Dr. Tim Lever, Angela Clemens, Dr. Jillian McKee and Dr. Shauna West. I am also grateful to Dr.J.R.Kusel for his friendship and advice throughout my postgraduate years. In addition to this, I would like to thank the staff of the Aberdeen University Biochemistry Department who were responsible for determining the amino acid analyses and sequences essential for this project. Furthermore, I am grateful to Miss Sharon Kelly for operating the spectropolarimeter at the University of Stirling, and for helping me understand the resulting CD spectra.

On a personal note, I would to thank the following people and organisations for their inspirational involvement in this thesis: my long-suffering parents; my fiancee Jennifer, for putting up with me while I was writing up; all the boys who lived in and loved (in) that glorious abode at 12, Kelvingrove Street; the Biochemistry Departmental football team, who won the league despite my managing and goalkeeping for them; and finally, the pride of Scotland, Rangers Football Club, although I would appreciate them winning the championship in a less coronary-inducing fashion next season.

I typed this thesis myself on an Apple Macintosh computer, using the MacWrite (v5.00 & v5.01) word processing package, although I would like to thank Dr. Frank Rinaldi for his invaluable help in mastering this software. I am further

indebted to Dr.Rinaldi, without whom many of the figures in this thesis would not have been possible.

SUMMARY

Protein-chemical and immunological studies were carried out on three mitochondrial proteins: the phosphate and adenine nucleotide inner membrane carriers (PTP and ANT), and the pyruvate dehydrogenase multienzyme complex (PDC).

PTP was purified to homogeneity from both mammalian (rat liver and bovine heart) and yeast (*S.cerevisiae*) mitochondria. Antisera were raised against the rat liver and yeast carriers. It was observed that, by pre-incubating freshly-isolated mitochondria with the potent ANT inhibitor, carboxyatractyloside (CAT), it was possible to co-purify PTP and ANT from both mammalian and yeast sources. However, it was not possible to purify ANT to homogeneity by adopting the procedure outlined in the literature. The petite (rho^-) *S.cerevisiae* mutant strain D273-10B-1 was employed to investigate the biosynthesis of the putative yeast PTP. In agreement with Wohlrab's group (Phelps *et al.*, 1991), the yeast carrier was observed to lack an N-terminal-extended signal sequence.

Various chromatographic techniques were employed in an attempt to separate the co-purified mitochondrial carriers. It was observed that, after application to the Matrex gel Procion Red A at pH 8.8, mammalian PTP and ANT were differentially eluted with a salt (NaCl) gradient. Following separation, the identities of the bovine heart carriers were confirmed by N-terminal protein sequencing. Procion Red A column chromatography was also found to represent an assay for native mammalian PTP conformation, an important pre-requisite for physical analyses of the carrier and crystallisation trials. This Matrex gel was further employed to transfer bovine heart PTP from solution in Triton X-100 to detergents suitable for analysis by circular dichroism (CD). In this manner, the secondary structure of the carrier was analysed and found to contain 85-95% α-helix. Although possibly rather high, this value may reflect the 6-7 transmembrane domains of PTP and the carrier's resistance to proteolysis in the membrane. In one of the detergents tested, PTP was also found to contain ~5% β-sheet structure, which may correspond to the putative 7th transmembrane domain proposed for the 'tripartite' family of membrane carriers. However, no further evidence was

found for the presence of this secondary structure in PTP. CD spectra were also obtained for bovine PTP in the presence of increasing concentrations of the denaturing reagent guanidine-HCl (GdnHCl).

The putative phosphate carrier isolated from yeast mitochondria was identified on the bases of its purification characteristics and immunological properties. Characterisation of yeast ANT was based on the protein's purification properties and its stabilisation with CAT. Although it was not possible to separate the yeast co-isolate with Procion Red A, the binding and elution characteristics of the proteins further suggested that they represent yeast PTP and ANT. Preliminary results also indicated that Procion Red A represents an assay for native PTP conformation. Further to this, chromatographic analysis revealed the presence of an unbound mitochondrial polypeptide with a near-identical M_r value to the putative yeast PTP. Subsequent research identified this contaminant as the outer membrane protein, porin.

The SERC-funded amino acid sequencing facility at the University of Aberdeen was employed to determine the N-terminus of the putative yeast PTP. This initially indicated that the protein isolated in this study was distinct from that encoded by the putative yeast PTP gene cloned and sequenced by Phelps *et al.* (1991). However, it was subsequently demonstrated that the amino acid sequence obtained was the N-terminus of contaminating porin in the PTP preparation. The N-terminal of PTP was shown to be blocked, confirming prior observations (A. Phelps, personal communication). Another group had previously isolated and characterised the putative yeast PTP gene (Murakami *et al.*, 1990), but claimed that it encodes a yeast mitochondrial import receptor. The possible *in vivo* function(s) of both this protein and the polypeptide isolated in this study are speculated upon, although further research is clearly required to fully characterise them and confirm that they are identical.

In addition to studying the mitochondrial carriers PTP and ANT, a specific problem relating to the control of yeast PDC activity was addressed. Whereas PDC activity is regulated by a phosphorylation/dephosphorylation cycle on the E1α subunit in all other eukaryotic organisms, no equivalent mechanism has ever been detected in yeast. In this project, *in situ* ATP-radiolabelling studies with freshly-prepared yeast mitochondria were employed to provide strong evidence for the presence of PDC

kinase activity in this microorganism. Further to this, immune precipitation studies with yeast PDC E1 antiserum illustrated that the ^{32}P-phosphoprotein in yeast mitochondria corresponds to PDC E1α.

CHAPTER 1: INTRODUCTION

1.1 THE MITOCHONDRION

1.1.1 Introduction

The average mammalian mitochondrion has similar dimensions to the bacterium *Escherichia coli*. It exists commonly as an oval particle 1-2μm long and 0.5-1μm wide. Mitochondria can thus be observed in the light microscope, and the earliest descriptions of this organelle were based on the analysis of fixed and stained tissue. Although initially observed microscopically towards the end of the last century, it was not until the late 1940's that intact mitochondria were successfully isolated (Hogeboom *et al.*, 1948).

Electron micrographs of negatively stained mitochondria in transverse section clearly show two membranes: a limiting outer membrane and a highly invaginated inner membrane. These membranes divide the mitochondrion into two distinct compartments creating a total of four locations; outer membrane, inner membrane, matrix and intermembrane space, where a protein can be situated in the organelle. The enzymes involved in oxidative phosphorylation are located in the inner membrane, and thus those tissues, such as muscle, that require the greatest output of oxidatively derived ATP have mitochondria suited for this purpose, expressed morphologically as a highly invaginated inner membrane.

1.1.2 Composition of the inner and outer membranes

The outer membrane is 6-7nm thick, smooth and unfolded. In addition to being rich in phosphatidyl choline (see Table 1.1.1), this membrane contains relatively high amounts of cholesterol (Parsons and Yano, 1967; Levy *et al.*, 1969). Structurally, the membrane is relatively inelastic but freely permeable to molecules of M_r value below 10,000. Further analysis indicates that this membrane contains a heterogeneous group of enzymes involved in lipid metabolism and hydroxylation reactions. However, as these enzymes do not constitute an integrated pathway, the function of this membrane is not clear. Monoamine oxidase is the marker enzyme most commonly used for the outer

Table 1.1.1: Lipid composition of mitochondrial membranes (Adapted from Parsons *et al.*, 1967)

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

membrane.

In contrast, the inner mitochondrial membrane shows evidence of substructure on its matrix-facing surface and exhibits differential solute permeabilities. This 6-8nm thick membrane thus represents the major permeability barrier between the cytoplasm and the matrix. Small uncharged molecules (*eg* H_2O, O_2, CO_2, NH_3 and ethanol) are freely permeable but specific transport systems (see Section 1.1.4) control the influx and/or efflux of hydrophilic ions (*eg* H^+, P_i and adenine nucleotides). This membrane has a low phospholipid to protein ratio and its highly invaginated nature offers a very large surface area. Characteristic features of the inner membrane include its high content of the phospholipid cardiolipin, and low levels of cholesterol which render it relatively resistant to the detergent digitonin (see Table 1.1.1). In addition to a variety of solute carriers, this membrane also contains the respiratory chain enzymes and ATP-synthesising machinery.

The intermembrane space is generally 6-8nm wide except at translocation contact sites where the two membranes meet to facilitate protein import (see Section 1.1.6.4). Relatively few enzymes are housed in this space, and most catalyse the interconversion of adenine nucleotides. In contrast, the matrix is extremely viscous and contains the vast array of enzymes that compose the tricarboxylic acid cycle (except succinate dehydrogenase, which is a component of the inner membrane) and fatty acid oxidation. These catabolic pathways provide the respiratory chain with NADH, its major oxidisable substrate. The matrix also houses the enzymes which catalyse the synthesis of mitochondrial DNA and RNA, together with part of the urea cycle in some tissues. The ribosomes and enzymes involved in the synthesis of mitochondrially-encoded proteins are also situated in the matrix.

1.1.3 The electron transfer chain and oxidative phosphorylation

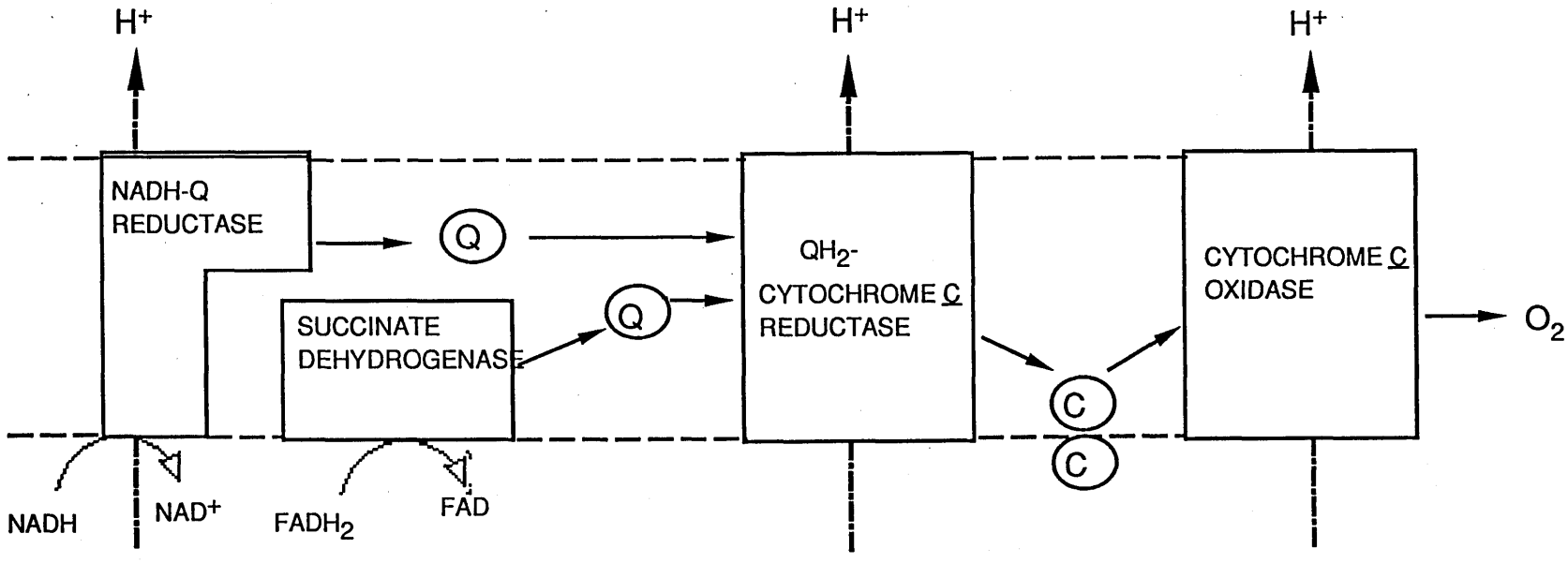
The primary function of mitochondria is to carry out oxidative phosphorylation. In this process, the electrons generated by the oxidation of the reduced coenzymes NADH and $FADH_2$ are ultimately transferred to molecular oxygen with the concomitant production of ATP.

While the protons generated during this process are readily soluble in aqueous

solution, the free electrons are passed from NADH or $FADH_2$ to oxygen along a chain of electron carriers which are components of the inner mitochondrial membrane. A series of electron carriers are grouped into four multiprotein intramembranous complexes, all of which exhibit lateral mobility in the plane of the membrane. As no stable contacts have been detected between these enzymes, transport of electrons from one complex to another may simply involve random collisions of the two. Figure 1.1.1 outlines the mitochondrial electron transfer chain. The lipid-soluble coenzyme Q shuttles electrons from the NADH-Q reductase and succinate dehydrogenase complexes to QH_2-cytochrome *c* reductase. Cytochrome *c* interacts with specific sites on both QH_2-cytochrome *c* reductase and cytochrome *c* oxidase and acts as a mobile carrier, transferring electrons from one complex to the other. Each of these complexes contains a variety of subunits and prosthetic groups. NADH-Q reductase consists of approximately 25 subunits with 8-9 FeS centres and a FMN group. Succinate dehydrogenase, which has 4 subunits, also contains 3 FeS centres as well as a FAD group and cytochrome b_{558}. QH_2-cytochrome *c* reductase, also known as the bc_1 complex, contains 11 subunits, cytochromes b_{566} and b_{562}, cytochrome c_1 and the Rieske FeS centre. Cytochrome *c* oxidase, which reduces molecular oxygen, consists of 13 subunits and contains cytochromes *a* and a_3 and 2-3 copper atoms.

Oxidation and ATP production are believed to be coupled by a proton motive force generated across the inner mitochondrial membrane during electron transport. First postulated by Mitchell (1961), the Chemiosmotic Hypothesis is now generally accepted as the model for oxidative phosphorylation. Protons are pumped out of the matrix at three of the electron transport complexes (NADH-Q reductase, QH_2-cytochrome *c* reductase and cytochrome *c* oxidase) creating a pH gradient and electrochemical potential across the membrane. The exact number of electrons transferred and protons pumped per complex is the source of much debate. When protons are shuttled back into the matrix through the transmembranous F_1F_0ATPase, ATP is synthesised from ADP and P_i.

Figure 1.1.1: Mitochondrial electron transport



1.1.4 Mitochondrial transport systems

The specialised role of mitochondria in intermediary metabolism requires that only certain substrates, cofactors and ions are accessible to the inner compartment. Of the molecules which must enter the matrix, the most important are the substrates for oxidation and phosphorylation, namely ADP, P_i, O_2, pyruvate and fatty acids. Equally important, the products of mitochondrial oxidation, including citrate, CO_2 and ATP, must have means of exiting the matrix. While mitochondria are freely permeable to CO_2 and O_2, the inner membrane, which represents the major permeability barrier between the cytoplasm and matrix, contains specific transport systems which accommodate the efficient influx or efflux of the remainder of these metabolites (see Table 1.1.2).

From Table 1.1.2, it is apparent that mitochondria possess a wide spectrum of transport systems, the majority of which are electroneutral exchange diffusion carriers. This movement of certain metabolites between the cytoplasm and matrix influences and regulates the overall metabolic balance of the cell (see Section 1.2; for review see Chappell, 1968).

1.1.5 The mitochondrial genome

Mitochondria contain their own genome which is responsible for the synthesis of about 5% of the organelle's proteins in addition to all the ribosomal and transfer RNAs of its separate transcription/translation system. The mitochondrial genome is autonomously replicated and is expressed within the confines of the organelle. Within a cell, each mitochondrion contains several copies of the closed circular DNA genome. The M_r value of the DNA varies between species, from approx. 10^7 in animal tissues to $7x10^7$ in higher plants.

The complete sequence and organisation of three mammalian mitochondrial genomes have been elucidated (Bibb *et al.*, 1981; Anderson *et al.*, 1981; Anderson *et al.*, 1982), and all demonstrate extreme economy in organisation with very few or no non-coding bases between adjacent genes (Figure 1.1.2). There are genes for two ribosomal RNAs (12S and 16S), 22 tRNAs and 13 proteins. Encoded on the heavy strand of the genome (H-strand) are the two ribosomal RNA genes, 14 tRNA genes and 12 proteins. The remainder of the tRNA and protein-coding genes are expressed on

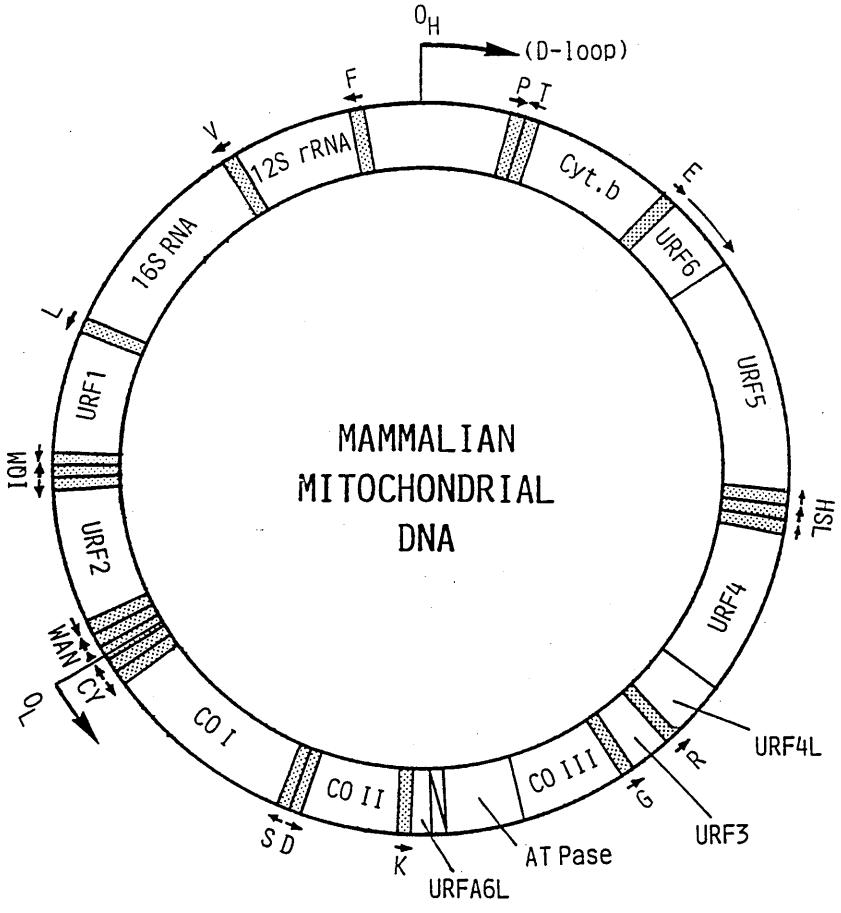
Table 1.1.2: Specific transport systems of the inner mitochondrial membrane

Transporter	*in vivo* function (IN/OUT)	Inhibitors	Biological importance
Phosphate	P_i/OH^-	N-ethylmaleimide	1)Mitochondrial ATP synthesis 2)Allows flux of dicarboxylates and tricarboxylates
Adenine nucleotide	ADP^{3-}/ATP^{4-}	atractyloside carboxyatractyloside bongkrekic acid	Mitochondrial ATP synthesis
Pyruvate	pyruvate/OH^-	α-cyano (4, OH) cinnamate	Link between glycolysis and the TCA cycle
Dicarboxylate	malate/P_i	*n* -butyl malonate 2-phenyl succinate	1)Transfer of reducing equivalents into and out of the matrix 2)Provides carbon skeleton for PEP
Malate/succinate	malate/succinate	-	-
Tricarboxylate	citrate/malate or isomalate (citrate/isocitrate)	citrate analogues	1)Transfer of acetyl CoA into cytosol 2)Control of PFK 3)Provision of NADPH to cytosol
Oxoglutarate	oxoglutarate/malate or malonate	aspartate	1)Transfer of reducing equivalents across the membrane 2)Transport of amino groups into the matrix
Glutamate	glutamate/OH^-	N-ethylmaleimide	Urea formation in the matrix
Glu/Asp	glutamate+H^+/aspartate	-	1)Transfer of reducing equivalents 2)Urea synthesis in the cytosol 3)In gluconeogenesis
Glu/Gln	glutamine/glutamate	-	NH_3 production in the kidney
Ornithine/citrate (liver only)	ornithine/citrate	-	Urea cycle
Acyl carnitine/carnitine	acyl / carnitine carnitine	carnitine derivatives	Movement of fatty acids across the membrane
Ca^{2+}	Ca^{2+} (in)	ruthenium red	Ca^{2+} storage in the matrix
Ca^{2+}/Na^+	$Ca^{2+}/2Na^+$	-	-

Figure 1.1.2: Genomic map of mammalian mitochondrial DNA (Reproduction from Clayton, 1984)

The stippled areas represent tRNA genes designated by the single-letter amino acid code, with polarity indicated by the arrows. All protein-encoding genes are on the H-strand (with counterclockwise polarity), with the exception of URF6, which is on the L-strand. COI, II and III: 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. URF's have now been assigned (Chomyn *et al.*, 1986) as encoding seven subunits of NADH-Q reductase and a further ATPase subunit.

Figure 1.1.2: Genomic map of mammalian mitochondrial DNA



the light strand (L-strand). The 13 reading frames have been assigned protein products of known function, all involved in oxidative phosphorylation. They were identified from the amino acid sequence of purified protein and by homology with the DNA sequence of known genes in other mitochondrial systems (Thalenfeld and Tzagaloff, 1980; Bibb *et al.*, 1981; Anderson *et al.*, 1981; Chomyn *et al.*, 1986). The only significant non-coding region of mammalian mitochondrial DNA is the displacement loop (D-loop). This region contains the origin of H-strand replication and sequences relevant to the promotion of transcription from each template strand (Montoya *et al.*, 1982; Montoya *et al.*, 1983).

In contrast, the yeast mitochondrial genome is approximately five times the size of mammalian mitochondrial DNA and appears to be less economically organised. It encodes two ribosomal RNAs, 25 tRNAs and 16 proteins. In addition, several yeast genes have been shown to contain intervening sequences (introns) in their structure, a phenomenon absent from mammalian mitochondrial genes.

Although there are marked differences in the overall organisation of genes in various eukaryotic mitochondria, the information available does suggest that the mitochondrial genome has been stripped of those elements that could be moved to the nucleus or dispensed with completely. This lends support to the popular hypothesis that these organelles have arisen as symbiotic prokaryotes providing oxidative metabolism to their pre-eukaryotic hosts. Southern Blotting has been employed as a technique to identify regions of mitochondrial-like DNA within the nuclear genomes of organisms as diverse as yeast (Farelly and Butow, 1983), insects (Gellisen *et al.*, 1983) and rat (Hadles *et al.*, 1983). A striking example of gene transfer between the mitochondrion and nucleus has been identified in *Neurospora crassa* (Van den Boogart *et al.*, 1982). The gene for mitochondrial ATPase subunit *IX* is nuclear-encoded though a copy is found within the mitochondrial DNA. This copy is apparently dormant and neither its origin nor physiological significance is known. However, in *S.cerevisiae* and plants, this protein is encoded by the mitochondrial genome and synthesised without a presequence (Viebrock *et al.*, 1982). Considerable evidence thus exists for a mechanism of gene transfer from the prokaryotic invader to the nucleus of the proto-eukaryotic host during the endosymbiotic process.

1.1.6 Mitochondrial Biogenesis

As previously discussed (Section 1.1.1), the mitochondrion possesses a double membrane system which divides the organelle into two compartments: the intermembrane space and matrix, bounded by the outer and inner membranes respectively. These compartments each have a special set of proteins, necessary for normal mitochondrial function.

Although the mitochondrion has its own genetic material (Section 1.1.5), this has a very limited coding potential. Most mitochondrial proteins (about 95%) are nuclear-encoded and synthesised on cytoplasmic ribosomes prior to transport to the organelle (For reviews, see Reid, 1985 and Hartl *et al.*, 1989). However, polypeptides destined for the inner mitochondrial compartments must translocate across one or both of the organelle's membranous barriers. In addition, some polypeptides must be targeted to positions within one of the mitochondrial membranes. Therefore, as four potential locations exist for each targeted polypeptide, the import process must be highly specific.

1.1.6.1 Mitochondrial protein import

Incorporation of nuclear-encoded proteins into mitochondria can be divided conceptually into five steps (shown schematically in Figure 1.1.3):-

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

2) recognition and binding of the precursor to the cytoplasmic surface of the mitochondrion;

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

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

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

Two principal mechanisms exist for the translocation of proteins across biological membranes. In both cases, protein synthesis starts on free cytoplasmic polysomes. During cotranslational transport, the nascent polypeptide chain is directed

Figure 1.1.3: Biogenesis of an imported mitochondrial protein

The steps in biogenesis are indicated as follows:-

1) Precursor synthesis

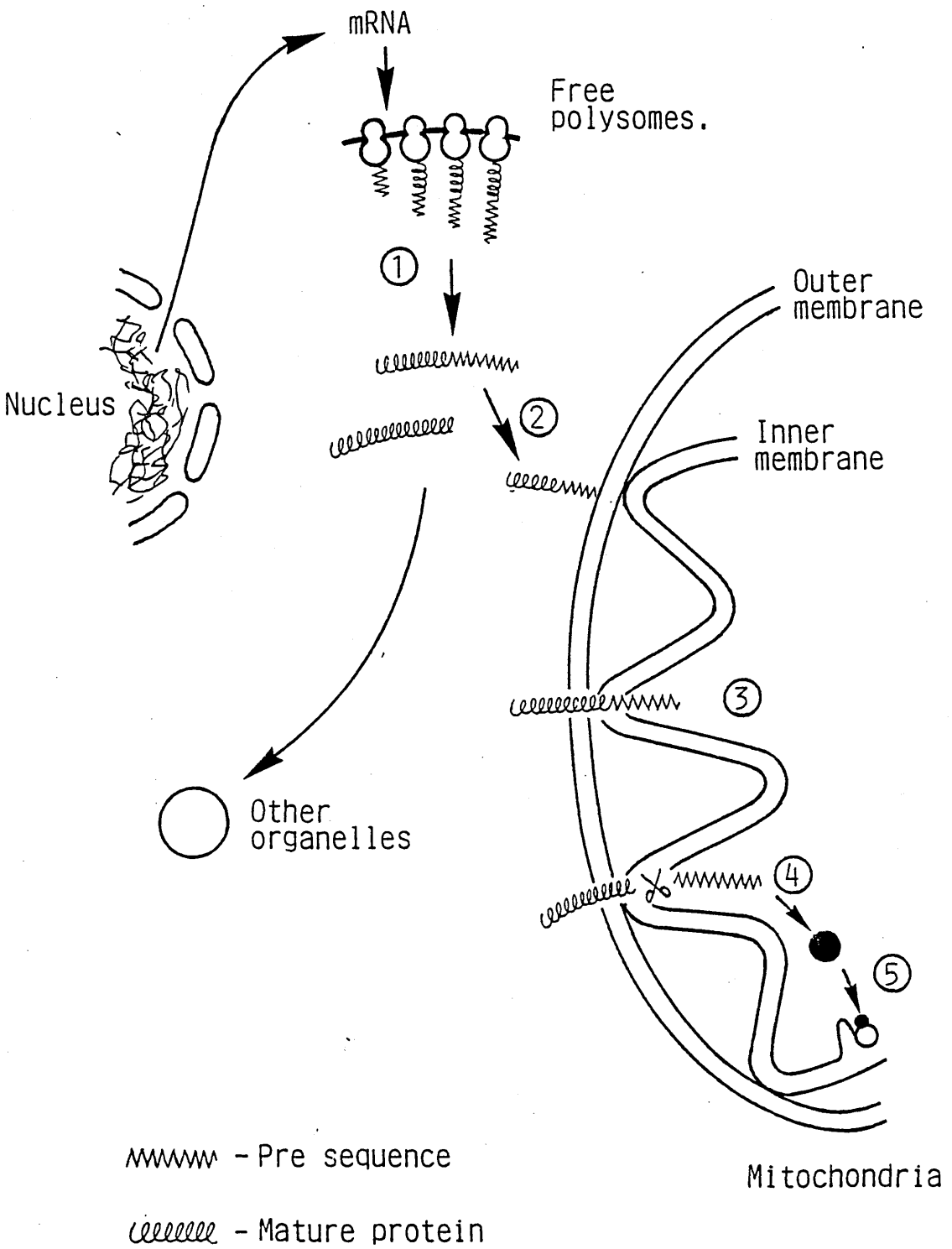
2) Delivery and binding of precursors to the mitochondrial surface

3) Translocation across membrane(s)

4) Processing of precursor polypeptides

5) Refolding and assembly into functional units

Figure 1.1.3: Biogenesis of an imported mitochondrial protein



to the target membrane as it emerges from the ribosome. Translocation is then initiated on resumption of protein synthesis after a temporary pause during initial membrane transfer. Translocation across the endoplasmic reticulum (ER) membrane of proteins which associate with the signal receptor particle (SRP) occurs in this fashion. However, the transport of some precursors across the ER membrane is stimulated by 70,000-M_r heat shock proteins (hsps; see Section 1.1.6.7) and occurs post-translationally. In post-translational transport, proteins bind to and are inserted into the target membrane after their synthesis is completed and the polypeptide has been released from the polysome. This type of membrane insertion is predominant in chloroplasts, peroxisomes, glyoxisomes and possibly mitochondria. The first evidence for a mitochondrial post-translational import mechanism came from *in vivo* experiments with intact cells. At various times after a pulse with radioactive amino acids, cells were fractionated and specific labelled precursor proteins analysed by immune precipitation. It was observed that labelled proteins first appeared in a cytosolic pool, in soluble form, and were detected in mitochondria only after a certain lag period, which varied for different precursors. In addition, mitochondrial import continued during chase periods after the inhibition of protein synthesis with cycloheximide.

These findings have been substantiated by similar studies with pulse-labelled yeast spheroplasts (Mori *et al.*, 1981; Reid and Schatz, 1982; Janussi *et al.*, 1982). The post-translational uptake of specific mitochondrial precursor polypeptides has also been demonstrated initially using *in vitro* translation assays. Precursors were synthesised in a cell-free translation system, derived from *Neurospora crassa* or rabbit reticulocytes, in the presence of radioactive amino acids. It was found that isolated mitochondria were capable of importing these radiolabelled, ribosome-free polypeptides (Harmey *et al.*, 1977; Korb and Neupert, 1978; Maccecchini *et al.*, 1979; Zimmerman and Neupert, 1980).

There is evidence that, in some cases, translocation may follow a cotranslational mechanism *in vivo* . The existence of mitochondrially-bound polysomes has been demonstrated in yeast cells (Ades and Butow, 1980), but their role in protein import does not appear to be obligatory for this process (Suissa and Schatz, 1982). 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. However, although most of the evidence points to this, it may be strictly a kinetic phenomenon and it cannot be ruled out that a cotranslational import process also occurs under some physiological conditions.

1.1.6.2 Mitochondrial precursor proteins

Precursor polypeptides targeted to the mitochondria possess critical differences to their mature counterparts. These can be categorised as follows:-

1) precursors must be soluble in the cytosol;

2) they have to be specifically recognised by mitochondria;

3) they must assume a conformation which is competent for translocation into or across membranes.

Most mitochondrial precursors contain cleavable N-terminal peptide extensions (presequences), which direct them to the organelle. Specific proteolytic processing enzymes exist within mitochondria for the maturation of delivered precursors (Bohni *et al.*, 1980). There are however, several precursor proteins which are targeted to each of the mitochondrial compartments without exhibiting transient signal sequences. For such proteins, it has been proposed that an internal targeting sequence directs mitochondrial import (Gasser and Schatz, 1983). The absence of a cleavable presequence has been documented for, among others, the ADP/ATP carrier of the inner membrane (Zimmerman and Neupert, 1980) and the closely associated brown fat uncoupling protein (Freeman *et al.*, 1983). These will be discussed more fully in Section 1.2.

In some cases, these N-terminal presequences might serve to maintain the precursor in a conformation that prevents premature assembly outside the mitochondrion. There is also evidence that precursors which lack a transient N-terminal extension still differ from the mature protein in conformation. The extramitochondrial form of the ADP/ATP carrier binds to hydroxylapatite while the mature form does not (Schleyer and Neupert, 1984). The precursor to the ADP/ATP carrier also occurs in the cytoplasm in higher molecular weight complexes of 120 and 500 kDa (Zimmerman and Neupert, 1980). It has been proposed that these aggregates may protect the precursors from proteolysis in the cytosol, ensure they remain in a soluble state or be involved in proper presentation to the mitochondrial surface.

1.1.6.3 Mitochondrial receptors, cytosolic cofactors and protein-lipid interactions

The specificity of mitochondrial import and sorting implies a receptor-mediated process. Several lines of evidence suggest that proteinaceous receptors on the mitochondrial surface perform the key functions of recognition (Hennig *et al.*, 1983; Riezman *et al.*, 1983; Zwizinski *et al.*, 1983). In these studies, isolated mitochondria were pre-treated with low concentrations of proteases (insufficient to degrade the outer membrane barrier). This inhibited the subsequent import of mitochondrial precursor proteins. Although various outer membrane proteins have subsequently been tentatively identified as import receptors, the only polypeptide to date which is known to be essential for mitochondrial protein import *in vivo* is the yeast import site protein 42 (ISP 42; Vestweber *et al.*, 1988). In *N.crassa*, the equivalent polypeptide, termed MOM 38 (Kiebler *et al.*, 1990), is thought to be identical to the previously detected 'general insertion protein' (GIP, see Section 1.1.6.4; for review, see Glover and Lindsay, 1991).

However, the relative scarcity of proteins associated with the outer membrane suggests that other factors are involved in the initial binding of precursor molecules to the mitochondrial surface. Several studies have suggested a requirement of cytosolic cofactors for mitochondrial protein import (Ohta and Schatz, 1984; Chen and Douglas, 1987). These cofactors have been partially characterised and determined to be of proteinaceous structure (Chen and Douglas, 1987) and to possibly contain RNA (Firgaira *et al.*, 1984). The exact nature and function of these cytosolic cofactors remains elusive thus far. It is possible that they play a role in conferring solubility to cytosolic precursors, in keeping precursors in an import competent conformation and/or in the initial binding of precursors to the mitochondrial surface. However, it is likely that at least some of these cytosolic cofactors represent heat shock proteins (hsps; see Section 1.1.6.7), which stimulate the translocation of precursors across the mitochondrial and ER membranes.

A further suggestion is that precursor proteins, prior to binding to import receptors, interact with low affinity with lipids of the outer membrane. Studies of the interaction of chemically synthesised presequences with artificial lipid membranes (Espand *et al.*, 1986; Roise *et al.*, 1986; Tamm, 1986; Skerjank *et al.*, 1987) imply the existence of such a pathway.

1.1.6.4 Membrane contact sites and the role of the 'general insertion protein'

It is now thought that, after the binding of precursors to the mitochondrial surface, the next steps in the import pathway are carried out by the protease-protected 'general insertion protein' (GIP), located in the outer membrane (For reviews, see Hartl *et al.*, 1989 and Baker and Schatz, 1991). This protein appears to bind precursors, interpret their signal sequences, and specifically target them to the correct mitochondrial location (Pfaller *et al.*, 1988). Outer membrane proteins are released into this membrane by GIP, while precursors destined for the other mitochondrial locations (inner membrane, matrix, intermembrane space) are released at contact sites between the outer and inner membranes (see Figure 1.1.4). The one apparent exception to this translocation pathway is cytochrome *c* , the import of which differs from other mitochondrial proteins in several respects (see Hartl *et al.*, 1989).

In *N.crassa*, GIP is now thought to be identical to the MOM 38 outer membrane polypeptide which has been isolated as a multimeric complex containing several other proteins including two import receptors, MOM 19 and MOM 72 (Kiebler *et al.*, 1990).

It has long been accepted that import of precursors occurs at these translocation contact sites between the two mitochondrial membranes. From electron microscopic studies, points of membrane contact have been known about for many years (Hackenbrock, 1968). More recently, import via translocation contact sites has been demonstrated for the precursors of F_1ATPase subunit β, cytochrome c_1 (Schleyer and Neupert, 1985), the Rieske Fe/S protein of the bc_1 complex (Hartl *et al.*, 1986), the ADP/ATP carrier (Pfanner and Neupert, 1987), and cytochrome b_2 (Pfanner *et al.*, 1987).

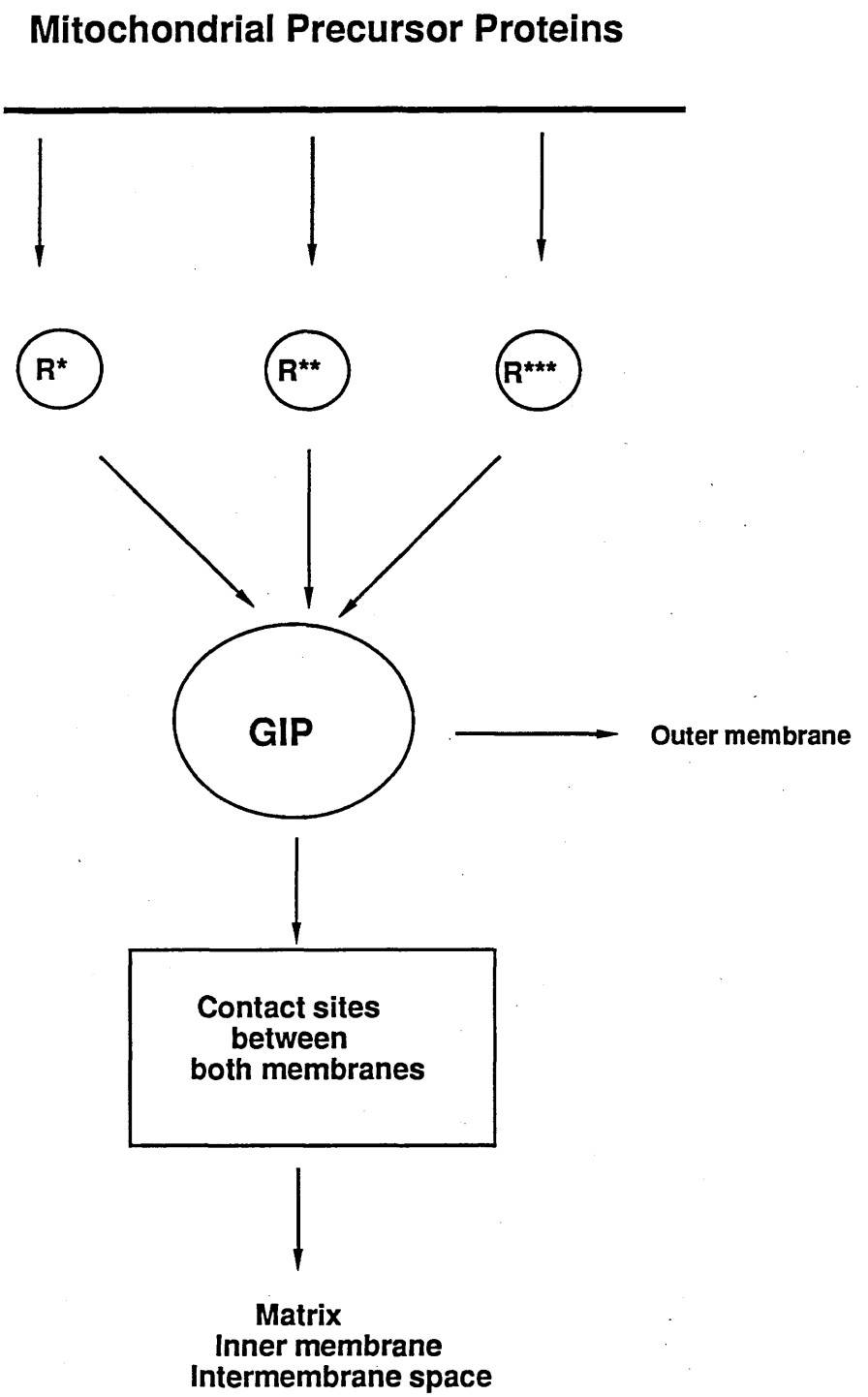
1.1.6.5 Energy requirement of mitochondrial import

Early studies on mitochondrial protein import showed that it was an energy-requiring process (Hallermayer and Neupert, 1976). Initial experiments indicated that ATP, and not the electrochemical potential across the inner membrane, was the primary energy source (Nelson and Schatz, 1979). However, later studies demonstrated that it was an energised inner membrane which was essential for mitochondrial protein import. Specifically, the electrochemical potential is necessary for the initial interaction of precursors with the inner membrane, and their subsequent

Figure 1.1.4: Working hypothesis for the binding and membrane translocation of mitochondrial precursor proteins

GIP represents the general insertion protein, while R*, R** and R*** represent different classes of outer membrane receptors. The model is adapted from Pfaller *et al.* (1988). In *N.crassa* GIP is now thought to be identical to the MOM 38 protein, which may exist as a multimeric outer membrane complex containing several import receptors (see Section 1.1.6.4).

Figure 1.1.4: Binding and membrane translocation of mitochondrial precursor proteins



transport into or across this membrane (Schleyer *et al.*, 1982). Those proteins which do not interact with the inner mitochondrial membrane do not require the membrane potential for import. However, several intermembrane space proteins do possess this energy requirement since their precursors transiently interact with the inner membrane and/or the matrix during distinct phases of their mitochondrial uptake (Gasser *et al.*, 1982; Reid *et al.*, 1982).

In addition to a membrane potential, mitochondrial protein import requires nucleoside triphosphates (NTPs). This was demonstrated by manipulating the levels of NTPs and electrochemical potential independently of each other (Pfanner and Neupert, 1986). Specifically, NTPs are necessary in most cases for both the initial interaction of precursors with the mitochondrial surface, and the completion of translocation into the organelle. Even protein import which does not require a membrane potential is usually dependent on NTPs.

1.1.6.6 Molecular basis of mitochondrial import

In recent years, the highly developed state of classical and molecular genetics in yeast has provided the foundation for analysing the molecular aspects of mitochondrial import. Gene fusion studies have demonstrated that the information required to direct a precursor to the mitochondrion is contained within its primary sequence (Douglas *et al.*, 1984; Hase *et al.*, 1984). This was established by observing that gene constructs encoding a mitochondrial amino terminus could import hybrid polypeptides containing a non-mitochondrial protein at the carboxyl terminus. Hurt *et al.* (1985) extended these studies to demonstrate that, in one specific case, the first twelve amino acids were sufficient to direct import to the correct mitochondrial compartment. This dodecapeptide retained the properties of the complete cleavable presequence in being hydrophilic, rich in basic and hydroxylated amino acids and devoid of acidic residues.

Based upon such elegant gene fusion studies, various models have been proposed for the targeting and intramitochondrial sorting of imported proteins (Roise *et al.*, 1986; Hurt and van Loon, 1986). The common theme running through these models is of mitochondrial presequences which can form amphiphilic α-helices, enabling them to interact directly with energised mitochondrial membranes. The ultimate intramitochondrial location of each precursor is determined by the

matrix-targeting sequence (as described above) and the presence or absence of a stop-transport domain (long interrupted sequence of uncharged amino acids followed by charged residues). If such a stop-transfer sequence is present, protein transport across the outer or inner membrane is prevented and the precursor molecule becomes an outer membrane, inner membrane or intermembrane space protein, depending on the disposition of the stop-transfer signal. The absence of such a domain results in the protein being targeted to the matrix. Cleavage sites exist for the proteolytic removal of presequences.

1.1.6.7 Assembly of imported proteins

The final stage of the import pathway remains the least understood, *i.e.* the assembly of these proteins into oligomeric complexes. The utilisation of genetic techniques in yeast as well as other organisms however, has led to a greater understanding of this process. A family of stress proteins or heat shock proteins (hsps) has been identified and found to be involved in several steps of mitochondrial protein import. These hsps have been classified according to similarities in their primary structure (For review, see Lindquist and Craig, 1988). The largest hsp family is composed of proteins of M_r values ~70,000. Cytosolic hsp70s, termed Ssa 1-4p in yeast (Werner-Washburne *et al.*, 1987), stimulate translocation of precursor proteins into mitochondria and, in some cases, the endoplasmic reticulum (ER), suggesting they are involved in conferring transport-competence to precursors. However, a mitochondrial hsp70 (Ssc 1p) has also been identified in yeast (Kang *et al.*, 1990). Located in the matrix, this hsp70 is involved in both translocation of precursors across the mitochondrial membranes and folding of imported proteins in the matrix. The 60,000-M_r family of hsps ('chaperonins') also includes a mitochondrial protein factor in yeast (Cheng *et al.*, 1989). Mitochondrial hsp60 is localised in the matrix as a high molecular weight complex, and is required for the assembly into oligomeric complexes of proteins imported into the matrix. Mitochondrial hsp60 is thought to function as an ATP-dependent 'foldase'.

The further application of genetic techniques, particularly in yeast, will fully clarify the functions of these hsps, and ultimately elucidate much of the remaining mystery of mitochondrial biogenesis.

1.1.7 Review of mitochondrial research

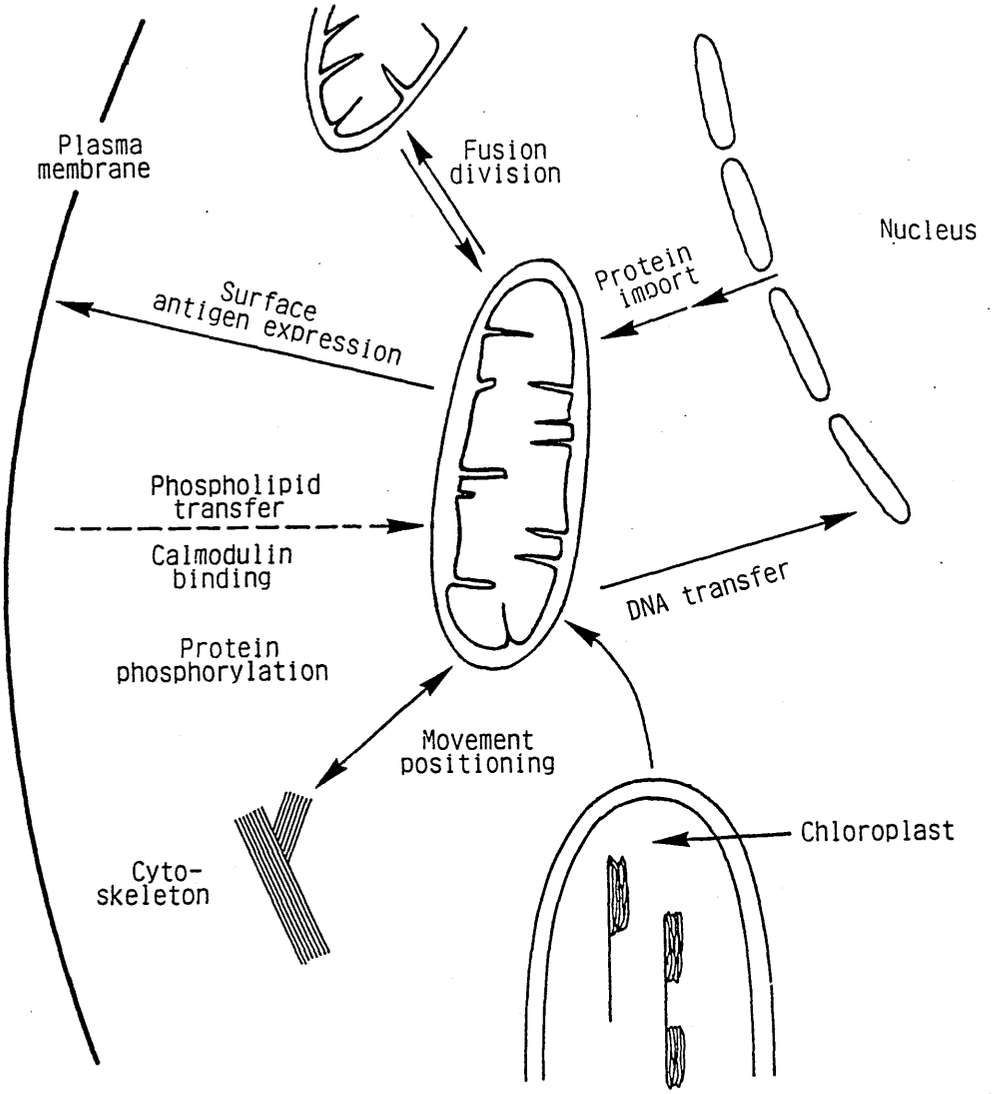
This introductory section (Section 1.1) illustrates the major advances in mitochondrial research over the last thirty years. Initially, mitochondria were thought to be self-contained organelles 'fed' by small molecules from the cytoplasm. This classical model originated from studies which demonstrated the ability of mitochondria to carry out all their major functions in the absence of cytoplasmic proteins and co-factors (Hogeboom *et al.*, 1948).

The discovery that mitochondria contain their own genome (Section 1.1.5) further supported this idea of autonomy, which was first disputed by Wildman *et al.* (1962) with the observation that these organelles are continually breaking up and fusing with each other. More recent observations, including the discovery that the overwhelming majority of mitochondrial proteins are nuclear-encoded, suggest that mitochondria interact extensively with other structures. Birkby (1978) noted the high level of genetic recombination between mitochondrial genome populations in yeast cells during somatic growth; Adams (1982) demonstrated that mitochondria exhibit saltatory motion; while Ball and Singer (1982) suggested that these organelles are tethered to the cytoskeleton, with this association dictating their movement. Previously it was believed that mitochondria were static organelles or moved via random diffusion.

Increasingly, a view of the eukaryotic cell is emerging in which mitochondria interact structurally, functionally and biosynthetically with other cellular components (Figure 1.1.5). This agrees with the endosymbiotic hypothesis of mitochondrial evolution, a phenomenon further supported by the ability of these organelles to transfer DNA to the host nucleus (see Section 1.1.5). This hypothesis receives further support from the hsp60 'chaperonin' class of protein factors. Structurally related hsp60s have been located in organelles of endosymbiotic origin (chloroplasts: Barraclough and Ellis, 1980; Ellis and van der Vies, 1988, mitochondria: see Section 1.1.6) and also in the bacterial cytosol (Georgopoulos *et al.*, 1973; Sternberg, 1973).

Interactions between mitochondria and the nucleus are not limited to the occasional exchange of genetic material. Mitochondrial biogenesis requires a continuously operating regulatory system between the two organelles. Knowledge of this system is still lacking and it represents a major area of future mitochondrial research. In addition to this, the further application of genetic techniques in the highly

Figure 1.1.5: Interactions between mitochondria and other subcellular structures



developed yeast system should answer the remaining questions regarding hsp function and mitochondrial biogenesis (see Section 1.1.6.7).

1.2 THE ADENINE NUCLEOTIDE AND PHOSPHATE TRANSPORT SYSTEMS

1.2.1 Introduction

In the aerobic cells of eukaryotic organisms, ATP is synthesised via two distinct routes, with some being generated via substrate-level phosphorylation reactions which occur predominantly in the cytoplasm. However, the vast majority of ATP is synthesised in mitochondria during oxidative phosphorylation. Once synthesised, mitochondrial ATP must be transported into the cytoplasm to be used in the many endergonic reactions of cellular metabolism, generating ADP, AMP and P_i. Conversely, ADP and P_i require to be transported into the mitochondria for ATP synthesis. Thus two mitochondrial transport systems are required which must be directly linked to ATP synthesis: specifically those for adenine nucleotides (ADP, ATP) and inorganic phosphate (P_i).

The transport of ADP and ATP is catered for by the adenine nucleotide translocase (ANT), the existence of which was first proposed by Klingenberg and Pfaff (1966). Subsequently, the carrier was defined by the binding of its substrates, ADP and ATP, and the highly specific inhibitors, atractyloside (ATR), carboxyatractyloside (CAT) and bongkrekic acid (BKA; Klingenberg *et al.*, 1970; Klingenberg *et al.*, 1971; Vignais *et al.*, 1973; Klingenberg *et al.*, 1975).

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

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

2) an electroneutral P_i/H^+ symporter or P_i/OH^- antiporter (For review, see Wohlrab, 1986) which catalyses the influx of 90% of mitochondrial P_i (Coty and Pedersen, 1975b). This carrier is now commonly referred to as the phosphate transport protein (PTP).

The latter system is dependent on the transmembranous pH gradient (see Section 1.1.3) and is inhibited by sulphydryl group reagents such as NEM, mersalyl and pCMB (Fonyo and Bessman, 1968; Tyler, 1969; Coty and Pedersen, 1974, 1975a; Wohlrab and Flowers, 1982). The P_i/dicarboxylate carrier is insensitive to NEM but is inhibited by mersalyl, pCMB or substrate analogues such as n-butyl malonate or 2-phenylsuccinate (Meyer and Tager, 1969; Meijer *et al.*, 1970; Coty and Pedersen, 1974).

1.2.2 Importance of adenine nucleotide and phosphate transport in cell physiology

The importance of adenine nucleotide and phosphate transport in cell physiology is evident from the various regulatory processes to which they are linked. Consequently, ANT and the major phosphate carrier, PTP are assigned as primary exchange carriers of the inner mitochondrial membrane:-

1) they are responsible for maintaining a continuous supply of substrates (ADP and P_i) for oxidative phosphorylation in the mitochondrial matrix-inner membrane compartment;

2) they are directly linked to the proton motive force generated across the membrane during electron transport (see Section 1.2.3);

3) their operation permits the functioning of secondary and tertiary exchange carriers of the inner membrane. PTP allows flux of dicarboxylic and tricarboxylic acids via the P_i/dicarboxylate (secondary) and dicarboxylate/tricarboxylate (tertiary) exchange carriers, thus controlling the distribution of a certain number of Krebs cycle intermediates.

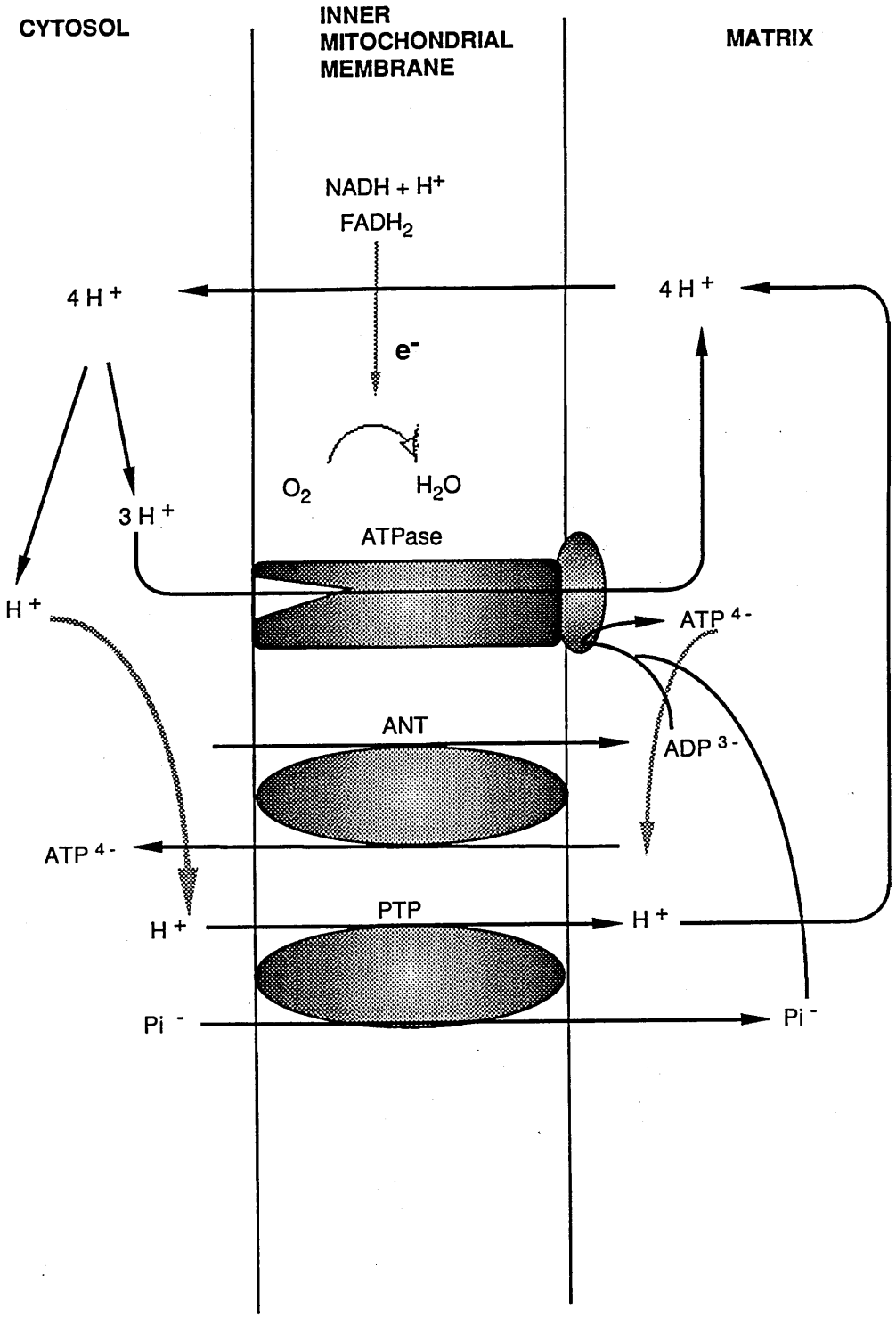
1.2.3 Role of ANT and PTP in oxidative phosphorylation

Both ANT and PTP are intimately involved in oxidative phosphorylation (see Figure 1.2.1). As well as controlling the influx of ADP and P_i for ATP synthesis, they

Figure 1.2.1: The role of ANT and PTP in oxidative phosphorylation

The diagram summarises the current standing of the Chemiosmotic Hypothesis. Four H^+ ions are illustrated being pumped out of the matrix at each proton-translocating complex although the exact number is the matter of some debate (see Section 1.2.3). However, only three protons are required to shuttle back into the matrix for the production of an ATP molecule. The resultant discrepancy in the electrochemical potential is corrected by the electrogenic ANT, while the extra proton is accounted for by the electroneutral PTP. ANT is thus driven by the membrane potential and PTP by the pH gradient.

Figure 1.2.1: The role of ANT and PTP in oxidative phosphorylation



are directly linked to the membrane potential and pH gradient components of the proton motive force, respectively.

The current standing of Mitchell's (1961) Chemiosmotic Hypothesis is the subject of much debate, and it now appears that the number of protons pumped out of the matrix varies for each of the proton-translocating complexes (NADH-Q reductase, QH_2-cytochrome *c* reductase and cytochrome *c* oxidase). Nevertheless, the apparent average is 3-4 protons per complex for each molecule of H_2O produced. However, only three protons are required to shuttle back into the matrix through the F_1F_0ATPase for the synthesis of one ATP molecule. This causes a discrepancy in both the electrochemical potential and the pH gradient. The charge discrepancy is corrected by the electrogenic ANT, which exchanges ADP^{3-} for ATP^{4-}, while the extra proton is accounted for by the electroneutral PTP (see Figure 1.2.1). ANT is thus driven by the membrane potential component of the proton motive force while PTP is driven by the pH gradient. The net effect (on the proton motive force) of PTP behaving as a P_i/OH^- antiporter rather than a P_i/H^+ symporter (as shown in Figure 1.2.1) is nil.

1.2.4 Identification and purification of ANT

The earliest evidence for the existence of an adenine nucleotide carrier in the inner mitochondrial membrane came from the observation that ADP/ATP transport through this membrane was specific (Klingenberg and Pfaff, 1966). As described earlier (Section 1.2.1), the carrier was subsequently defined by the binding of its substrates, ADP and ATP, and its inhibition by highly specific ligands (ATR, CAT and BKA).

The availability of the specific inhibitor, CAT, subsequently shown to be the most effective ligand for stabilising the carrier (Aquila *et al.*, 1978), in the ^{35}S-radiolabelled form has proved to be the key to identifying and purifying the translocase. The procedure commonly applied for the isolation of a membrane protein consists of solubilisation with a suitable detergent and subsequent identification by its capacity to bind specific ligands added to the crude mixture of solubilised proteins. Initially however, the identification of ANT by ^{35}S- CAT binding was unsuccessful since no specific binding of CAT was found after solubilisation of the membranes with

a variety of detergents. A breakthrough was achieved by loading the carrier with CAT in the parent mitochondria prior to extraction (Klingenberg *et al.*, 1974). It was then found that the CAT-protein complex could be solubilised with a variety of detergents (Riccio *et al.*, 1975a) and subsequently purified by applying standard fractionation procedures (Riccio *et al.*, 1975b).

Various detergents, both ionic and non-ionic, were used to solubilise mitochondria pre-loaded with ^{35}S-CAT; with the release of protein, free ^{35}S-CAT and protein-bound ^{35}S-CAT being assessed (Riccio *et al.*, 1975a). Detergents were applied in amounts giving near maximum solubilisation of total protein. Although ionic detergents were quite effective in solubilising CAT, it was found that the released ligand was in a free form, unbound to protein. Among the non-ionic detergents of the poly-glycol group, only Triton X-100 was found to be effective. However, Triton X-100 was unique among all the detergents tested, being the only case where the solubilised CAT remained largely bound to protein. In contrast, the ionic detergents appeared to denature the CAT-inhibited carrier. Riccio *et al.* (1975a) also determined that solubilisation of the CAT-ANT complex was enhanced by the presence of salt, the optimum conditions being 4% (w/v) Triton X-100, 0.5M NaCl (or KCl). Furthermore, it was established that the CAT-binding protein could be enriched in the mitochondrial membrane by extracting the more soluble proteins with 4% (w/v) cholate, 0.1M $(NH_4)_2SO_4$ or 0.5% (w/v) Triton X-100, 0.4M NaCl (Riccio *et al.*, 1975a).

The solubilisation of the CAT-complexed ANT from mitochondria facilitated the purification of this protein to apparent homogeneity, by standard fractionation techniques (Riccio *et al.*, 1975b). The Triton X-100 mitochondrial extract was directly applied to a hydroxylapatite (HTP) column and eluted with 0.5% (w/v) Triton X-100, 0.1M NaCl.The major ^{35}S-CAT containing fractions were pooled and tested electrophoretically. A single polypeptide of estimated M_r value 29,000 was detected and tentatively identified as the ADP/ATP carrier. Riccio *et al.* (1975b) also carried out a preliminary characterisation of the isolated protein, involving the displacement of CAT by ADP or ATP. Subsequently, the transporter has been further characterised by reconstitution studies in artificial liposomes (Schertzer and Racker, 1976; Schertzer *et al.*, 1977; Kramer *et al.*, 1977; Kramer and Klingenberg, 1977a,b). The subunit M_r

value of mammalian ANT is approx. 30,000 according to SDS-PAGE, although the carrier may exist as a homodimer in its functional state (Kramer and Klingenberg, 1979).

The purification scheme for ANT has remained essentially the same since the pioneering work by Klingenberg's group (Klingenberg *et al.*, 1974; Riccio *et al.*, 1975a; Riccio *et al.*, 1975b). However, it has been found that the critical purification step, adsorption chromatography with HTP, can be performed more quickly and efficiently by adopting a batch procedure rather than running a column (Kramer *et al.*, 1977). Kramer *et al.* (1977) also report that the carrier can be isolated in an unliganded form by replacing Triton X-100 with 3-lauramido-*N,N*-dimethylpropylamine *N*-oxide (LAPAO), as the extracting detergent. The same group have subsequently claimed that unliganded ANT can also be purified in Triton X-100 by application to HTP prepared in their own laboratory, rather than material obtained commercially (Kramer and Klingenberg, 1979).

1.2.5 Identification and purification of PTP

On the basis of the observations noted in Section 1.2.1, any protein which is responsible for the majority of mitochondrial phosphate transport must have an affinity for its substrate, P_i; it must react with sulphydryl group reagents which inhibit transport; and it must be capable of catalysing phosphate transport when incorporated into artificial liposomes in the correct orientation. PTP purification has however, been hindered by the lack of a specific inhibitor as exists for ANT (CAT, ATR and BKA).

An affinity for P_i was first observed in chloroform extracts from rat liver mitochondria (Kadenbach and Hadvary, 1973). These extracts were also found to contain proteins with free sulphydryl groups, with a reduced affinity for P_i being noted upon the addition of sulphydryl group reagents. Similarly, a 10,000-M_r cardiolipin-associated proteolipid isolated from yeast was found to contain sulphydryl groups and exhibit an affinity for $[^{32}P]P_i$ (Guerin and Napias, 1978). However, subsequent studies have failed to identify a protein of M_r value 8-10,000 involved in phosphate transport.

Coty and Pedersen (1975a) found that if phosphate transport in rat liver

mitochondria was inhibited by the addition of limiting amounts of [^{3}H]NEM, at least 10 different radiolabelled polypeptides could be detected by SDS-PAGE, demonstrating the non-specific manner by which NEM reacts with proteins of the inner mitochondrial membrane. In the same study, Coty and Pedersen (1975a) developed an elegant procedure for labelling the sulphydryl groups involved in phosphate transport more specifically, the basis for which was their observation that mercurial compounds inhibit PTP in a specific and reversible manner. The phosphate transport system (PTS) was initially protected by inhibition with the mercurial mersalyl, with all free sulphydryl groups not involved in transport then being blocked by the addition of non-radiolabelled NEM. Mersalyl inhibition of PTP was reversed by treatment with DTT, restoring about 65-75% of original carrier activity. When phosphate transport was then inhibited by [^{3}H]NEM, a marked increase in specificity was observed, with only five polypeptides in the M_r range 30-32,000 being modified. These prominent NEM-modified bands were subsequently localised in the inner membrane of mitochondria from various sources (Briand *et al.*, 1976; Hadvary and Kadenbach, 1976; Touraille *et al.*, 1977; Wohlrab and Greaney, 1978; Wohlrab, 1980). Coty and Pedersen (1975a) also established that mitochondrial phosphate transport is controlled by the transmembranous pH gradient, by demonstrating that uptake of [^{32}P]P_i was strongly stimulated by the ionophore, valinomycin.

PTP was further characterised by studies on the flight muscle of the adult blowfly, *Sacrophaga bullata*, a membrane system well suited to such studies as it is highly specialised, containing only the NEM-sensitive transporter and not the P_i/dicarboxylate carrier. Only two protein bands of M_r values 32,000 and 45,000 were labelled by [^{3}H]NEM in this membrane system (Wohlrab and Greaney, 1978). Since no equivalent to the 45,000-M_r band was detected in rat liver mitochondria (Coty and Pedersen, 1975a), Wohlrab and Greaney (1978) suggested the 32,000-M_r polypeptide as the most likely NEM-binding component of the PTS. Further studies by Wohlrab (1980) confirmed this result by identifying an NEM-sensitive 32,000-M_r polypeptide, via high resolution SDS-PAGE, in blowfly flight muscle and rat heart submitochondrial particles (SMP) as the strongest candidate for PTP.

PTP has since been isolated from bovine and pig heart mitochondria and reconstituted into artificial liposomes (Kolbe *et al.*, 1981; Touraille *et al.*, 1981). Purification involves the solubilisation of isolated mitochondria with the detergent Triton X-100 in a phosphate-containing buffer, followed by adsorption chromatography with HTP. The protein was identified by conventional SDS-PAGE analysis as a single band, M_r value 34,000, which could be labelled with [^{3}H]NEM (Wohlrab, 1980; Kolbe *et al.*, 1981; Touraille *et al.*, 1981). Wohlrab (1980) and Wohlrab and Flowers (1982) reported co-purification of unliganded ANT in their PTP preparations. This can be removed by a further fractionation step, adsorption chromatography with Celite (Kolbe *et al.*, 1981) or mersalyl-ultrogel (Touraille *et al.*, 1981).

One of the main criteria to be fulfilled by a purified component of the PTS is that it should catalyse a P_i-P_i exchange when reconstituted into artificial liposomes. The 34,000-M_r polypeptide, as isolated by Kolbe *et al.* (1981) and Touraille *et al.* (1981), could maintain only 2% of the activity achieved in isolated mitochondria by Coty and Pedersen (1975b). This exchange was however, found to be sensitive to NEM and mercurials but insensitive to the ANT inhibitors, CAT and *n* -butyl malonate. Nevertheless, these low levels of phosphate exchange observed *in vitro* may indicate that PTP as isolated merely represents the NEM-sensitive, transmembrane component of a more complex PTS as exists in bacteria.

Kolbe *et al.* (1984) attempted to further characterise PTP by employing a large scale purification scheme from bovine heart. After silver-staining of the SDS-polyacrylamide gel, two bands were identified in the ratio 1:1, and assigned as the putative α and β subunits of PTP. However, peptide maps of the two subunits are essentially identical following fragmentation with either CNBr or HCl/dimethyl sulphoxide/HBr. In addition to this, carboxymethylation of the sample prior to SDS-PAGE resulted in the detection of only a single band (M_r 34,000), suggesting that apparent subunit heterogeneity is caused by the formation of transient disulphide bonds during electrophoresis (Kolbe and Wohlrab, 1985). More recently, Gibb *et al.* (1986) reported an improved scheme for the purification of PTP from rat liver mitochondria,

involving a modification of the method of Kolbe *et al.* (1981). SDS-PAGE revealed a single polypeptide, subunit M_r 34,000, after adsorption chromatography on Celite, in general agreement with the value quoted from other mammalian sources (Wohlrab, 1980; Kolbe *et al.*, 1981; Touraille *et al.*, 1981). Gibb *et al.* (1986) also reported a method for characterising the carrier using an antiserum raised against the 34,000-M_r protein. Specific partial inhibition of phosphate uptake, as measured by iso-osmotic swelling in the presence of $(NH_4)_2HPO_4$, was achieved when mitoplasts (mitochondria minus outer membrane) were incubated with this antiserum.

1.2.6 Relationship of cardiolipin to PTP activity

Bisaccia and Palmieri (1984) have obtained evidence that cardiolipin can be used to improve the purification of PTP to such an extent that only one major protein band is detectable by gradient SDS-PAGE. Previous studies have demonstrated that both total protein purified and reconstituted transport activity can be significantly increased by the inclusion of cardiolipin in the solubilisation buffer (Kadenbach *et al.*, 1982; Mende *et al.*, 1982). The specific effect of cardiolipin was corroborated by results showing a reversal of enhanced PTP activity in the presence of doxorubcin, which forms a specific complex with this lipid (Cheneval *et al.*, 1983). This apparent affinity for cardiolipin strongly supports the idea of a specific interaction of the carrier protein with this lipid which, in reconstitution studies, may maintain PTP in its native conformation. This specific effect has not been observed with any other phospholipids tested.

1.2.7 Further characterisation of ANT and PTP: structural and functional studies on the carriers

Attempts to further understand the modes of action of the primary mitochondrial exchange carriers, ANT and PTP, have concentrated on elucidating their transmembrane arrangements and identifying their substrate-binding sites. Such studies have concentrated principally on the ADP/ATP carrier, the first of the two to be successfully purified, which has the added advantage of being susceptible to a range of highly specific inhibitors, ATR, CAT and BKA (see Section 1.2.4).

A greater understanding of how ANT functions at the molecular level has come from reconstitution studies with the purified bovine heart carrier (Schertzer and Racker, 1976; Schertzer *et al.*, 1977; Kramer *et al.*, 1977; Kramer and Klingenberg, 1977a,b; Kramer and Klingenberg, 1979). In particular, the ability of Klingenberg's group to purify ANT in both the liganded and unliganded forms (Kramer *et al.*, 1977; Kramer and Klingenberg, 1979) has facilitated characterisation of the carrier. After addition of unliganded ANT to lecithin liposomes, ADP/ATP exchange is activated by sonication. By then correlating CAT (or ATR) and BKA binding with adenine nucleotide transport, three populations of the inserted protein were identified (see Figure 1.2.2):-

1) a portion (up to 5%) with high exchange activity, comparable to the original transport rates, was differentiated. This class of the protein is inhibited by both CAT (or ATR) and BKA. Separate binding studies with these ligands indicate that this portion is orientated in the same direction as in mitochondria;

2) a population of very weakly exchanging, though still active, carrier molecules (about 25%), which are inhibited only by BKA and not by CAT (or ATR), was differentiated. This portion of transporters appears to be orientated in the opposite direction as in mitochondria and reaches only about 0.2% of the activity of the carrier in the first population;

3) the remainder of the carrier protein (about 70%) was irreversibly denatured during isolation and reconstitution.

Separate binding studies demonstrated that CAT and ATR are unable to permeate the mitochondria, and bind exclusively to the cytosolic surface of the inner membrane (Klingenberg *et al.*, 1973). In contrast, BKA is a permeant ligand capable of binding to the matrix side of the membrane. In addition to this, these ligands appear to demonstrate half-site reactivity ie. one molecule of ATR, CAT or BKA binds to two ANT subunits. This data justifies the interpretations explained above and illustrated in Figure 1.2.2. It thus appears possible that native ANT exists as a homodimer, as previously suspected, with matrix ('m') and cytosolic ('c') binding sites. CAT and ATR bind at the 'c' site, which is also the location of ADP binding, whereas BKA binds at the 'm' site.

These results also support Klingenberg's (1976) 'gated pore' model for membrane carriers (see Figure 1.2.3) which proposes that a single channel is formed

Figure 1.2.2: Binding and exchange properties of three reconstituted ANT populations in liposomes

The carrier protein in its functional state is shown as a dimer with the 'c' (cytosolic) or 'm' (matrix) binding site exposed to the external ('e') or internal ('i') side of the liposomal membrane. The diagram is adapted from Kramer and Klingenberg (1979). For further details see Section 1.2.7.

Figure 1.2.2: Binding and exchange properties of three reconstituted ANT populations

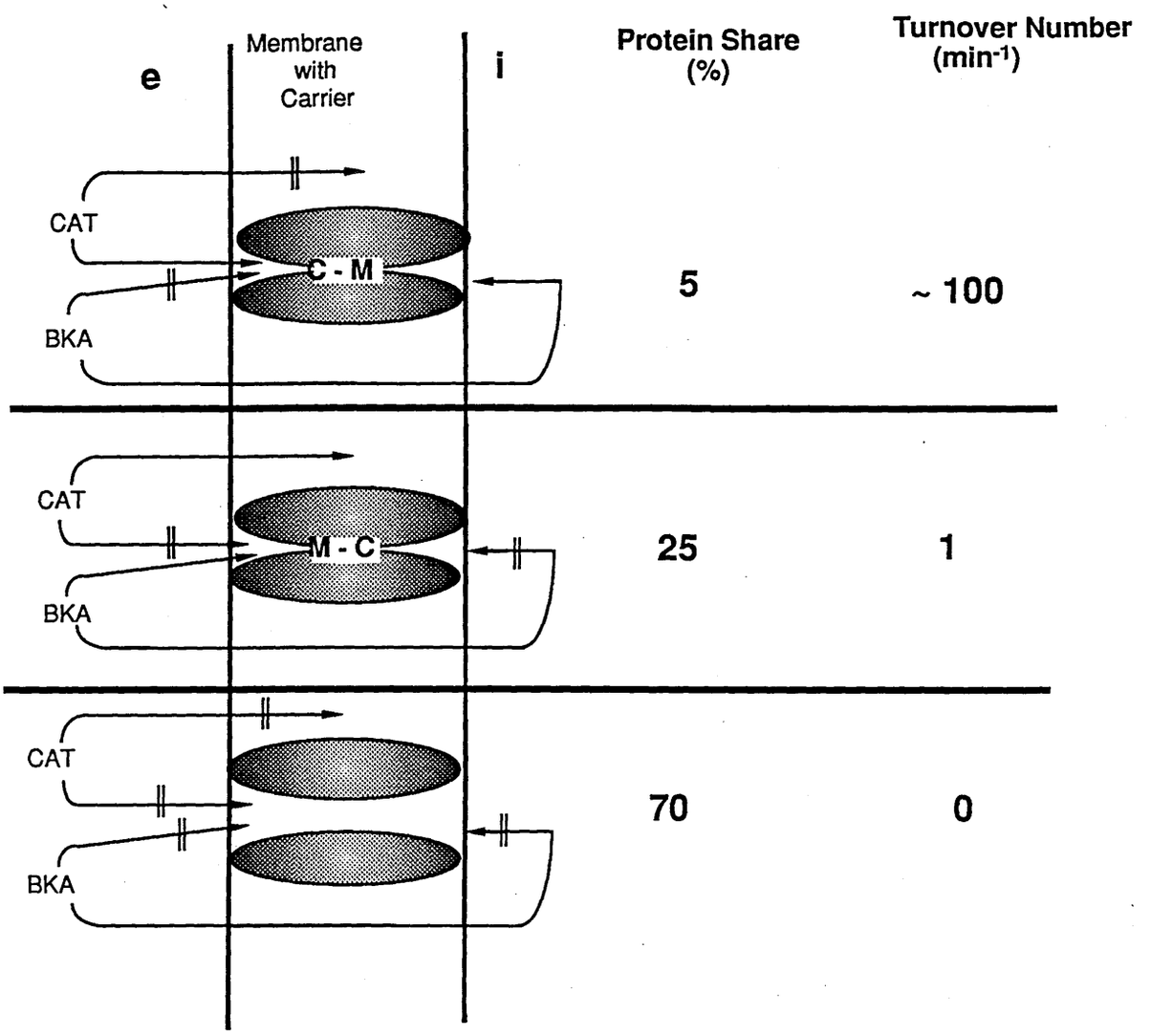
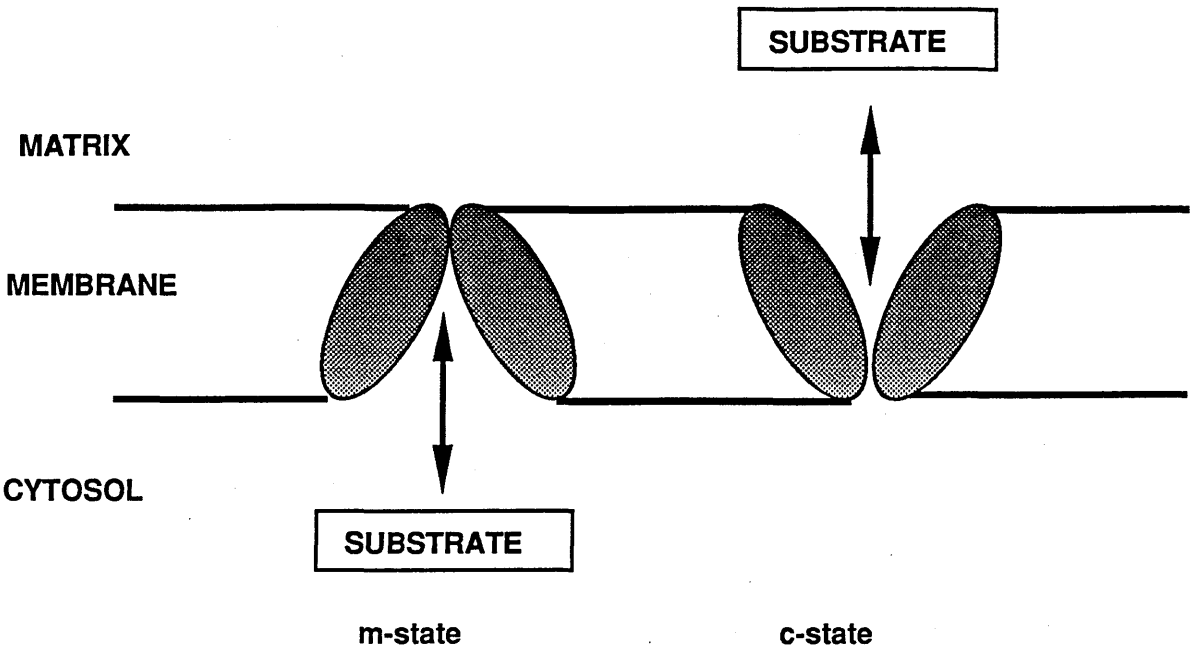


Figure 1.2.3: 'Gated Pore' model of transmembrane carriers

The carrier is shown as a dimer with a central channel in the 'm-state' or 'c-state'. Substrate-binding (as shown) induces the conformational change necessary for translocation (Adapted from Klingenberg, 1976).

Figure 1.2.3: 'Gated Pore' model of transmembrane carriers



from the two subunits, with the carrier capable of existing in either an 'm-state' or 'c-state'. Binding of the substrate induces a conformational change which opens the channel gate to facilitate translocation. The gate can only open to one side of the membrane at a time (Klingenberg, 1981). Further studies with CAT, BKA and the sulphydryl group reagent NEM appear to offer further support to this model of ANT function. NEM binding to ANT in the presence of ADP is inhibited by CAT but not BKA (Aquila *et al.*, 1982a). The BKA-ANT complex incorporates about one molecule of NEM per subunit more than the CAT-ANT complex. It was concluded that the single reactive sulphydryl group is masked in the 'c-state', but unmasked due to re-orientation as the 'm-state' is formed upon the addition of ADP. CAT, which binds at the cytosolic side of the carrier, inhibits NEM binding by stabilising ANT in the 'c-state'. In contrast, BKA binds at the matrix side of the carrier and stabilises ANT in the 'm-state'.

With the primary structure of bovine ANT being elucidated (Aquila *et al.*, 1982b), attempts have been made to characterise the exact binding sites of the various ligands. Boulay *et al.* (1983) positioned the CAT/ATR binding site in the centre of the molecule between Cys^{159} and Met^{200}. More recently, photolabelling work by Dalbon *et al.* (1988) using non-permeant derivatives of ADP, in conjunction with Boulay's data, indicated that the region Phe^{153}-Met^{200} represents the common binding centre for CAT/ATR and ADP, with Tyr^{250}-Met^{281} also being involved.

Bogner *et al.* (1986) used the lysine reagent pyridoxal 5-phosphate to investigate the transmembrane arrangement of the ADP/ATP carrier. The high proportion of lysines present in the bovine ANT sequence meant that this residue could be used to probe the folding, sidedness and distribution of the amino acid chain with respect to the membrane. Furthermore, lysine groups were expected to participate in the cationic binding areas for the negatively charged substrates, ADP and ATP and for the inhibitors, ATR, CAT and BKA. Several lysines possibly involved in the translocation pathway were identified (Lys^{22}, Lys^{42}, Lys^{48}, Lys^{106}, Lys^{162}), and a transmembrane folding model for the carrier was presented (see Section 1.2.9).

Further characterisation of PTP has proved more difficult owing to the lack of equivalent specific inhibitors to this carrier and the low level of phosphate exchange

observed in reconstitution studies. However, Tommasino *et al.* (1987) attempted to identify the substrate-binding site of PTP by utilising the photoreactive phosphate analogue, 4-azido-2-nitrophenyl phosphate (ANPP). They successfully demonstrated that [^{32}P]ANPP competitively inhibits phosphate transport in the dark, and that upon photoirradiation the probe irreversibly binds to the carrier. Inhibition is dependent on both the pH of the medium and the concentration of the probe. ANPP thus appears to be a suitable reagent for characterising the phosphate-binding site of PTP, assuming it is possible to localise the modified amino acids in the primary structure of the carrier.

Another possible probe which may prove useful in the characterisation of the PTP substrate-binding site is *N,N'*-dicyclohexylcarbodiimide (DCCD), a reagent which inhibits proton-translocating carrier proteins by covalently binding to residues in their pores (Beechey *et al.*, 1967). Previously, Houstek *et al.* (1981) proposed that the mitochondrial DCCD-reactive 33,000-M_r protein is identical with PTP. However, subsequent studies have indicated that this protein may represent porin (De Pinto *et al.*, 1985).

Topographical studies have been used to elucidate the membrane organisation of PTP. Phelps (1987) demonstrated that the bovine carrier's membrane orientation rendered it essentially resistant to proteolysis, indicating that no part of PTP is exposed on the membrane surface. However, the non-specific protease, proteinase K cleaves the carrier into two segments with M_r values 11,000 and 23,000. The potential thus exists for further topographical studies involving the analysis of these proteolytic products by immune precipitation and N-terminal sequencing, since the primary structure of bovine PTP is now known (Runswick *et al.*, 1987).

From the above, it is apparent that the further characterisation of ANT and PTP has been facilitated in recent years by the elucidation of their primary structures from both amino acid sequencing and nucleotide sequencing of cDNA clones encoding the proteins. The complete amino acid sequence of bovine heart ANT was described by Aquila *et al.* (1982b) after liquid-phase sequencing of tryptic peptides. Subsequently, the primary structure of ANT from three other sources, *Neurospora crassa* (Arends and Sebald, 1984), *Saccharomyces cerevisiae* (Adrian *et al.*, 1986) and *Zea mays* (Baker and Leaver, 1985), has been established by cDNA sequencing. In the case of

PTP, the first information on primary structure came from Kolbe and Wohlrab (1985) who purified and sequenced a 47-residue N-terminal formic acid fragment of the bovine heart carrier, identifying the NEM-binding site as Cys^{42} (see Section 1.2.8). Subsequently, Aquila *et al.* (1987) elucidated the full primary structure of bovine PTP, bar 28 residues, by sequencing chemically-cleaved peptides. More recently, the amino acid sequence of PTP from three sources, bovine heart (Runswick *et al.*, 1987), rat liver (Ferreira *et al.*, 1989) and *S.cerevisiae* (Phelps *et al.*, 1991), has been established from isolated cDNA clones. The implications of this primary structural information will be discussed in Section 1.2.9.

1.2.8 Relative location of sulphydryl groups of ANT and PTP

An important feature of ANT and PTP in mammalian mitochondria is that both are sulphydryl-containing proteins with one or more of these sulphydryl groups being required for function. The inhibition of PTP by sulphydryl group reagents is well established and led to the purification of the carrier (see Section 1.2.5). Phosphate transport is inhibited by both permeable inhibitors (NEM and mersalyl) and the inner membrane-impermeable sulphydryl group reagent, eosin-5-maleimide (EMA; Houstek and Pedersen, 1985), suggesting that the reactive cysteine residue is located on or near the cytosolic side of the inner mitochondrial membrane. As previously discussed, Kolbe and Wohlrab (1985) identified the sole NEM-reactive residue as Cys^{42}, after isolating and sequencing a 47-residue N-terminal formic acid fragment of bovine PTP (see Section 1.2.7).

The ADP/ATP carrier was originally thought to be insensitive to sulphydryl-reactive reagents. However, sensitivity can be demonstrated if mitochondria are preincubated with a low concentration of either ADP or ATP. Under these conditions, the sulphydryl groups are unmasked and become reactive towards NEM (see Section 1.2.7). Houstek and Pedersen (1985) found that in intact mammalian mitochondria, the impermeable probe EMA is unreactive with ANT, even under conditions which promote maximal labelling by NEM (*i.e.* ADP present). However, EMA labels ANT in 'inverted' inner membrane vesicles, even in the presence of NEM. Labelling is prevented if the vesicles are prepared from mitochondria pretreated with CAT. It was interpreted from this data that, with respect to EMA, sulphydryl groups of

the ADP/ATP carrier occur in two distinct classes, both of which are inaccessible in intact mitochondria. Only one class, depending on conditions, can be exposed in SMP. Houstek and Pedersen (1985) propose that EMA and NEM label different sulphydryl groups of ANT. The single NEM-alkylated residue has been identified as Cys^{56} by chemical and enzymatic cleavage techniques (Boulay and Vignais, 1984), but the functional role, if any, of the other three sulphydryl groups (Cys^{129}, Cys^{159}, Cys^{256}) is unknown.

1.2.9 ANT and PTP: prominent members of a family of structurally related mitochondrial exchange systems

As previously discussed, the primary structures of the mitochondrial adenine nucleotide and phosphate carriers have been elucidated from various sources (see Section 1.2.7). In addition to this, the amino acid sequence of a third solute carrier involved in energy transfer has been elucidated: the uncoupling protein (UCP) of brown fat mitochondria (Aquila *et al.*, 1985). UCP has a function which is somewhat contrary to that of the other two carriers (ANT and PTP), as it dissipates oxidative energy into heat. It functions as an H^+ carrier and short-circuits the proton motive force generated by electron transport, thus bypassing ATP generation (For review, see Nicholls and Locke, 1984). Like ANT, UCP has an adenine nucleotide-binding site, but whereas ADP and ATP activate transport by ANT, they inhibit proton translocation by UCP. The uncoupling protein is a highly specialised carrier found only in brown adipose tissue, which is potentially present in all mammalian organisms (Locke *et al.*, 1982).

Analysis of the sequences of these three mitochondrial carriers has led to the belief that they form a structurally related protein family with a common evolutionary origin (Aquila *et al.*, 1987). The elucidation of primary structures has also highlighted various similarities and differences between the carriers:-

1) they all contain 300-350 amino acids, with subunit M_r values 30-35,000, although it is possible that they exist functionally as homodimers, as previously described for ANT (see Section 1.2.8);

2) although all three carriers are nuclear-encoded and synthesised on cytoplasmic

ribosomes (Hackenberg *et al.*, 1978), there appear to be differences with respect to their modes of insertion into the inner mitochondrial membrane. cDNA sequencing of ANT from various sources indicates that the precursor form of this carrier does not contain a cleavable presequence (Arends and Sebald, 1984; Adrian *et al.*, 1986), supporting the view that the information required to target ANT to the mitochondria is contained within its primary structure (see Section 1.1.6.2). This also appears to be the case for UCP (Freeman *et al.*, 1983). However, the nucleotide sequences of cDNA clones encoding PTP from bovine heart (Runswick *et al.*, 1987) and rat liver (Ferreira *et al.*, 1989) indicate that this protein is synthesised as a higher-M_r precursor with an N-terminal signal sequence. More recently, Phelps *et al.* (1991) have cloned and sequenced the gene encoding PTP in *S.cerevisiae*. In contrast to the mammalian carrier, no presequence has been detected. Therefore, although these three carriers form a family of mitochondrial exchange systems, they appear to differ in their modes of biosynthesis;

3) these proteins are very highly conserved over long evolutionary distances. This is particularly true for ANT, which has been sequenced from four sources (bovine, *N.crassa, S.cerevisiae, Z.mays;* see Section 1.2.7). In the case of PTP, 116 amino acids are conserved between the yeast and bovine proteins (Phelps *et al.*, 1991).

The most striking feature noted about these three carriers upon analysis of their amino acid sequences is the high degree of structural homology which exists between them (Runswick *et al.*, 1987; Aquila *et al.*, 1987). They all contain a three-fold repeated sequence element 100-125 amino acids in length, with the repeats in each of the three proteins related to each other (tripartite structure). This implies that the carriers have related three-dimensional structures and mechanisms of action. In order to investigate the secondary structure and transmembrane folding of the proteins, the average hydrophobicity distribution (Kyte and Doolittle, 1982) of ANT and UCP has been analysed (Aquila *et al.*, 1985; Aquila *et al.*, 1987). For ANT, only one transmembrane α-helix was initially detected, whereas three such domains could be visualised in UCP. However, both proteins contain about 41% α-helical structure according to circular dichroism (CD) measurements (Aquila, unpublished data). Also, the fact that these

carriers are integral membrane proteins strongly suggests that the number of transmembrane α-helices must be considerably greater. Subsequently, further analysis of sided hydropathy profiles allowed for the prediction of amphipathic helices in all three carriers (Aquila *et al.*, 1987). In this manner, the similarity in the hydrophobicity distribution of the three carriers became obvious and, in particular, the tripartite structure of the proteins could be clearly detected.

Further hydropathy profiles, using the HYDROPLOT computer programme (Runswick *et al.*, 1987), confirmed the tripartite structure and indicated that each repeated sequence element consists of two membrane-spanning amphipathic α-helices linked by an extensive hydrophilic domain (see Figure 1.2.4). Some of the putative membrane-spanning domains are less hydrophobic than others, containing basic and acidic residues which are thought to be involved in pore formation. Alignment of the hydropathy plots with the primary structures of the carriers shows that the most highly conserved residues are located in the proposed α-helical segments (I-VI; see Figure 1.2.4) and also in the regions of the hydrophilic domains (A,B and C) that connect them to the α-helices. The central parts of domains A-C are poorly conserved, as are the N- and C-termini and the regions between the repeats. It is possible that these regions contribute to the specificities of the carriers.

Aquila *et al.* (1987) used their hydropathy profiles to suggest a topographical arrangement for UCP in the inner mitochondrial membrane, which they propose as a common carrier model for this family of proteins (see Figure 1.2.5). The transmembrane components of each repeat are visualised as consisting of two α-helices,the first being more hydrophobic and exposed to the lipid bilayer, with the second being more amphipathic and involved in pore formation. The structure of the hydrophilic domains remains unknown, although protease resistance (see Section 1.2.7) suggests that they may be involved in pore formation rather than being exposed as shown in Figure 1.2.5. The presence of a transmembrane amphipathic β-strand in these carriers was first suggested by Aquila *et al.* (1985) from hydrophobicity distribution studies on ANT, and subsequently proposed as a common feature. For

Figure 1.2.4: Hydropathy plots of UCP, ANT and PTP (Adapted from Runswick *et al.*, 1987)

A comparison is shown of the hydrophobic profiles of the mitochondrial carriers UCP (Uncoupler), ANT (ADP/ATP Translocase) and PTP (Phosphate carrier). The calculations were made using HYDROPLOT with a window of 11 amino acids. The horizontal bar represents an average hydrophobicity as defined by Kyte and Doolittle (1982); A, B and C are hydrophilic domains; and I-VI represent putative membrane-spanning α-helices.

Figure 1.2.4: Hydropathy plots of UCP, ANT and PTP

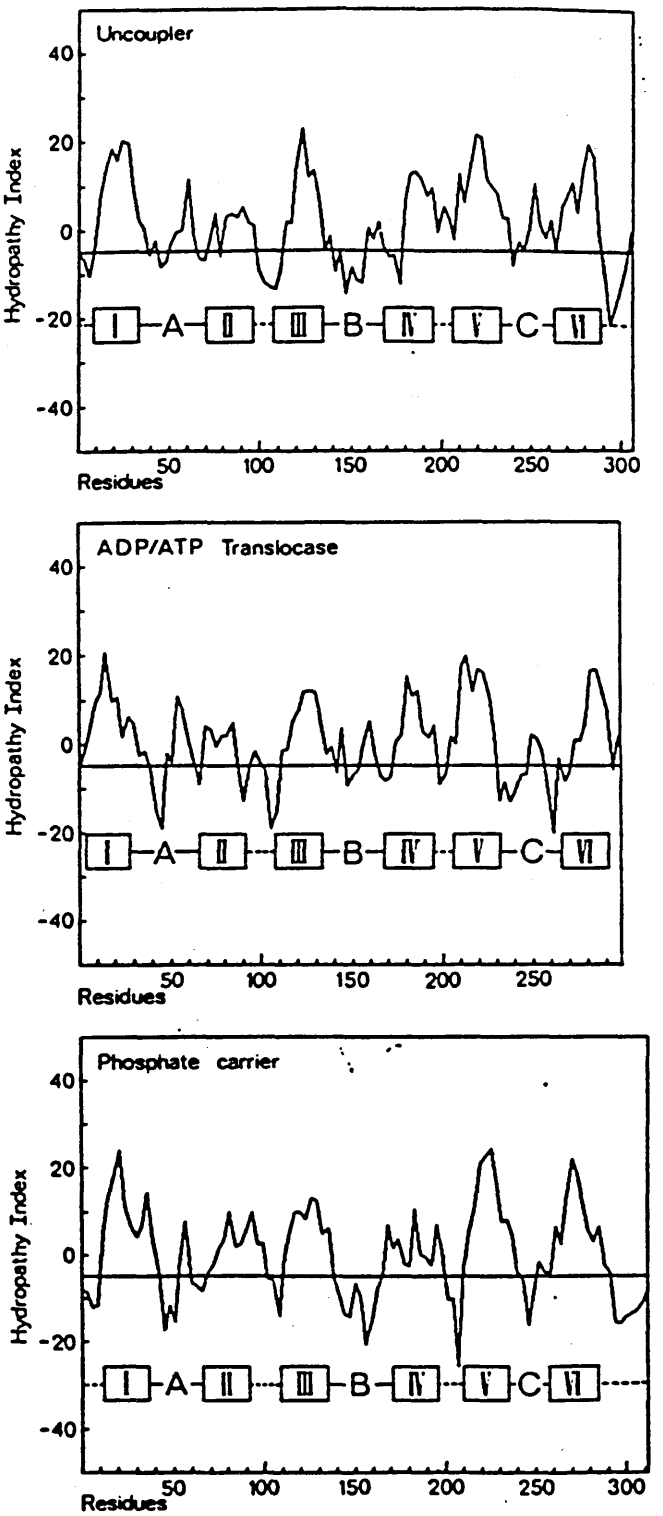
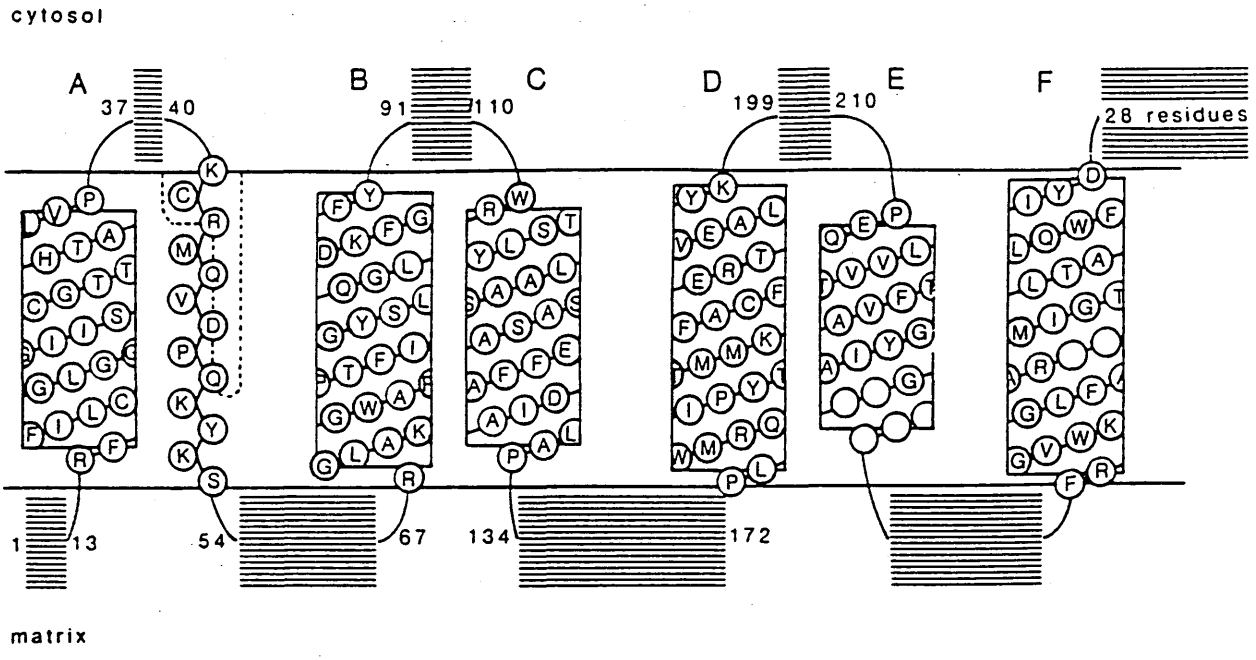


Figure 1.2.5: Proposed arrangement of PTP in the inner membrane, as representation for the family of mitochondrial carriers. The six membrane-spanning α-helices are shown as boxes and are labelled A-F starting from the N-terminus. A possible transmembrane β-strand (dashed line) is shown between helices A and B. The intervening hydrophilic segments are indicated by hatched boxes as their structure is unclear.
(Adapted from Aquila *et al.*, 1987)

Figure 1.2.5: Proposed arrangement of PTP in the membrane



UCP, there is evidence that the C-terminus is situated on the cytosolic surface (Aquila, unpublished data) which agrees with an uneven number of membrane-spanning domains with the N-terminal on the matrix side. More recently, Ferreira *et al.* (1991) have used anti-peptide directed antibodies, raised against the C-terminal of rat liver PTP, to show that this part of the carrier is exposed on the cytosolic surface of the membrane. This offers further support to the concept of a 7th transmembrane domain. However, the presence of β-strands is a rare phenomenon within transmembrane proteins and this aspect of the common carrier model requires further research.

Bogner *et al.* (1986) used the lysine reagent pyridoxal 5-phosphate to elucidate the transmembrane arrangement of ANT, by assessing which lysines were accessible and which were inaccessible in the presence or absence of CAT, ATR or BKA. As well as identifying several lysines possibly involved in the translocation pathway (see Section 1.2.7), a transmembrane folding model of ANT was presented, which agrees with the putative PTP structure described above and proposed as a common carrier model.

The elucidation of the primary structures of ANT, PTP and UCP has thus led to a greater understanding of the transmembrane arrangement of this family of mitochondrial exchange carriers as well as their modes of action at the molecular level. Nevertheless, the most striking observation upon comparison of the amino acid sequences is the occurrence of three repeats, and the recurrence of the tripartite structure in all three proteins. This observation is supported by hydrophobicity data, and suggests that each carrier is composed of three structurally similar domains with a common evolutionary origin. If the similarity of the repeats is based upon a true homology, it can be further speculated that other mitochondrial anion metabolite carriers, *eg.* the P_i/dicarboxylate carrier, are also derived from the same ancestral gene. Subsequent research has indeed indicated that further mitochondrial proteins contain the tripartite structure: the malate/2-oxoglutarate carrier and a yeast protein implicated in mitochondrial RNA splicing (Gargouri, 1988). Following the ancestral gene hypothesis, a hierarchical family of metabolite carriers can be derived, to which the primary mitochondrial transporters, ANT and PTP, belong. Furthermore, it is likely that PTP appeared first, and contained the single gene from which the other carriers

evolved. This hypothesis stems from the fact that PTP, unlike ANT, UCP and other proposed members of the 'tripartite' transporters family, is known to be present in prokaryotes. Further support has been provided by Pratt *et al.* (1991), who have clearly demonstrated that optimal import of rat liver mitochondrial PTP, unlike ANT and UCP, is dependent on the presequence absent from the other carriers. This indicates that the ancestral gene first appeared in PTP, and that subsequent evolutionary events leading to other mitochondrial carriers eliminated the presequence.

1.3 PYRUVATE DEHYDROGENASE COMPLEX

1.3.1 Multienzyme complexes

Enzymes are biological catalysts which normally function sequentially, promoting consecutive chemical reactions linked by intermediate products. When a number of enzymes are associated to form a defined entity capable of catalysing a series of linked biochemical conversions, this is termed a multienzyme complex. Well characterised examples include: tryptophan synthase from *E. coli* (Yanofsky and Crawford, 1972); fatty acid synthase from both *S.cerevisiae* (Lynen, 1972) and mammalian sources (Stoops *et al.*, 1978); and the 2-oxo acid dehydrogenase complexes present in all organisms studied to date (Reed, 1974; Perham, 1975). A full description of several multienzyme complexes can be found in reviews by Reed and Cox (1966) and, more recently, by Hardie and Coggins (1985).

Organisation into a multienzyme complex can provide a number of functional advantages including enhancement of catalytic or regulatory efficiency and substrate channelling (Perham, 1975). As a form of compartmentalisation, intermediates may be positioned on or within the complex in such a manner as to exclude competition by other biochemical pathways, or prevent decomposition in aqueous solution of unstable intermediates. Interaction of the component polypeptides, as in the case of tryptophan synthase, may produce aggregates with catalytic properties not present in the separate chains. Assembly of interrelated enzymes also makes it physically easier for the product of one enzyme to act as the substrate for the next in sequence. This can increase the efficiency of the overall process, although the intrinsic catalytic activity of each

component may not be altered by the association.

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

1.3.2 2-oxo acid dehydrogenase complexes

The 2-oxo acid dehydrogenase multienzyme complexes are associations of three enzymatic activities, a substrate-specific dehydrogenase (E1), a distinct dihydrolipoyl acyltransferase (E2) and dihydrolipoamide dehydrogenase (E3), a flavoprotein which is common to all three complexes.

Each complex catalyses a coordinated sequence of steps which can be represented by the following overall reaction:-

$R\text{-}CO\text{-}CO_2H + CoASH + NAD^+ \text{----> } R\text{-}CO\text{-}S\text{-}CoA + NADH + H^+ + CO_2$

$R=CH_3$ (pyruvic acid) for pyruvate dehydrogenase complex (PDC), and $HO_2C\text{-}(CH_2)_2$ (2-oxoglutarate) for the 2-oxoglutarate dehydrogenase complex (OGDC). In the case of the branched-chain 2-oxo acid dehydrogenase complex (BCOADC), $R\text{-}CO\text{-}CO_2H$ represents a 2-oxo acid derived from the metabolism of the branched-chain amino acids, valine, isoleucine and leucine (α-oxoisovaleric, α-oxoisocaproic and α-oxo-3-methylvaleric acids).

The acyltransferase (E2) constitutes the core of the complexes to which the E1 and E3 component enzymes are attached by non-covalent bonds. The total number of subunits is large and, depending on the type of complex and its source, generally in the range 60-100. These complexes may be self-assembling and form ordered, symmetrical structures. There are six coenzymes and prosthetic groups involved in the sequence of reactions catalysed by the complexes:- Mg^{2+}, thiamine pyrophosphate (TPP), lipoic acid, coenzyme A (CoASH), flavin adenine dinucleotide (FAD) and nicotinamide

adenine dinucleotide (NAD^+).

In different organisms the complexes are subject to regulation by ligand binding (Reed, 1974), covalent modification (Linn *et al.*, 1969), control of gene expression (Langley and Guest, 1978), proteinase action (Linn, 1974), product inhibition (Garland and Randle, 1964), hormonal control (Denton and Hughes, 1978; McCormack and Denton, 1984; Reed and Yeaman, 1987) and glucose oxidation *i.e.* in starvation or diabetes (Wieland, 1983). The specific reactions catalysed by PDC, the functionally analogous OGDC, and BCOADC are shown in Table 1.3.1.

In the eukaryotic cell PDC, OGDC and BCOADC are located in the mitochondrial matrix, and all three complexes play important roles in metabolism. PDC represents the critical link between glycolysis in the cytoplasm and the citric acid cycle in the matrix. In most aerobic organisms, the oxidation of acetyl CoA (via the citric acid cycle) is a major source of reducing power in the form of NADH and metabolically-utilisable energy in the form of ATP. The provision of acetyl CoA by PDC is also essential for the synthesis of fatty acids, leucine and steroid hormones in eukaryotes. OGDC is a major constituent of the citric acid cycle, regulating the flux of this pathway in its latter stages. This complex catalyses the formation of succinyl CoA, an important biosynthetic intermediate of porphyrins, lysine and methionine. BCOADC catalyses the committed step in the degradation of branched-chain amino acids. In mammals this is nutritionally important in dealing with excess dietary valine, leucine and isoleucine (Goldberg and Chang, 1978). These amino acids are ultimately converted to acetyl CoA (ketogenic), succinyl CoA (glucogenic) and propionyl CoA (glucogenic). BCOADC may also be involved in the catabolism of methionine and threonine since 2-oxobutyrate (Pettit *et al.*, 1978) and 4-methylthio-2-oxobutyrate (Jones and Yeaman, 1986) can be used as substrates by the complex. A deficiency in BCOADC activity in humans causes Maple Syrup Urine disease.

The major contributor to organisation and catalytic efficiency of the respective 2-oxo acid complexes is their specific E2 components. This enzyme serves three major roles:-

1) it forms a central symmetrical core;

2) it is an acyltransferase, catalysing the formation of the acyl CoA product;

3) it provides attachment sites for the lipoic acid prosthetic groups which are required to

Table 1.3.1 Specific reactions catalysed by 2-oxo acid dehydrogenase multienzyme complexes

(Basic substrate R-CO-CO_2H)

Complex	R	Substrate	Product
PDC	CH_3	pyruvate	acetyl CoA
OGDC	$CO_2(CH_2)_2$	2-oxoglutarate	succinyl CoA
BCOADC	$(CH_3)_2CH_2CH_2$	2-oxoisocaproate	isovaleryl CoA
	$(CH_3CH_2)CH_3CH$	2-oxo-3-methylvalerate	2-methyl butyryl CoA
	$(CH_3)_2CH$	2-oxoisovalerate	isobutyryl CoA
	CH_3CH_2	2-oxobutyrate	propionyl CoA
	CH_3-S-$(CH_2)_2$	4-methylthio 2-oxobutyrate	3-methylthio-propionyl CoA

interact with the different active sites of all the constituent enzymes of the complex.

The 2-oxo acid dehydrogenase complexes have been purified from a variety of prokaryotic and eukaryotic sources. PDC has been isolated from *E.coli* (Reed and Mukerjee, 1969; Danson *et al.*, 1978), *Pseudomonas aeruginosa* (Jeyasselan *et al.*, 1980), *Bacillus stearothermophilus* (Henderson and Perham, 1980), *Bacillus subtilis* (Hodgson *et al.*, 1983), *S.cerevisiae* (Kresze and Ronft, 1981a), bovine kidney (Kresze and Steber, 1979; Cate and Roche, 1979), porcine liver (Roche and Cate, 1977) and bovine heart (Stanley and Perham, 1980). OGDC has been purified from *E.coli* (Reed and Mukerjee, 1969), bovine kidney (Reed and Oliver, 1968) and porcine heart (Koike and Koike, 1976). BCOADC has been isolated from *Pseudomonas putida* (Sokatch *et al.*, 1981b), bovine liver and heart (Danner *et al.*, 1979; Pettit *et al.*, 1978) and *Ascaris suum* (Sokatch *et al.*, 1981a).

1.3.3 Structure, function and composition of PDC

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

PDCs isolated from bovine kidney and heart have M_r values of approximately 7.0×10^6 and 8.5×10^6, respectively (Linn *et al.*, 1972). The M_r values of the various polypeptide chains and the proposed stoichiometry of the bovine heart complex are presented in Table 1.3.2. The E1 component consists of non-identical subunits α, M_r

Table 1.3.2: Subunit composition of bovine heart PDC (Adapted from Stepp *et al.*, 1983)

Enzyme	M_r	Subunits		Subunits per molecule of complex
		No.	M_r	
Native complex	8.5×10^6			
E1	154,000	4		
E1α		2	41,000	60
E1β		2	36,000	60
E2	3.1×10^6	60	52,000	60
E3	110,000	2	55,000	12
Kinase	~100,000	1	47,000	1-3
		1	45,000	1-3
Phosphatase	~150,000	1	97,000	(not tightly associated
		1	50,000	with complex)
Protein X	51,000	1	51,000	8 - 12

41,000, and β, M_r 36,000, which form $\alpha_2\beta_2$ tetramers of M_r 154,000. The E2 core enzyme consists of 60 identical polypeptide chains of M_r 52,000. E3 has an overall M_r of 110,000, comprising two identical 55,000-M_r subunits, each containing a molecule of FAD. The exact stoichiometries of PDC kinase and phosphatase are not known. The kinase and phosphatase have however been purified to homogeneity, and each of these enzymes consists of two subunits with M_r values of 47,000 and 45,000 for the kinase (designated α and β, respectively), and 97-110,000 and 50,000 for the phosphatase (Pratt *et al.*, 1982; Stepp *et al.*, 1983).

PDC has also been highly purified from baker's yeast (*S.cerevisiae*), and its molecular structure studied in some detail (Kresze and Ronft, 1981a,b). The complex was found to be similar in size (M_r ~8-9x10^6) and FAD content to those from mammalian mitochondria. The M_r values of the constituent polypeptides, as determined by SDS-PAGE, are: E2, 58,000; E3, 56,000; E1α, 45,000; E1β, 35,000. Thus, the organisation and subunit M_r values are similar to the mammalian complexes.

In mammalian PDC, a distinct polypeptide of unknown function has been observed in recent years. Although this component had been noted previously (Stanley and Perham, 1980), it was originally considered to be a degradation product of E2 or possibly responsible for the PDC-associated kinase activity. However, De Marcucci and Lindsay (1985) demonstrated that the 51,000-M_r polypeptide is immunologically distinct and named it component X. The presence of this polypeptide appears to be unique to eukaryotic PDC, as no similar component has been detected in the analogous 2-oxo acid complexes, OGDC and BCOADC. However, a similar polypeptide is present in yeast PDC (Kresze and Ronft, 1981a), and the gene encoding protein X from *S.cerevisiae* has been cloned and sequenced (Behal *et al.*, 1989). Although the relationship between the mammalian and yeast components has not been confirmed conclusively, several similarities between the two polypeptides suggest they may be functionally equivalent. Independent research indicates that both mammalian and yeast component X polypeptides are involved in E3 binding in the respective complexes (Neagle *et al.*, 1990; Reed and Hackert, 1990).

1.3.4 Regulation of PDC

Mammalian PDC is subject to product inhibition by acetyl CoA and NADH, with reversal of inhibition occurring in the presence of CoASH and NAD^+ respectively (Garland and Randle, 1964; Tsai *et al.*, 1973). Mammalian PDC is also regulated by ATP-dependent phosphorylation (Reed, 1974; Denton *et al.*, 1975) in which the E1α subunit is covalently modified by a specific PDC kinase, with concomitant loss of overall complex activity. In PDC from pig heart (Sugden *et al.*, 1978) and bovine sources (Yeaman *et al.*, 1978) inactivation is correlated with the phosphorylation of a specific serine residue in the E1α chain. A specific phosphatase, loosely associated with the complex, removes the phosphate group and restores activity (Reed, 1974). The activities of the PDC kinase and phosphatase enzymes are regulated by various metabolites (see Figure 1.3.1). The kinase is activated by Mg^{2+}, acetyl CoA and NADH, and inhibited by ADP, pyruvate and CoASH. The phosphatase is activated by Mg^{2+} and Ca^{2+}, and inhibited by NADH (Reed *et al.*, 1980). Ca^{2+} also inhibits kinase activity (Denton *et al.*, 1975).

The steady state activity of PDC is sensitive to the ratios [ADP]/[ATP], [AcSCoA]/[CoASH] and [NAD^+]/[NADH]. Pyruvate dehydrogenase (E1) and its two converter enzymes, kinase and phosphatase, comprise a monocyclic interconvertible enzyme cascade. However, the mechanism by which acetyl CoA, CoASH, NAD^+ and NADH modify kinase activity is not fully understood and is the subject of some controversy.

During ATP-induced inactivation of PDC, phosphorylation occurs at three serine residues on the E1α subunit of the pyruvate dehydrogenase (E1) component (Yeaman *et al.*, 1978; Sugden *et al.*, 1978). These sites were identified by tryptic digestion of [^{32}P]PDC from bovine kidney and heart. A small monophosphorylated peptide containing site 1 was isolated, as well as a diphosphorylated tetradecapeptide (sites 1 and 2) and a monophosphorylated nonapeptide (site 3). Sites 1 and 2 are located in the same region of the peptide chain (see Figure 1.3.2). Phosphorylation at site 1 occurs most rapidly, results essentially in total loss of E1 enzymatic activity, and closely parallels inactivation of the complex (Sugden *et al.*, 1978; Yeaman *et al.*, 1978).

Figure 1.3.1: Regulation of mammalian PDC by a phosphorylation/dephosphorylation mechanism on the E1α component of the complex

The covalent modification of PDC by kinase and phosphatase, and its regulation by various metabolites is represented schematically (Adapted from Reed *et al.*, 1980).

Figure 1.3.1: Regulation of mammalian PDC by phosphorylation/ dephosphorylation

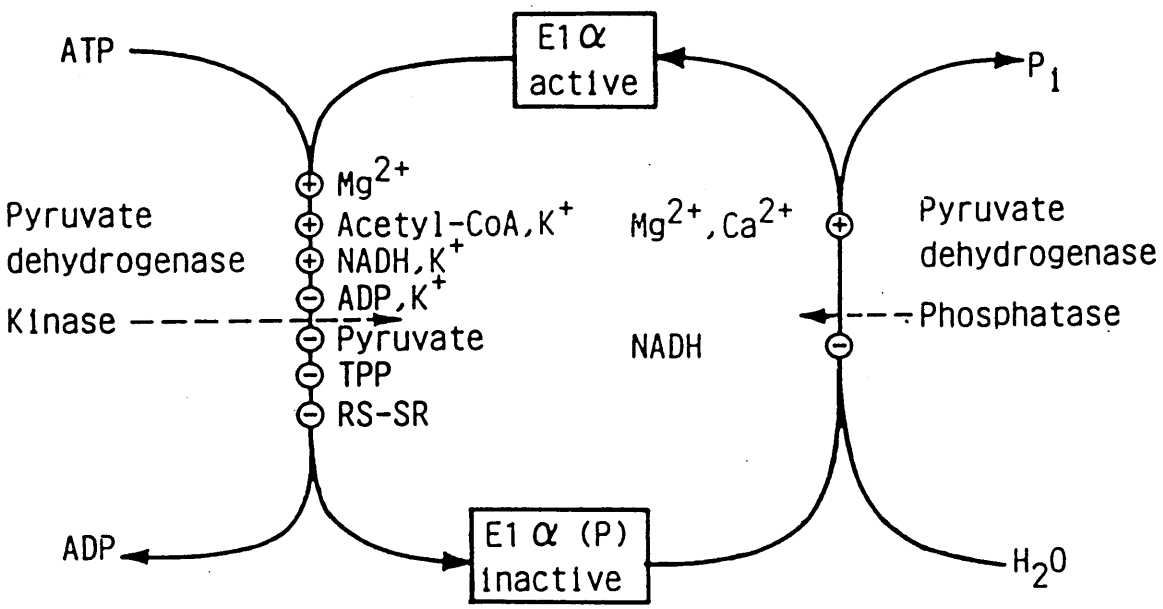


Figure 1.3.2: Phosphorylation sites on the E1α subunit of bovine PDC (Adapted from Yeaman, 1989)

Site 1 Site 2

Tyr-His-Gly-His-Ser(P)-Met-Ser-Asp-Pro-Gly-Val-Ser(P)-Tyr-Arg-Tyr-

Site 3

-Gly-Met-Gly-Thr-Ser(P)-Val-Glu-Arg

The role of the additional sites of phosphorylation on PDC has still to be established. It has been suggested that phosphorylation at sites 2 and 3, in addition to site 1, markedly inhibits the rate of reactivation of the complex by PDC phosphatase (Sugden *et al.*, 1978; Kerbey and Randall, 1979). On this basis, Randle *et al.* (1981) proposed a multi-site phosphorylation mechanism for regulating the conversion of inactive PDC into active complex. However, this model has been criticised by Reed *et al.* (1980) who concluded that the presence of phosphate groups on sites 2 and 3 in bovine kidney PDC did not significantly affect the rate of reactivation by the phosphatase. Evidence was also obtained that the relative rates of dephosphorylation on sites 1, 2 and 3 was different, in the order site 2, site 3, site 1. In addition to this, phosphorylation at site 2 was found to function as well as site 1 phosphorylation in inactivating the complex while phosphorylation at site 3 did not cause inactivation.

Mammalian BCOADC is also subject to regulation by phosphorylation/dephosphorylation (Reed and Yeaman, 1987) but unlike PDC, has only two serine residues on the E1α subunit which are acted upon by the kinase. In common with PDC however, phosphorylation at site 1 in BCOADC is responsible for inactivation of the complex, while the role of the additional phosphorylation site has still to be firmly established.

Regulation of PDC activity by a phosphorylation/dephosphorylation mechanism on the α subunit of the E1 component of the complex is a common theme in eukaryotic cells (For reviews, see Reed, 1974; Denton *et al.*, 1975; and Wieland, 1983). Activity of PDC from eukaryotic sources including mammalian, avian and plant tissues, and *Neurospora crassa* is regulated by such a phosphorylation/dephosphorylation cycle. A possible exception to this is yeast, with all attempts thus far to demonstrate PDC kinase activity being unsuccessful (Kresze and Ronft, 1981a). However, Uhlinger *et al.* (1986) demonstrated the phosphorylation and inactivation of purified PDC from *S.cerevisiae* by bovine kidney PDC kinase. Conversely, it was found that the inactive complex can be dephosphorylated and reactivated with purified PDC phosphatase from bovine heart.

1.4 AIMS OF THE THESIS

1.4.1 Mitochondrial exchange carriers

The proteins chosen for investigation, ANT and PTP, are major exchange carriers of the inner mitochondrial membrane. By following on from previous work done on these transmembranous proteins, it was intended to achieve the following aims:-

1) to develop a procedure for co-purifying ANT and PTP from mammalian sources and efficiently separating the proteins into homogeneous populations. It was initially hoped to carry this out on a small scale, using rat liver mitochondria, then apply the procedure to large-scale preparations of ANT and PTP from bovine heart mitochondria;

2) to design a method for transferring large-scale preparations of the carriers from their isolating detergent, Triton X-100, into detergents suitable for physical analysis by circular dichroism (CD), and ultimately for obtaining crystals prior to X-ray diffraction studies. It was hoped that by binding co-purified ANT and PTP to suitable columns, it would be possible to both separate them by differential elution and, in doing so, switch detergents;

3) to develop an assay for isolating PTP in its native conformation. Because this protein is not an enzyme, no simple assay exists for assessing its activity. Reconstituting PTP into artificial liposomes represents a laborious technique for studying the carrier and, in this case, particularly unsatisfactory in view of the low affinity of the PTP for its substrate, P_i. Isolation of the carrier in the native state is an important prerequisite for CD and X-ray crystallographic studies;

4) to develop and optimise an isolation procedure for PTP from baker's yeast (*S.cerevisiae*). This organism was chosen primarily because large quantities were made available to us (see Section 2.1.4). The highly developed state of classical and molecular genetics in yeast was also taken into account. Subsequently, it was hoped to apply some of the procedures described above to a yeast PTP purification scheme (co-purification of ANT, separation of co-purified ANT and PTP, assay for native PTP conformation).

In summary, it was hoped to carry out a series of purification and

conformational studies on PTP and ANT from both mammalian sources and *S.cerevisiae*, with the ultimate target being to purify PTP of sufficient quality and quantity to obtain crystals suitable for X-ray diffraction studies. It was felt that such studies would help elucidate the manner by which PTP functions at the molecular level, and increase our understanding of this family of related mitochondrial exchange carriers.

1.4.2 Pyruvate dehydrogenase complex

As well as studying the primary mitochondrial exchange carriers, it was decided to address a specific problem relating to the control of PDC activity in yeast. A general feature of PDCs from eukaryotic sources is their regulation by a phosphorylation / dephosphorylation mechanism on the E1α subunit of the complex. However, PDC kinase activity has never been detected in yeast (see Section 1.3.4). It was hoped to demonstrate the phosphorylation of yeast PDC E1α *in vivo* by isolating intact mitochondria from *S.cerevisiae*, and incubating the organelles with [γ^{32}P]ATP. It would then be possible to confirm yeast PDC kinase activity by immune precipitation and the use of mammalian kinase inhibitors.

CHAPTER 2: MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Chemicals and Biochemicals

The following reagents were obtained from Sigma Chemical Co., Poole, Dorset, UK: substrates and coenzymes for enzymatic assays, phenylmethylsulphonyl fluoride (PMSF), *NNN'N'*- Tetramethylethylene-diamine (TEMED), benzamidine-HCl, Coomassie Brilliant Blue type R250, 3-(*N*- Morpholino) propane-sulphonic acid (MOPS), Leupeptin, Tween 20, carboxyatractyloside (CAT), cardiolipin (sodium salt), CCCP, oligomycin, *n*- octyl β-D-glucopyranoside (*n*-octylglucoside) and *n*- dodecyl β-D-maltoside (*n*- laurylmaltoside). Millmaster-Onyx Ltd., UK supplied lauryl dimethyl amine oxide (LDAO), while Triton X-100 was purchased from Rohme and Haas (UK) Ltd.

Poly (ethylene glycol) 6000 was obtained from Serva, Heidelberg, Germany. Iodgen^{TM} was from Pierce Labs., Rockford, Illinois, USA, while DTT was purchased from Koch-Light Labs., Colnbrooke, Berks., UK. Sodium salicylate and dichloroacetic acid were obtained from Aldrich Chemical Co., Ltd., Gillingham, UK. Yeast extract, peptone and agar were purchased from Difco Labs., Detroit, Michigan, USA.

All other chemicals used, including reagents for sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), were Analar grade from BDH Chemicals Ltd., Poole, Dorset, UK, or were of the highest grade commercially available.

2.1.2 Radiochemicals

[γ^{32}P]ATP (100-130 Ci/mmol) and Na[^{125}I]I (carrier-free) were supplied by Amersham International, Bucks., UK.

2.1.3 Enzymes and proteins

Bovine serum albumin (BSA) and protein A (*Staphylococcus aureus*, Cowan I

strain) were obtained from Sigma Chemical Co., Poole, Dorset, UK. Yeast lytic enzyme (Zymolyase-100) was purchased from Seikagaku Kogyo Co., Ltd., Tokyo, Japan.

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

2.1.4 Biological materials

Bovine hearts were obtained from Paisley Abbatoir, Sandyford Road, Paisley. Hearts were removed from the animals within 1h of slaughter, chilled immediately on ice and used in the laboratory within 2h of slaughter. New Zealand White Rabbits and 150-250g rats (Albino Wistar strain, male or female) were provided by the departmental animal house.

Baker's yeast (*S.cerevisiae*) was supplied by DCL Ltd., Menstrie, Clackmannanshire, UK. 1kg tablets of freshly-grown yeast were chilled immediately on ice, and subsequently stored at either 4°C (for use within 7 days) or -80°C (for long-term storage). Specific strains of *S.cerevisiae* (D273-10B and S288C) were cultured and grown routinely in the laboratory under suitable conditions.

2.1.5 Photographic materials

"Lighting Plus" intensifying screens were purchased from Du Pont Cronex, Stirling, UK. Plast-X cassettes, for exposure of X-ray films, were obtained from Anthony Monk (England) Ltd., Sutton-in-Ashfield, UK.

X-OMAT S and XAR-5 X-ray films , Kodak FX-40 liquid fixer and Kodak LX-24 X-ray developer were supplied by Kodak Ltd., Dallimore Road, Manchester, UK.

2.1.6 Chromatography materials

Biogel Hydroxylapatite (HTP) was supplied by Bio-Rad Labs., Holywell Industrial Estate, Watford, Herts., UK., and Celite (Type 535) by Serva, after distribution by Uniscience, Cambridge, UK. Matrex™ Gels (Procion Red A and Procion Blue A) were purchased from Amicon Ltd., Upper Mill, Gloucestershire, UK.

DEAE Affi-Gel Blue was supplied by Bio-Rad Laboratories, Richmond, California, while CM Cellulose and DEAE Cellulose were purchased from Whatman Ltd., Maidstone, Kent.

Pre-packed columns (MonoQ^{TM}, MonoS^{TM} and Superose^{TM}12) for FPLC (Fast protein liquid chromatography) were obtained from Pharmacia (GB) Ltd., Milton Keynes, Bucks., UK, who also supplied Sepharose CL-2B and DEAE Sephacel.

2.1.7 Miscellaneous

Nitrocellulose paper (0.45 μm pore size) was obtained from either Schleicher and Schull, Dassel, Germany, or Amersham International, Bucks., UK (Hybond C extra). Novacell^{TM} stirred cells were purchased from Filtron Technologies UK Ltd., Warwickshire, UK.

Non-immune rabbit and donkey sera were supplied by the Scottish Antibody Production Unit (SAPU), Lanarkshire, UK. Pansorbin, standardised 10% (w/v) formalinised *S.aureus* cells, was obtained from Calbiochem-Behring Corp., Bishop's Stortford, Herts., UK.

2.2 METHODS

2.2.1 Determination of protein concentration

The concentration of protein was routinely determined by the method of Lowry *et al.* (1951), as modified by Markwell *et al.* (1976). Standard curves were constructed using bovine serum albumin (BSA) as the standard, and absorbances were measured at 660nm. Alternatively, where appropriate, the Biuret method (Gornall *et al.*, 1949) was employed. With this method BSA was used as the standard and absorbances were measured at 540nm.

When highly accurate protein concentrations were required, the SERC-funded amino acid analysing facility at the University of Aberdeen was utilised. Analyses were carried out using an Applied Biosystems 420A amino acid analyser with automatic hydrolysis and derivatisation.

2.2.2 Concentration of protein samples

Protein samples were concentrated by sealing in dialysis tubing, followed by exposure to excess poly (ethylene glycol) for 30-60min. Alternatively, Filtron Novacell^{TM} stirred cells were employed to reduce sample volumes under high pressure (55psi).

Frequently, samples for SDS-PAGE were concentrated by the addition of 4 volumes of acetone, followed by overnight storage at -20°C. The protein precipitates were then pelleted by centrifugation at 500-1,000 xg.

2.2.3 N-terminal analysis of proteins

The SERC-funded amino acid sequencing facility at the University of Aberdeen was employed to determine the N-terminal sequence of a protein when required. The methodology involves an improvement on the degradation technique of Edman *et al.* (1981). Purified protein samples were resolved by SDS-PAGE according to the conditions described in Applied Biosystems User Bulletin no.25. The gel was then blotted onto Immobilon membrane (Millipore, Harrow, Middlesex) as described by Matsudaira (1987). Protein bands were carefully excised with a scalpel and placed in the cartridge block of an Applied Biosystems model 470A protein sequencer equipped with a 120A on-line PTH analyser, using the standard 03R PTH programme.

2.2.4 Polyacrylamide gel electrophoresis

(a) Preparation of polypeptides prior to resolution by SDS-PAGE

Protein solutions were mixed with an equal volume of Laemmli sample buffer (0.0625M Tris-HCl, pH 6.8, 2% [w/v] SDS, 10% [w/v] sucrose and 0.001% [w/v] Pyronin Y) containing 5% (v/v) 2-mercaptoethanol or 10mM DTT, and boiled for 5min prior to electrophoresis. Protein samples in pellet form were solubilised in an appropriate volume of Laemmli sample buffer and boiled as above.

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

Proteins were electrophoresed on SDS-containing polyacrylamide gels using the discontinuous Tris-glycine system according to Laemmli (1970). Analytical gels were cast using a home-made apparatus in slabs 19.0cm x 14.0cm x 0.15cm, or when greater resolution was required, using vertical gel electrophoresis apparatus (Life

Technologies Inc.) in slabs 16.0cm x 14.0cm x 0.15cm. Resolving gels (pH 8.8) usually contained 10.0% or 12.5% (w/v) acrylamide, while the stacking gel (pH 6.8) was cast using 4.5% (w/v) acrylamide. Polymerisation was catalysed by TEMED and initiated by ammonium persulphate.

(c) Staining of gels with Coomassie blue

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

(d) Destaining of gels

Unbound stain was removed by washing the gel several times in 20% (v/v) methanol, 10% (v/v) acetic acid solution.

(e) Processing of gels for fluorography

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

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

(f) Processing of gels for autoradiography

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

(g) Determination of M_r value by SDS-PAGE

The M_r values of polypeptides resolved by SDS-PAGE were determined by calibrating with a set of standard proteins on 10% or 12.5% (w/v) slab gels. The standard proteins (and apparent M_r values) were as follows: phosphorylase b (M_r 94,000); BSA (M_r 67,000); ovalbumin (M_r 43,000); carbonic anhydrase (M_r 30,000); soybean trypsin inhibitor (M_r 20,000), and lysozyme (M_r 14,000).

Relative mobility (R_f) was calculated for each protein as the ratio: distance migrated by the protein/distance migrated by the tracking dye. A plot of R_f versus log

(M_r) of the standards yields a curved line which can be used for calibration.

2.2.5 Radioiodination of proteins

Iodogen™ (1,3,4,6- tetrachloro-3α,6α-diphenylglycoluril) is a mild solid phase reagent that efficiently reacts with aqueous mixtures of I^- and tyrosine groups on proteins to produce iodinated proteins (Fracker and Speck, 1978). Iodogen™ was used to iodinate protein A for immune replica analyses, and also to radiolabel standard calibration proteins for determination of M_r values on immunoblots, autoradiographs and fluorographs.

Iodogen™ (1mg) was dissolved in 1ml of chloroform. The solvent was then removed with a gentle air stream, in the process producing a thin film of Iodogen™ on the base of the reaction container. Proteins for iodination (1mg of protein A or 600µg of M_r standards) were dissolved in 0.5ml 0.1M Tris-HCl pH 7.2, 0.15M NaCl and transferred into the reaction container with the Iodogen™. After the addition of 200-400µCi [^{125}I]NaI, the reaction mixture was incubated for 15min at room temperature with occasional mixing. The reaction was terminated by removing the mixture from the container and passing it through a 10ml Sephadex G-25 column equilibrated in 0.1M Tris-HCl pH 7.2, 0.15M NaCl. 1ml fractions were collected in disposable Eppendorf tubes, with the iodinated protein generally being found between fractions 4 and 7. This was confirmed by counting 5µl aliquots in a liquid scintillation counter. Peak fractions were pooled and stored at -20°C. Protein A was dispensed in 10-30µl aliquots, each containing 3 x 10^6cpm, for immunoblotting analyses.

2.2.6 Immunological techniques

(a) Preparation of antisera

Protein purity was assessed initially by Coomassie blue staining of polyacrylamide gels. The acetone-precipitated protein pellet was dissolved in 0.9% (w/v) NaCl and mixed thoroughly with an equal volume 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°C, the antiserum was removed with a pasteur pipette, aliquoted (0.5-1ml fractions) and stored at -20°C. In some instances the antiserum had to be clarified by centrifugation at 2500 xg for 10min to remove contaminating red blood cells. Before subsequent bleedings, the rabbit was injected 10-12 days previously with antigen in incomplete adjuvant. All injections and bleedings were carried out by Dr. J. G. Lindsay.

(b) Immune blotting (Immune replica analysis, Western blotting)

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

Polypeptides were resolved by SDS-PAGE (see Section 2.2.4) and transferred electrophoretically onto nitrocellulose paper at 350ma for 3h or 40ma for 16-20h using a Trans-Blot^{TM} cell. The transfer buffer comprised 25mM Tris-HCl pH 8.3, 192mM glycine, 0.02% (w/v) SDS, 20% (v/v) methanol and was stored at 4°C before use. After transfer the nitrocellulose paper was removed and incubated, face up, in blocking buffer (20mM Tris-HCl pH 7.2, 0.15M NaCl, 0.5% [v/v] Tween 20) for 1h at room temperature or 4°C overnight.

Nitrocellulose paper was then incubated in fresh blocking buffer supplemented with antiserum at a dilution of 1/50-1/100, and 5% (v/v) heat-inactivated donkey serum for 90min at room temperature. Excess antibody was then removed by washing with five changes of blocking buffer over a period of 1h, before incubation for 90min at room temperature with blocking buffer containing ^{125}I-labelled protein A (approx. $3x10^6$ cpm per blot). Excess radiolabelled protein was removed by several washes with blocking buffer as above. The processed nitrocellulose paper was then allowed to dry at room temperature before analysis by autoradiography. On some occasions, immune blots were further analysed by scanning densitometry, using an LKB 2202 Ultroscan Laser Densitometer.

(c) Immune precipitation of [^{32}P]phosphoproteins from mitochondria

Phosphoproteins were immune precipitated from [γ^{32}P]ATP-labelled

mitochondria by adapting the method as used by Jones and Yeaman (1986a). The labelled mitochondrial pellet was used fresh or frozen instantly on dry ice and stored at -80°C prior to use.

The pellet was resuspended in 80µl of ice-cold dilution buffer (20mM $NaPP_i$ pH 7.0, 0.5% [v/v] Triton X-100, 0.5% [w/v] SDS, 1% [w/v] sodium deoxycholate, 1mM EDTA, 1mM EGTA, 5mM NaF, 1mM DTT, 0.5mM PMSF, 0.5mM benzamidine-HCl, 0.3µM leupeptin), and the mitochondria lysed by vortex-mixing for 15s. This mitochondrial solution was then centrifuged at 1,000 xg for 20s (to remove oil) and 10,000 xg for 30s (to remove lipid). The infranatant was extracted and incubated with 5-50µl of antiserum for 1h at room temperature, then overnight at 4°C. Pansorbin (10µl) was added, and the solution incubated for 1h at room temperature with occasional end-over-end mixing. Following a 2.5min centrifugation at 10,000 xg, the supernatant was discarded and the pellet washed three times in wash buffer (50mM Tris-HCl pH 7.5, 0.9% [w/v] NaCl, 0.1% [w/v] SDS, 1% [v/v] Triton X-100). The pellet was then given a fourth and final wash in detergent-free wash buffer. The immune precipitate was dissociated by boiling in Laemmli sample buffer, subjected to SDS-PAGE and analysed by autoradiography.

2.2.7 Isolation of mitochondria

(a) Preparation of rat liver mitochondria

Rat liver mitochondria were isolated by differential centrifugation (Chance and Hagihara, 1963). Rats (150-250g) were starved overnight and killed by cervical dislocation. Livers were removed, chopped finely with scissors and transferred into ice-cold isolation medium (0.225M mannitol, 0.075M sucrose, 0.5mM EGTA, 2mM MOPS, pH adjusted to 7.4 with NaOH). Chopped livers were homogenised in isolation medium, initially with a loose-fitting, then with a tight-fitting Potter-Elvehjem homogenizer. Subsequently, mitochondria were isolated by differential centrifugation. Nuclei, cell debris and red blood cells were removed by centrifuging at 800 xg for 7min, prior to spinning for 15min at 6,500 xg to sediment the crude mitochondrial fraction. The low-speed spin was then repeated on the resuspended mitochondrial pellet

to remove subcellular contaminants. Subsequent centrifugation of this supernatant at 6,500 xg for 15min pelleted the mitochondria.

The final pellets, containing highly purified mitochondria, were used fresh or stored at -20°C until required.

(b) Preparation of bovine heart mitochondria

Bovine heart mitochondria were isolated by differential centrifugation as described by Smith (1967). The homogenisation step was however, replaced by blending the heart tissue at high speed for 1min in a Philip's (UK) Ltd. blender. Approx. 1.5-1.8g of mitochondrial protein was obtained from 300g of tissue.

Mitochondrial pellets were used fresh or stored at -20°C until required.

(c) Preparation of yeast mitochondria

Yeast mitochondria were isolated by adapting the method as described by Rickwood *et al.* (1988). Yeast cells were harvested by centrifuging at 1,000 xg for 10min at 4°C, washed once in cold distilled water, and resuspended in 2 vol 0.1M Tris-SO_4 pH 9.3, 10mM DTT. After a 30min incubation at 30°C, the suspension was re-centrifuged and the pelleted cells washed once in 1.2M sorbitol, 20mM KP_i pH 7.4. The cells were then resuspended to 0.15g wet weight/ml in the sorbitol/KP_i buffer, and the cell walls digested by the action of yeast lytic enzyme (0.25mg Zymolyase-100/g wet cell weight). After a 1h incubation at 30°C, spheroplasts were pelleted by centrifugation at 1,000 xg for 10min, and washed twice in the sorbitol/KP_i buffer. All subsequent steps were carried out at 4°C. The spheroplast pellet was suspended in 2 vol breaking buffer (0.6M mannitol, 20mM HEPES-KOH pH 7.4, 1mM PMSF) and homogenised at maximum speed using a Dounce homogenizer (pestle B, 10 strokes). 1 vol breaking buffer was added to the homogenate, and nuclear and cellular contaminants were removed by centrifugation at 1,000 xg for 10min. Mitochondria were pelleted by high speed centrifugation of the supernatant (6,500 xg for 15min), and washed once in 0.6M mannitol, 20mM HEPES-KOH pH 7.4 to remove PMSF.

Mitochondrial pellets were used fresh or stored at -20°C until required.

2.2.8 Preparation of PTP from bovine heart and rat liver mitochondria

The phosphate transport protein was purified from bovine heart and rat liver

mitochondria as described by Gibb *et al.* (1986), a modification of the method of Kolbe *et al.* (1981). This modification involved the pre-extraction of mitochondria with a low Triton X-100 buffer (20mM LiCl, 0.1mM EDTA, 0.5mM DTT, 20mM H_3PO_4, 0.5% [w/v] Triton X-100, adjusted to pH 7.0 with LiOH). After centrifugation for 1h at 105,000 xg, the resulting pellet was solubilised by incubation for 15min at 4°C in the above buffer supplemented with 8% (w/v) Triton X-100 to extract the PTP. 1 vol ice-cold distilled water was added, residual insoluble material was removed by centrifugation at 105,000 xg for 1h, and the supernatant fraction was subjected to adsorption chromatography on hydroxylapatite. A batch procedure was adopted for this step (0.5g HTP/ml solubilised membrane). In some cases, a further adsorption chromatography step was undertaken on the HTP-adsorbed supernatant, using Celite (type 535). A similar batch procedure was adopted for this step.

For some experiments, PTP was purified in the presence of cardiolipin which was added to the extraction buffer to a final concentration of 1mg/ml.

2.2.9 Chromatographic techniques

Adsorption chromatography was carried out on HTP and Celite as described above (Section 2.2.8). Manual chromatography was also carried out using the Matrex™ gels Procion Red A and Procion Blue A. 2.0-5.0cm x 1.0cm columns were poured and equilibrated in 20mM Tris-HCl pH 7.0-9.0. The exact size of the columns, pH of the buffer, and the inclusion of various detergents in the buffer were determined by the nature of the experiment and are described in the appropriate figure legends.

Column chromatography was also undertaken using the Pharmacia FPLC system. The following columns were used: Superose™12 and Sepharose CL-2B (poured manually), gel filtration columns; MonoQ™, an anion exchanger; MonoS™, a cation exchanger.

2.2.10 Circular dichroism

Circular dichroism (CD) spectra were recorded at 20°C in a Jasco J-600 spectropolarimeter at Stirling University by Miss Sharon Kelly. Spectra in the far UV (250-205nm) and the near UV (320-260nm) were recorded in cells of path lengths

1mm and 10mm respectively. Data was collected and analysed on data processors attached to the spectropolarimeter. The spectra were plotted as $[\sigma]_\lambda$ against wavelength (nm).

Samples of protein in the presence and absence of guanidine-HCl (GdnHCl) were prepared and the spectra were recorded 15min after addition of this reagent. Measurements were corrected by the inclusion of appropriate blanks.

2.2.11 Purification of PDC

(a) Purification of bovine heart PDC

PDC was purified from bovine heart essentially as described by Stanley and Perham (1980), with the modifications outlined below.

All operations were carried out at 4°C, starting with 250g of cubed bovine heart. Cubes of tissue were blended and extracted in 50mM MOPS, 2.7mM EDTA, 3% (w/v) Triton X-100, 0.1mM DTT, adjusted to pH 7.0 with NaOH. Residual material was discarded. The extracts contained assayable amounts of OGDC activity; however, the presence of lactate dehydrogenase interfered with the estimation of PDC activity at the early stages of purification.

PDC was precipitated by the addition of 0.12 vol of a 35% (w/v) poly (ethylene glycol) solution at pH 6.45. Pellets were resuspended in 50mM MOPS, 1% (w/v) Triton X-100, 2.7mM EDTA, 0.1mM DTT, 1.5μM leupeptin, adjusted to pH 6.8 with NaOH, by homogenisation with a loose-fitting teflon-glass homogenizer. This extract was adjusted to 13mM $MgCl_2$ and 50mM NaP_i before the addition of 0.12 vol poly (ethylene glycol) for a second precipitation.

The 2-oxo acid dehydrogenase complexes were separated by differential precipitation with poly (ethylene glycol). 0.04-0.06 vol 35% (w/v) poly (ethylene glycol) was required to precipitate 90% of OGDC activity. OGDC was pelleted at 18,000 xg for 10min, and the resulting supernatant fraction was found to contain 90-95% of the PDC activity. PDC was concentrated by centrifugation at 170,000 xg for 2.5h, or by the addition of 0.15 vol 35% poly (ethylene glycol) followed by centrifugation at 30,000 xg for 10min.

The PDC pellet was purified further by solubilising in column buffer (50mM

NaP_i, pH 7.0, 1% [w/v] Triton X-100, 2.7mM EDTA, 0.2mM TPP, 1mM $MgCl_2$) and separating residual OGDC by gel filtration chromatography on a Sepharose CL-2B column (106cm x 3.5cm) equilibrated in column buffer, at a flow rate of 24ml/h. Approx 60 x 12ml fractions were collected and assayed for PDC and OGDC activities. Fractions with maximal PDC activity were pooled and concentrated by centrifugation at 170,000 xg for 2.5h.

The yellow PDC pellets were resuspended by standing overnight in a small volume of column buffer supplemented with 0.01% NaN_3, 0.15μM leupeptin. The complex was stored until required at 4°C at a final concentration of 20-30mg/ml.

(b) Purification of yeast PDC

PDC was prepared from baker's yeast (*S.cerevisiae*) essentially as described by Kresze and Ronft (1981a). On some occasions the modifications introduced by Uhlinger *et al.* (1986) were also adopted for further purification of the complex.

Yeast PDC utilised in this study was prepared by Drs. J.McKee and S.West in our laboratory, and stored at 4°C until required.

2.2.12 Enzymatic assays

(a) PDC activity

Overall PDC activity was determined spectrophotometrically by measuring NADH formation at 340nm at 30°C (Brown and Perham, 1976). Assays were performed in a final volume 0.67ml of 50mM KP_i pH 7.4, 2mM TPP, 1mM $MgCl_2$, 2.5mM NAD^+, 0.13mM CoASH, 2.6mM cysteine-HCl, 2mM sodium pyruvate. A unit of activity (Kat) was defined as the amount of enzyme which produced 1mol NADH/sec at 30°C under the assay conditions.

(b) PDC kinase activity

The activity of bovine heart PDC kinase (EC 2.7.1.99) was assayed by measuring the rate of inactivation of the complex in the presence of 0.2mM ATP at 30°C (Hucho *et al.*, 1972), or by estimation of the rate of incorporation of ^{32}P-labelled phosphoryl groups from [$\gamma^{32}P$]ATP into serine residues on the E1α subunit of PDC (Stepp *et al.*, 1983).

2.2.13 Separation of PDC components

PDC was separated into its individual components by FPLC, a modification of the method of Kresze and Ronft (1981b). The complex was first separated into E1/E3 and the E2/X core assembly using a Superose^{TM} 12 column of 100ml bed volume. The column was equilibrated with 2 vol running buffer (50mM Tris-HCl pH 9.0, 1M NaCl, 1mM DTT, 0.01% [w/v] Triton X-100), and then preinjected with 4ml of running buffer containing 4M NaCl, in order to create a high molarity band onto which the dissociated complex was loaded. PDC (maximum 100mg) was loaded after a 1:1 dilution and incubation with 4M NaCl running buffer (to dissociate E1/E3 from E2/X), and 2ml fractions were collected.

A MonoQ^{TM} column was used to separate E3, E1 and the associated kinase. The column was equilibrated with running buffer (50mM imidazole pH 7.2, 1mM DTT, 0.01% Triton X-100), against which the E1/E3 fraction was also dialysed prior to loading. E1, E3 and kinase were differentially eluted with a salt gradient (0-1.0M NaCl). 1ml fractions were collected and analysed by SDS-PAGE and PDC kinase activity assays (see Section 2.2.12b).

2.2.14 Growth of cultured yeast cells

Cultured yeast cells (*S.cerevisiae*, strains D273-10B and S288C) were grown aerobically in the following media, all containing 1% (w/v) yeast extract and 2% (w/v) peptone, and buffered with 0.1M KP_i pH 6.0: YPD, 2% (w/v) glucose; YPG, 2% (w/v) galactose; YPEG, 2% (v/v) glycerol, 3% (v/v) ethanol; YPDG, 0.1% (w/v) glucose, 3% (v/v) glycerol; YPS, 2% (w/v) sodium succinate pH 6.0; YPL, 2% (w/v) sodium lactate pH 6.0.

Yeast stocks were stored at 4°C on 10ml solid slopes of YPG supplemented with 2% (w/v) agar. These slopes were replaced at three-monthly intervals to maintain viable colonies of the microorganisms.

2.2.15 Preparation of extracts from yeast cells grown in the presence of CCCP

The yeast cytoplasmic petite (rho$^-$) mutant *S.cerevisiae* D273-10B-1 was grown at 23°C in YPG to early exponential phase. The culture was then divided into

six aliquots and CCCP was added to five of them at final concentrations of 1, 2, 5, 10 and 20 μM. Growth was then continued at 23°C for 8h. Cell extracts were subsequently prepared as described by Reid and Schatz (1982). Cell suspensions were mixed with 0.2 vol of 100% (w/v) trichloroacetic acid, the denatured cells were broken by agitation with glass beads, and proteins were extracted with SDS buffer (2% [w/v] SDS, 2mM EDTA, 50mM Tris-HCl, 10% [v/v] glycerol). After neutralisation with 1M Tris base, the samples were boiled for 3 min and centrifuged for 10 min at 1000 xg. The supernatants were subjected to SDS-PAGE prior to immune replica analysis.

CHAPTER 3: PROTEIN-CHEMICAL STUDIES ON THE MAMMALIAN PHOSPHATE AND ADENINE NUCLEOTIDE MITOCHONDRIAL CARRIERS

3.1 INTRODUCTION

The inner membrane phosphate transport protein (PTP) represents a major protein component of the mitochondrion. The intimate involvement of this carrier in oxidative phosphorylation (see Section 1.2.3), the primary function of mitochondria, demonstrates the biological importance of PTP. In view of this, much research has been carried out in our laboratory, specifically aimed at the isolation and characterisation of mammalian PTP (Gibb, 1985; Gibb *et al.*, 1986; Phelps, 1987).

The aim of this part of the project was to follow on from the above research, extending our understanding of the structural and physical properties of PTP. The closely related adenine nucleotide translocase (ANT) was also included in the study since these two proteins (PTP and ANT), designated as the primary exchange carriers of the inner mitochondrial membrane, appear to be members of a structurally related family of mitochondrial exchange systems with a common evolutionary origin (see Section 1.2.9).

In view of the close similarities in the isolation procedures for PTP and ANT (see Sections 1.2.4 and 1.2.5), it was hoped to develop a method for co-purifying the two carriers and efficiently separating them in order to obtain maximum yields of both from a single mitochondrial preparation. Isolation of the carriers in their native conformations, an important pre-requisite for physical analyses and X-ray crystallography, was also studied.

The long-term aim of this project is to obtain crystals of PTP (and/or ANT) of sufficient quality for X-ray diffraction studies, thus greatly increasing our understanding of a family of important mitochondrial exchange carriers. Accordingly, many of the experiments in this study, although initially carried out on rat liver mitochondria, were subsequently applied to bovine heart mitochondria in order to significantly increase yields.

3.2 PURIFICATION OF MAMMALIAN PTP AND ANT

3.2.1 Purification of mammalian PTP

The phosphate carrier was isolated from rat liver and bovine heart mitochondria basically as described by Gibb *et al.* (1986; see Section 2.2.8). Pre-extraction with a 0.5% (w/v) Triton X-100 buffer was followed by extraction with 8% (w/v) Triton X-100 and batch chromatography with firstly hydroxylapatite (HTP), then Celite. The Coomassie blue-stained profile of the various stages of rat liver PTP purification is illustrated in Figure 3.2.1. Analysis by SDS-PAGE reveals an apparently homogeneous 34,000-M_r single polypeptide after Celite fractionation. Similarly, SDS-PAGE analysis of the various stages of bovine heart PTP isolation also reveals a single 34,000-M_r polypeptide band after the ultimate purification step. These observations contrast with the findings of Kolbe *et al.* (1984), who detected two protein bands at the final stage of bovine heart PTP purification. Historically, the isolation of this carrier has been hampered by the apparent heterogeneity of preparations. It should be noted that on a few occasions a single contaminating band of very similar M_r value (32-33,000) to PTP was also detected in the above preparations (this can be observed in various figures in this chapter). This phenomenon was ignored for subsequent experiments, as it can be explained by the formation of transient disulphide bonds during electrophoresis (see Section 1.2.5). However, it is also possible that the extra band represents minor contamination by the mitochondrial outer membrane protein, porin (T. Lever, personal communication) or proteolytically degraded PTP.

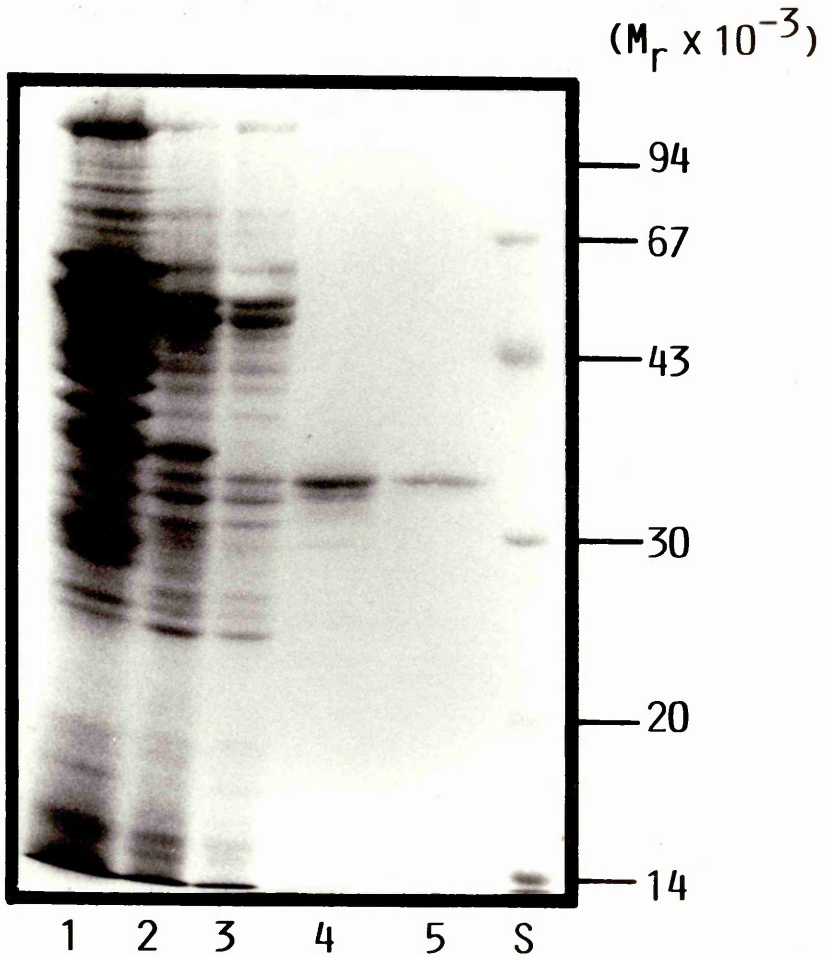
Figure 3.2.1 also demonstrates that the crucial step in mammalian PTP isolation is adsorption chromatography on HTP. Virtually all proteins present in the 8% (w/v) Triton X-100 extract remain bound to the column matrix, while only PTP and a few other proteins of similar M_r values are present in the eluate. Contaminating bands in this supernatant are then adsorbed out by Celite. However it is possible, with careful preparation, to obtain a homogeneous isolate of PTP following HTP extraction.

The lack of homogeneous preparations has presented obvious difficulties when raising antibodies to PTP, since any contaminating bands may be more immunogenic

Figure 3.2.1: Purification of PTP from rat liver mitochondria

Mitochondria were prepared as described in Section 2.2.7(a), and PTP was purified as outlined in Section 2.2.8. Samples were acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, rat liver mitochondria; 2, 0.5% (w/v) Triton X-100 pre-extracted pellet; 3, 8% (w/v) Triton X-100 extract; 4, HTP-adsorbed extract; 5, Celite-adsorbed extract; S, M_r standards.

Figure 3.2.1: Purification of PTP from rat liver mitochondria



than the chosen antigen. Antibodies represent a powerful biochemical tool and although their use is limited in this project, it was decided to raise antiserum against the rat liver phosphate carrier in view of the apparent homogeneity of the isolate. Anti-rat liver PTP serum was prepared by immunising a New Zealand White rabbit as described in Section 2.2.6(a). The antiserum was characterised by employing it for immune replica analysis of the various steps in the isolation of rat liver PTP, as illustrated in Figure 3.2.2. The monospecificity of the anti-PTP serum is clearly demonstrated, as no cross-reactivity with any other mitochondrial proteins is apparent.

3.2.2 Co-purification of mammalian PTP and ANT

The purification schemes for PTP and its closely related companion protein ANT contain many similarities, with the critical stage in both involving adsorption chromatography on HTP (see Sections 1.2.4 and 1.2.5). However, ANT will denature upon extraction and be adsorbed out by HTP unless it has been stabilised by one of its inhibitors, especially carboxyatractyloside (CAT; Klingenberg *et al.*, 1974). Accordingly, it was decided to attempt to co-purify mammalian PTP and ANT in the HTP-adsorbed extract by carrying out a standard PTP preparation on rat liver mitochondria which were pre-incubated (when fresh) with 50-100μM CAT for 30min at 4°C. Analysis by SDS-PAGE (Figure 3.2.3) reveals the presence of both PTP and a 30,000-M_r CAT-stabilised polypeptide following chromatography on HTP. The 30,000-M_r protein, assumed to be ANT, is adsorbed out by Celite, which agrees with previous observations by Wohlrab (1980) and Wohlrab and Flowers (1982) who detected contaminating ANT in their PTP isolates. Figure 3.2.4 illustrates the effect of CAT on the isolation of PTP from rat liver mitochondria. In this experiment, a single batch of fresh mitochondria was used, with half being pre-incubated with CAT (100μM) as described above, and half being treated as normal.

ANT will also co-purify with PTP when the above procedure is carried out on bovine heart mitochondria pre-loaded with CAT. It is thus possible to co-purify the two carriers on a relatively large scale (1-3mg of each).

Comparison of the above procedure for co-purifying PTP and ANT from

Figure 3.2.2: Western blot analysis of PTP purification from rat liver mitochondria

Mitochondria were prepared as described in Section 2.2.7(a), and PTP was purified as outlined in Section 2.2.8. Samples were acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was transferred to nitrocellulose paper and subjected to immune replica analysis with anti-rat liver PTP serum. Lanes: 1, rat liver mitochondria; 2, 0.5% (w/v) Triton X-100 pre-extracted pellet; 3, 8% (w/v) Triton X-100 extract; 4, HTP-adsorbed extract; 5, Celite-adsorbed extract; S, M_r standards (^{125}I-radiolabelled).

Figure 3.2.2: Western blot analysis of PTP purification from rat liver mitochondria

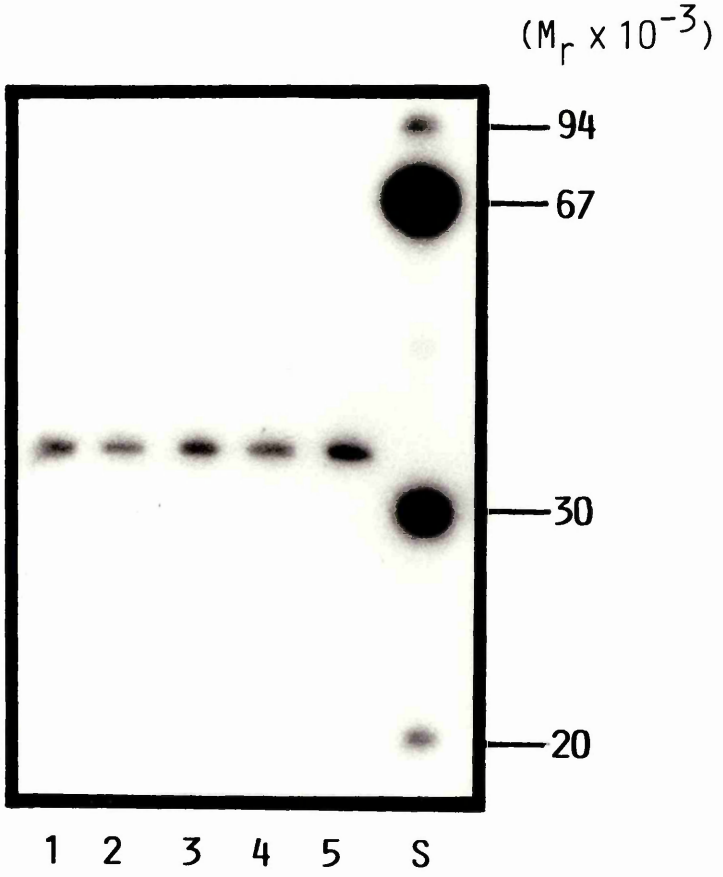


Figure 3.2.3: Purification of PTP (+ANT) from rat liver mitochondria pre-incubated with CAT

Mitochondria were prepared as described in Section 2.2.7(a), and the proteins were purified as outlined in Section 3.2.2. Samples were acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, rat liver mitochondria; 2, 0.5% (w/v) Triton X-100 pre-extracted pellet; 3, 8% (w/v) Triton X-100 extract; 4, HTP-adsorbed extract; 5, Celite-adsorbed extract; S, M_r standards.

Figure 3.2.3: Purification of PTP (and ANT) from rat liver mitochondria pre-incubated with CAT

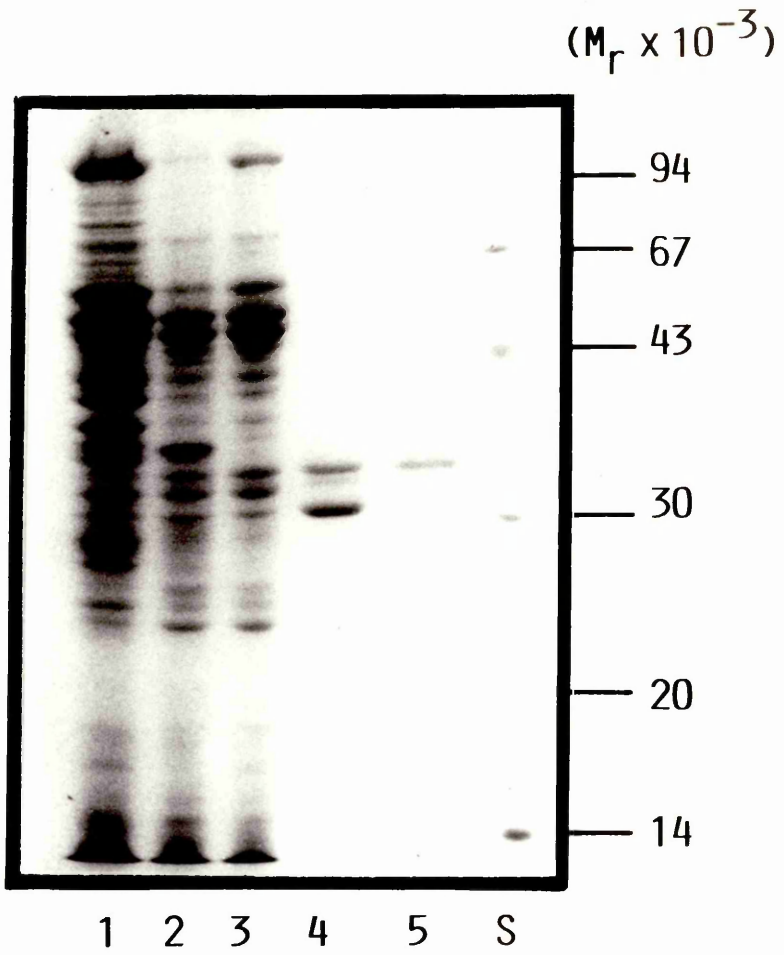
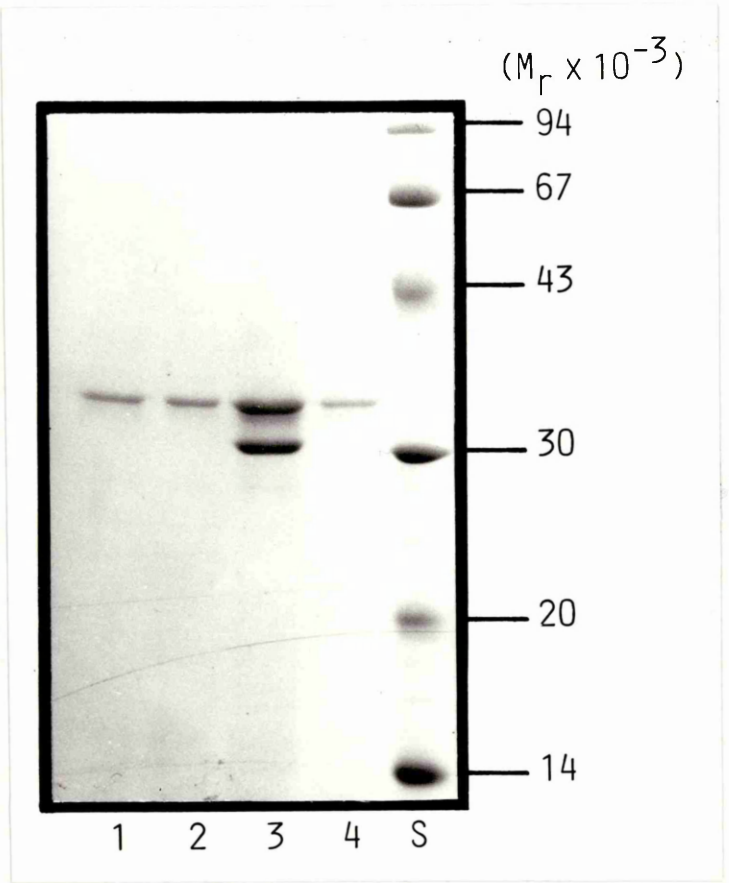


Figure 3.2.4: Effect of CAT on the purification of PTP from rat liver mitochondria

PTP was purified from rat liver mitochondria pre-incubated with the ANT inhibitor carboxyatractyloside (CAT; see Section 3.2.2), and also from mitochondria not subjected to CAT-pretreatment. In each case the HTP and Celite-adsorbed extracts were acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1 and 2, HTP and Celite extracts respectively; 3 and 4, HTP and Celite extracts (mitochondria pre-incubated with CAT); S, M_r standards.

Figure 3.2.4: Effect of CAT on the purification of PTP from rat liver mitochondria



mammalian mitochondria with the established method for the isolation of the ADP/ATP carrier (Riccio *et al.*, 1975a,b) reveals that the two methods are essentially identical. The only differences that exist are in the type of buffer used (20mM MOPS, pH 7.2 for ANT; 20mM $H_2PO_4^-$, pH 7.0 for PTP/ANT), and the inclusion of salt (0.4-0.5M NaCl or KCl) in the Triton X-100 solutions when isolating ANT. Accordingly, it was decided to assess the effect of investigating these factors in relation to the PTP/ANT co-purification procedure described above.

Changing from a phosphate to a MOPS buffer system had absolutely no effect on the co-purification of PTP and ANT in the HTP-adsorbed extract from rat liver or bovine heart mitochondria. The effect of KCl on the purification of rat liver PTP and ANT was analysed by SDS-PAGE and is illustrated in Figure 3.2.5. The inclusion of 0.5M KCl in the 0.5% (w/v) Triton X-100 pre-extraction buffer clearly did not influence the final protein content of the subsequent HTP-adsorbed extracts. However, the inclusion of 0.5M KCl in the 8% (w/v) Triton X-100 extraction buffer markedly increased the relative content of ANT in the subsequent co-isolate. Nevertheless, it was not possible to purify a homogeneous preparation of ANT without significant PTP contamination.

3.3 ANALYSIS OF MAMMALIAN PTP AND ANT BY COLUMN CHROMATOGRAPHY

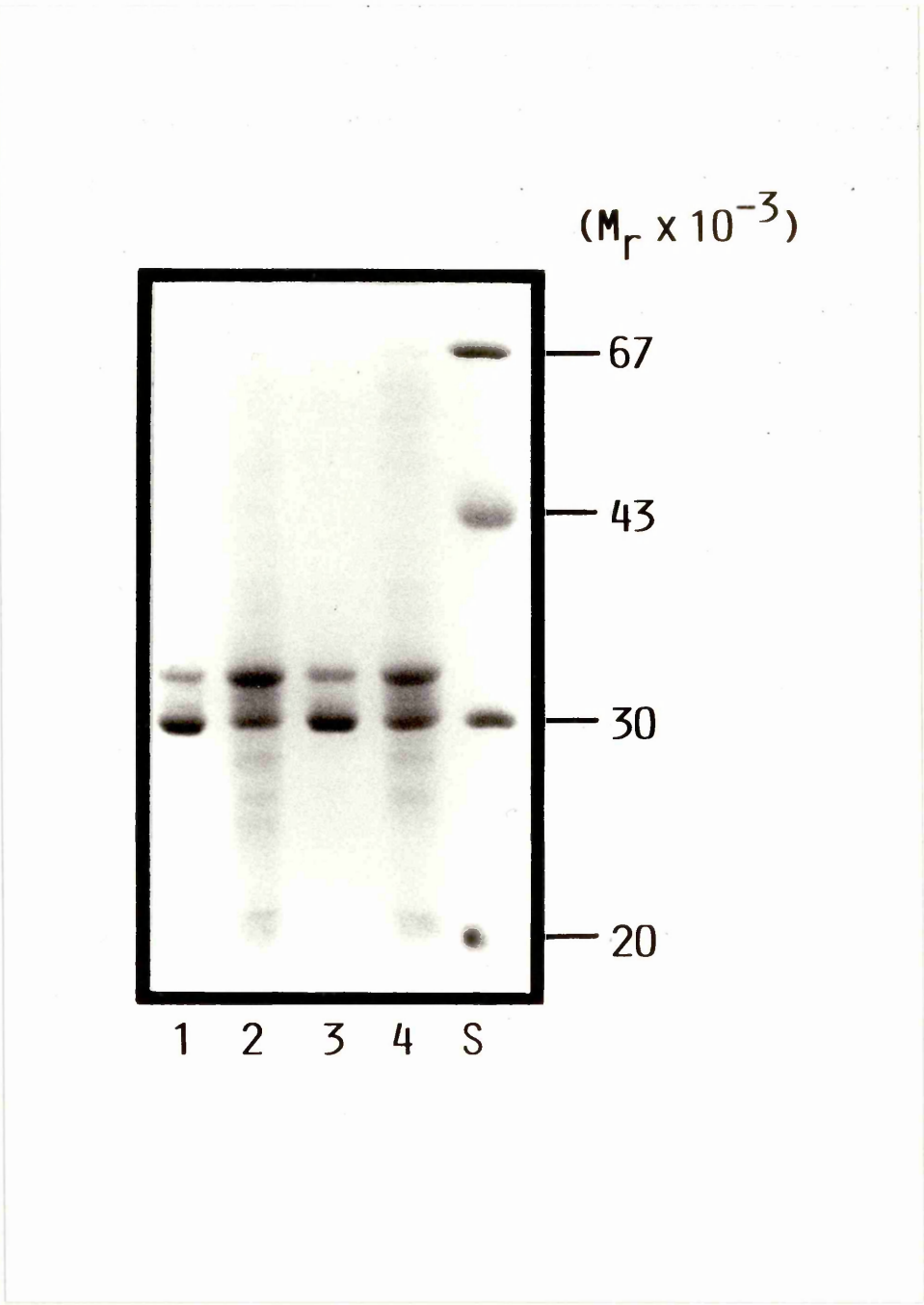
3.3.1 Introduction

The above section (3.2.2) describes the methodology for the co-isolation of PTP and the closely related ADP/ATP carrier. In order to obtain optimal yields of both carriers from a single mitochondrial preparation, it would be necessary to develop a procedure for efficiently separating them. Such a procedure would require to identify differences between two proteins with a high degree of structural and functional homology. In order to achieve this, various types of column chromatography were employed. The identification of a column to which only one of the carriers could bind and subsequently be eluted from under defined conditions would be sufficient to separate PTP and ANT. However, it was hoped to bind both proteins to a suitable

Figure 3.2.5: Effect of KCl on the purification of ANT and PTP

Rat liver mitochondria pre-incubated with CAT were pre-extracted in the 0.5% (w/v) Triton X-100 buffer in the presence and absence of 0.5M KCl. Subsequently, extraction of ANT and PTP was carried out with the 8% (w/v) Triton X-100 buffer, also in the presence and absence of 0.5M KCl. The extracts were subjected to adsorption chromatography on HTP and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, 8% (w/v) Triton X-100 extract + 0.5M KCl; 2, 8% (w/v) Triton X-100 extract (Extracts 1 and 2 prepared from mitochondria pre-extracted with 0.5% [w/v] Triton X-100); 3 and 4, identical to extracts 1 and 2, except prepared from mitochondria pre-extracted with 0.5% (w/v) Triton X-100 + 0.5M KCl; S, M_r standards.

Figure 3.2.5: Effect of KCl on the purification of ANT and PTP



column and obtain separation by differential elution. In this manner, it should be possible to replace the solubilising detergent Triton X-100 with detergents more suitable for physical analyses of the proteins and crystallisation trials.

Co-isolated mammalian PTP and ANT was analysed by various chromatographic techniques including FPLC (Fast protein liquid chromatography). Two pre-packed FPLC columns were used: the MonoQ anion exchanger, and the cation exchange MonoS column. In addition to this, the HTP-adsorbed extracts of mammalian CAT-loaded mitochondria were analysed using DEAE Affi-gel Blue, DEAE Sephacel, CM Cellulose and DEAE Cellulose as well as the Matrex gels, Procion Red A and Procion Blue A. Binding experiments were carried out in 20mM Tris HCl buffer pH 7.0-8.0, 0.5% (w/v) Triton X-100 and the columns (2.0 x 1.0cm in each case) were eluted with salt (0-1.0M NaCl). Following analysis of the unbound and NaCl-eluted fractions from each column by SDS-PAGE, it was found that, under the above conditions, optimal binding of both carriers was achieved using the Matrex gels. Although PTP appeared to bind more tightly to Procion Red A, it was readily eluted from both columns with NaCl (0.1M for Procion Blue A; 0.25M for Procion Red A). However, the binding of ANT to both these gels was observed to be considerably stronger, particularly in the case of Procion Blue A, where significant amounts of ANT remained bound to the column even after elution with 1% (w/v) SDS. Since it was ultimately hoped to obtain homogeneous ANT in its native state, all subsequent experiments were carried out using Procion Red A.

3.3.2 Analysis of mammalian PTP and ANT by FPLC

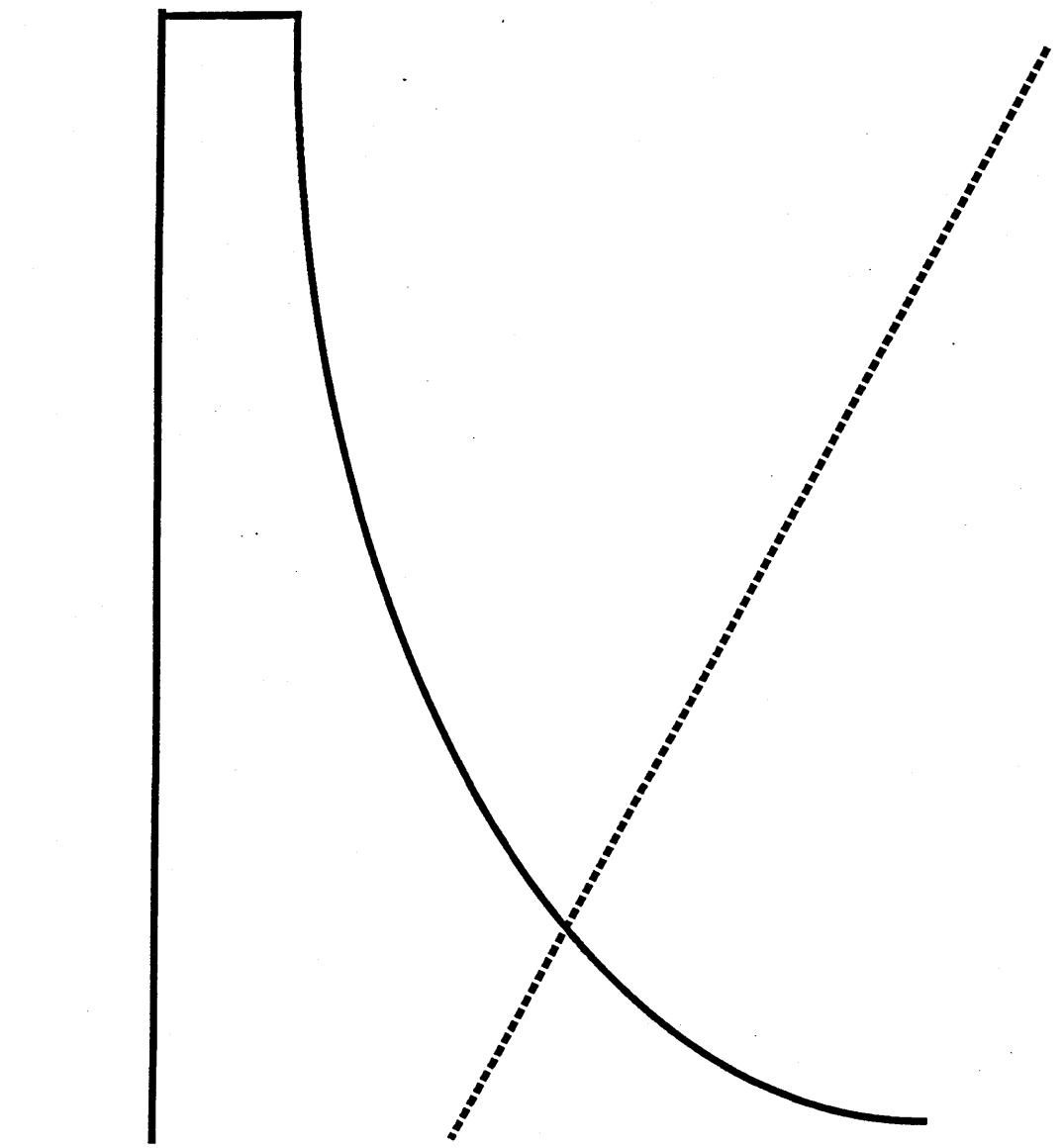
The HTP-adsorbed extract isolated from rat liver mitochondria pre-incubated with CAT was initially analysed by FPLC using the MonoQ anion exchange column. The PTP/ANT co-isolate was dialysed into 20mM Tris-HCl pH 8.0 or pH 7.0, 0.5% (w/v) Triton X-100, and loaded onto the column which was equilibrated in the same buffer. Elution was carried out using a salt gradient (0-1.0M NaCl). Under both conditions (pH 8.0 and pH 7.0), a large unbound peak was detected by the trace (see Figure 3.3.1), but no peaks were detected during elution with the salt gradient. The high levels of Triton X-100 present in the preparation are partly responsible for the absorbance of the unbound fraction. Analysis of the unbound fractions and the pooled

Figure 3.3.1: FPLC trace of MonoQ analysis of rat liver mitochondrial (+CAT) HTP extract

The HTP-adsorbed fraction containing PTP and ANT was dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100, loaded onto a MonoQ column equilibrated in the same buffer, and eluted with 0-1.0M NaCl.

Figure 3.3.1: FPLC trace of MonoQ analysis of rat liver mitochondrial (+CAT) HTP extract

SAMPLE : RAT LIVER PTP/ANT
COLUMN : MONOQ
BUFFER : 20 mM TRIS-HCl, pH 8.0
GRADIENT: 0.0 - 1.0M NaCl



NaCl-eluates by SDS-PAGE, as illustrated in Figure 3.3.2, revealed a more interesting result. At the higher pH, ANT binds so tightly to the column that an increase in the ionic strength as described above does not lead to its elution. However, a reduction in pH of only one unit results in the ADP/ATP carrier co-eluting with PTP in the unbound fraction. PTP does not bind to the column under these experimental conditions. The above experiment was then repeated at pH values of 7.2 and 7.5 in an attempt to identify conditions at which ANT would bind to the column and be eluted by increasing ionic strength, thus separating the carriers with PTP in the unbound fraction. However, at both pH values, the FPLC trace obtained was as illustrated in Figure 3.3.1. SDS-PAGE analysis of the fractions confirmed the absence of any protein in the salt eluates. In both cases, PTP and ANT were detected in the unbound fraction, as illustrated in Figure 3.3.2 (lanes 4-6).

Since the MonoQ is an anion exchange column, the above results suggest that PTP has a net positive charge over the pH range 7.0-8.0, while ANT is also positively charged at pH 7.0-7.5. However at pH 8.0, the increase in the negative charge on the ADP/ATP carrier appears to be such that it binds very tightly to the column. In view of this, it was decided to carry out further FPLC analysis of the rat liver PTP and ANT co-isolate using the MonoS cation exchange column. Under suitable conditions, such a column should bind only to PTP, leaving ANT in the unbound fraction. Assuming PTP could be eluted, separation of the carriers could thus be achieved. Co-purified rat liver PTP and ANT was loaded onto the MonoS column as described above for the MonoQ. Elution was carried out using a 0-1.0M KH_2PO_4 gradient. Analysis was carried out initially at pH 7.0, then at pH 8.0 and pH 6.0. However in all three cases, the trace obtained was identical to that illustrated in Figure 3.3.1, with a large unbound peak but no KH_2PO_4-eluted peaks. SDS-PAGE analysis of the unbound fraction and pooled salt-eluates at pH 7.0 is illustrated in Figure 3.3.3, with an identical pattern being observed for the corresponding fractions at pH 8.0 and pH 6.0. Thus, over the pH range 6.0-8.0, neither PTP nor ANT were found to bind to the MonoS column.

Figure 3.3.2: Analysis of rat liver mitochondrial (+CAT) HTP extract by FPLC (MonoQ column)

The HTP-adsorbed extract containing ANT and PTP was dialysed into 20mM Tris-HCl pH 8.0 or pH 7.0, 0.5% (w/v) Triton X-100, loaded onto a MonoQ column equilibrated in the same buffer, and eluted with a 0-1.0M NaCl gradient. At each pH a large unbound fraction was detected and collected, and in each case no peaks were detected after elution with the salt gradient. The NaCl-eluates were pooled, concentrated by exposure to poly (ethylene glycol) and, like the unbound fractions, acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1 and 4, rat liver HTP extract; 2 and 3, unbound fraction and NaCl-eluate respectively at pH 8.0; 5 and 6, unbound fraction and NaCl-eluate respectively at pH 7.0; S, M_r standards.

Figure 3.3.2: Analysis of rat liver mitochondrial (+CAT) HTP extract by FPLC (MonoQ column)

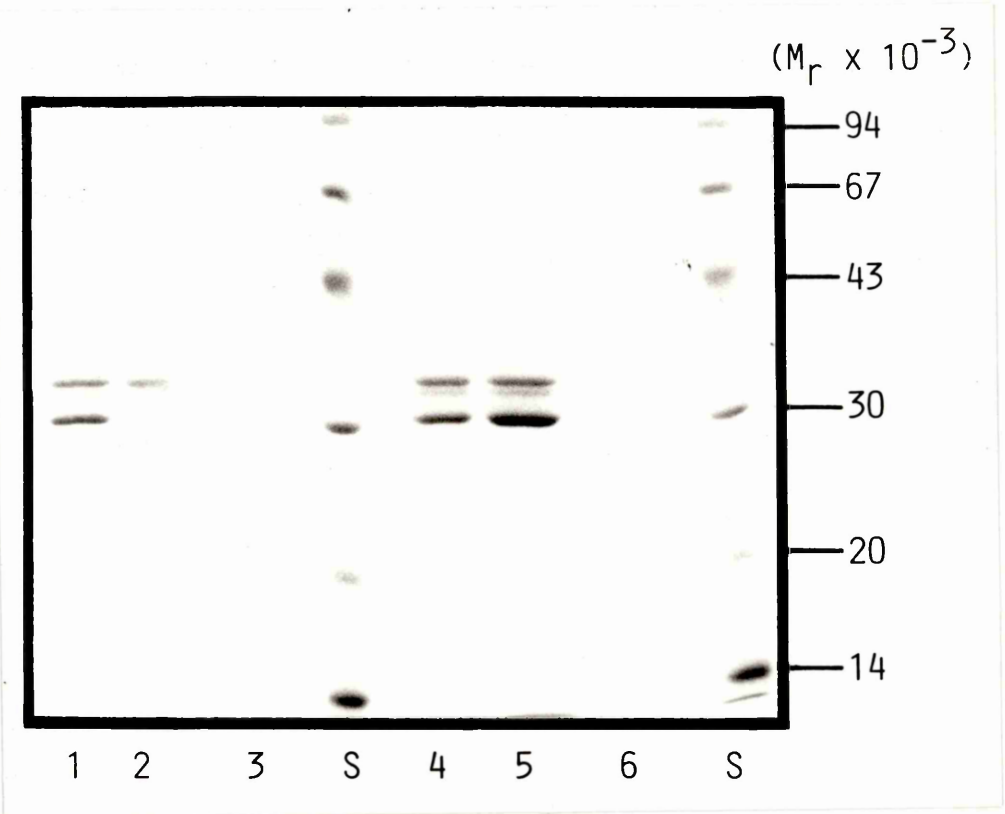
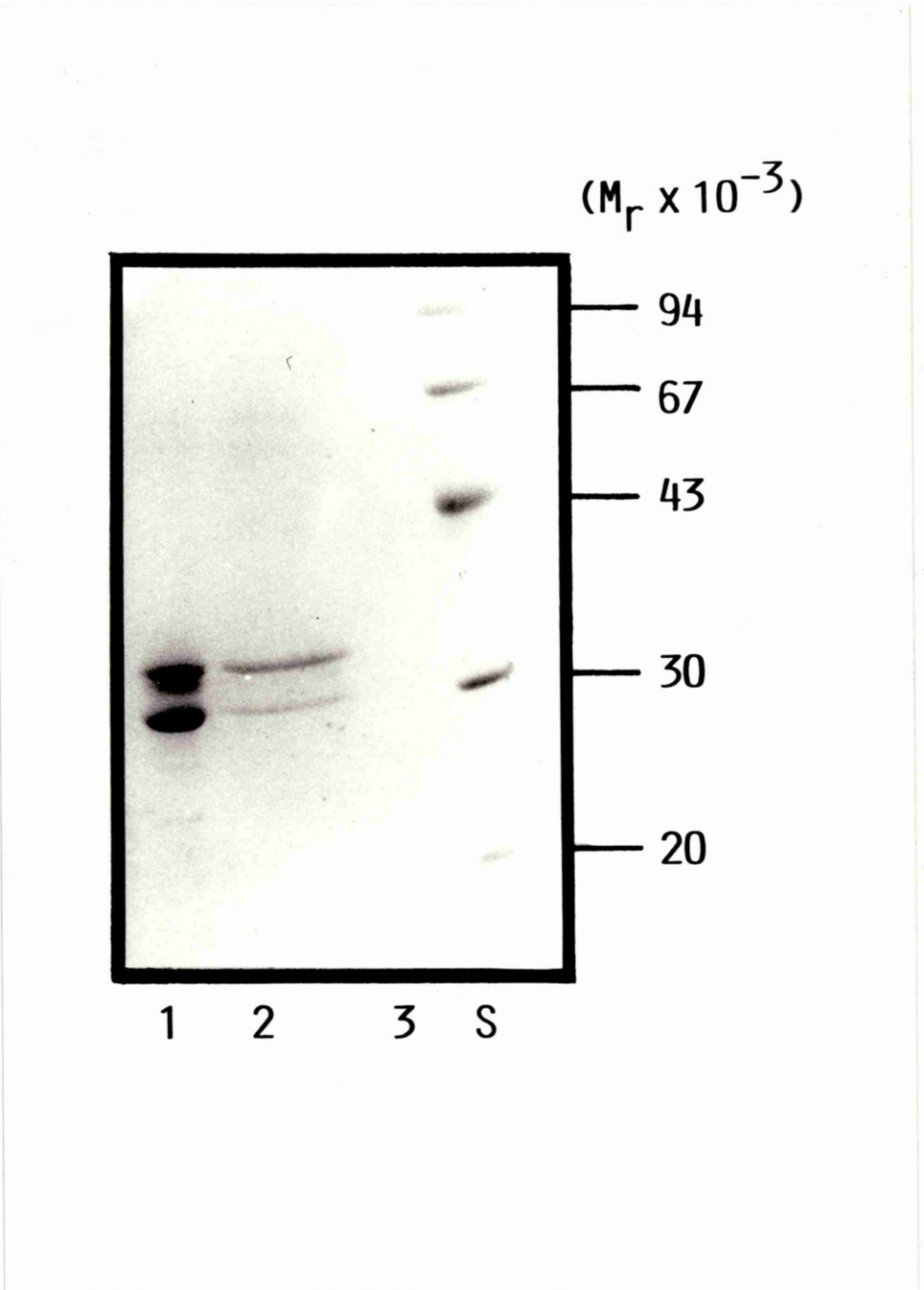


Figure 3.3.3: Analysis of rat liver mitochondrial (+CAT) HTP extract by FPLC (MonoS column)

The HTP-adsorbed extract was dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100, loaded onto a MonoS column equilibrated in the same buffer, and eluted with a 0-1.0M KH_2PO_4 gradient. A large unbound fraction was detected and collected and, although no peaks were detected during salt-elution, the KH_2PO_4 eluates were collected and pooled. The fractions were concentrated by exposure to poly (ethylene glycol) and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, rat liver HTP extract; 2, unbound fraction; 3, pooled KH_2PO_4 eluates; S, M_r standards.

Figure 3.3.3: Analysis of rat liver mitochondrial (+CAT) HTP extract by FPLC (MonoS column)



3.3.3 Analysis of mammalian PTP and ANT by Procion Red A column chromatography

The HTP-adsorbed extract isolated from rat liver mitochondria pre-loaded with CAT was analysed using 2.0 x 1.0cm columns of the Matrex gel Procion Red A. The PTP/ANT co-isolate was dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100, and loaded onto the column which was equilibrated in the same buffer. Elution was carried out using increasing concentrations of NaCl (0.1-1.0M) and finally with 1% (w/v) SDS. Unbound and eluted fractions were concentrated by exposure to poly (ethylene glycol) as described in Section 2.2.2, and prepared for analysis by SDS-PAGE (see Figure 3.3.4). As Figure 3.3.4 illustrates, both carriers readily bind to the Procion Red A column at pH 7.0. However, whereas PTP is eluted at 0.1-0.5M NaCl (optimally at 0.25M), ANT remains bound to the column during treatment with salt and is dislodged only upon elution with SDS, which will also result in denaturation of the carrier. A further observation was that a significant proportion of the loaded PTP behaves in a similar manner to ANT, remaining bound to the column until treatment with SDS. This will be discussed in a subsequent section (3.3.4). The apparent existence of PTP as a heterodimer in Figure 3.3.4 (Lane 7) is discussed in Section 3.2.1. The elution profiles of bovine heart PTP and ANT were found to be identical to those observed for the rat liver carriers, under the conditions described above (except using a 5.0 x 1.0cm column for the bovine heart co-isolate).

The above results indicate that the binding of PTP to Procion Red A is electrostatic, since an increase in ionic strength leads to elution of the carrier. However, the mode of binding of ANT to the column is unclear. Matrex gels consist of a highly complex molecular structure and, as a result, binding of proteins may involve electrostatic interactions, hydrophobic interactions and/or affinity binding. Consequently, it was decided to repeat the above experiment and attempt to dislodge ANT from the column by eluting with an increased concentration of NaCl (3.0M) as well as a collection of substrates and substrate analogues (ADP, ATP and NADP; all at 1mM). SDS-PAGE analysis of the unbound and eluted fractions is illustrated in Figure 3.3.5. Once again, ANT was observed to elute only upon treatment of the column with 1% (w/v) SDS. The absence of PTP in the SDS-eluate is discussed in Section 3.3.4.

The possibility that the ADP/ATP carrier binds electrostatically to Procion Red

Figure 3.3.4: Analysis of rat liver mitochondrial (+CAT) HTP extract by Procion Red A column chromatography

The HTP-adsorbed fraction containing ANT and PTP was dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100, loaded onto a 2.0 x 1.0cm Procion Red A column equilibrated in the same buffer, and eluted with 0.1-1.0M NaCl and 1% (w/v) SDS. Fractions were collected and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, rat liver HTP extract; 2, unbound fraction; 3-6, salt-eluates (0.1, 0.25, 0.5 and 1.0M NaCl); 7, 1% (w/v) SDS eluate; S, M_r standards.

Figure 3.3.4: Analysis of rat liver mitochondrial (+CAT) HTP extract by Procion Red A column chromatography (1)

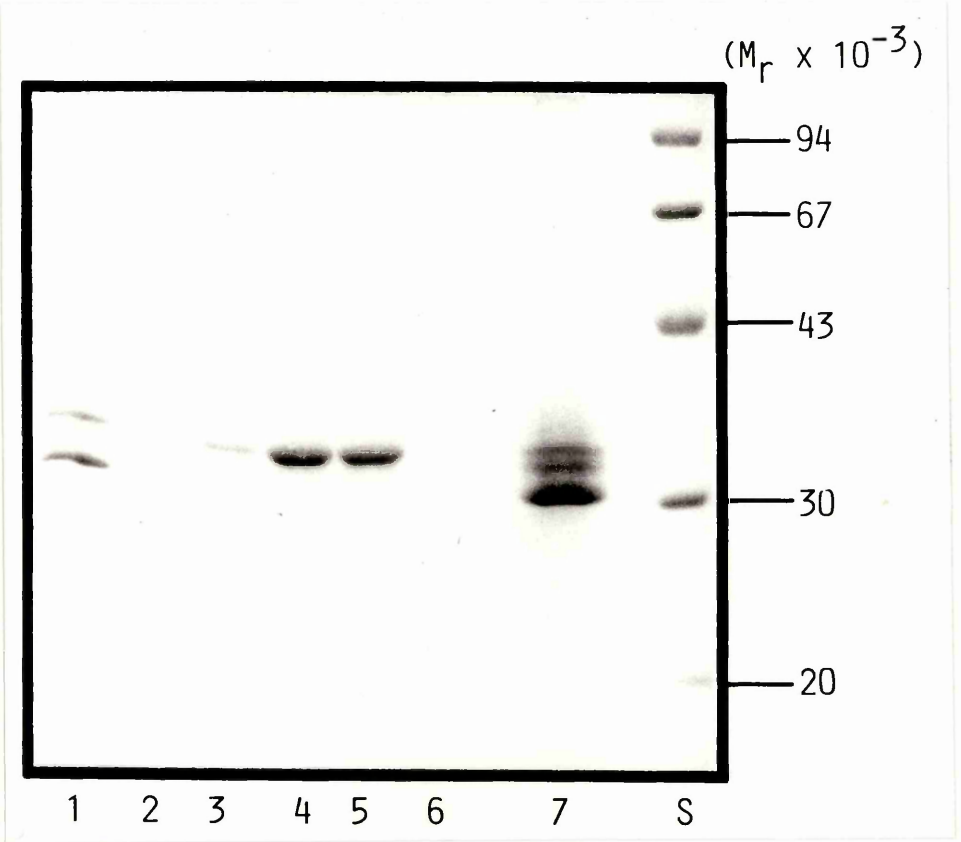
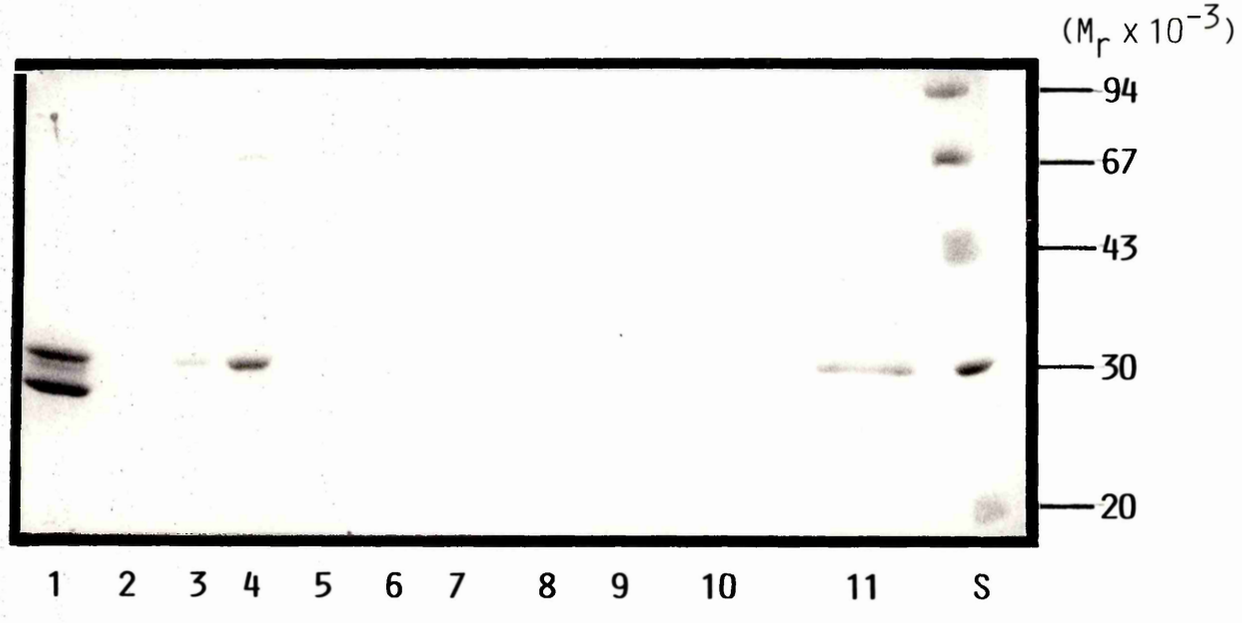


Figure 3.3.5: Analysis of rat liver mitochondrial (+CAT) HTP extract by Procion Red A column chromatography

The HTP-adsorbed fraction containing ANT and PTP was dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100, loaded onto a 2.0 x 1.0cm Procion Red A column equilibrated in the same buffer, and eluted with 0.1-3.0M NaCl, 1mM ADP, 1mM ATP, 1mM NADP and 1% (w/v) SDS. Fractions were collected and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, rat liver HTP extract; 2, unbound fraction; 3-7, salt-eluates (0.1, 0.25, 0.5, 1.0 and 3.0M NaCl); 8, ADP-eluate; 9, ATP-eluate; 10, NADP-eluate; 11, SDS-eluate; S, M_r standards.
(The co-isolate used in this experiment was extracted in the presence of 1mg/ml cardiolipin [see Section 3.3.4])

Figure 3.3.5: Analysis of rat liver mitochondrial (+CAT) HTP extract by Procion Red A column chromatography (2)



A was further investigated by determining the pH-dependence of the binding and elution profiles of the carriers with respect to the column. Accordingly, the analysis of the rat liver PTP and ANT co-isolate by Procion Red A column chromatography (as described above) was repeated at pH 6.0 (using KP_i as the buffer) and pH 8.0 (Tris-HCl). When the unbound and eluted fractions were analysed by SDS-PAGE the results obtained were identical to those illustrated in Figure 3.3.4 at both pH values. However, when the experiments were repeated with the HTP-adsorbed extract isolated from CAT-loaded bovine heart mitochondria, a small amount of ANT was detected in the 1.0M NaCl-eluate at pH 8.0 (see Figure 3.3.6). The elution profile of PTP was not affected, with optimal dislodgement of the carrier occuring at 0.25M NaCl, as illustrated in Figure 3.3.6. The data thus suggested that, by increasing the pH from 7.0 to 8.0, the net negative charge on bovine heart ANT is increased sufficiently (or the net positive charge is sufficiently decreased) for a proportion of the bound carrier to elute upon increasing ionic strength. The failure of rat liver ANT to elute under the same conditions could be explained by a slightly different net charge on the protein.

In an attempt to increase the yield of NaCl-eluted ANT and separate the carriers more effectively, the analysis of the bovine heart PTP and ANT co-isolate by Procion Red A column chromatography was repeated at pH 8.8. SDS-PAGE analysis of the fractions is illustrated in Figure 3.3.7, and clearly demonstrates the efficient separation of homogeneous populations of PTP (optimally eluted at 0.25M NaCl) and ANT (eluted at 1.0M NaCl). At pH 8.8, all bound ANT was salt-eluted, with none of the carrier being detected in the SDS-eluate (see Figure 3.3.7). When this experiment was repeated with rat liver PTP and ANT, and the fractions analysed by SDS-PAGE, the pattern observed was identical to that illustrated in Figure 3.3.7. Therefore, although ANTs from bovine heart and rat liver sources have slightly different elution profiles at pH 8.0, this discrepancy is corrected by a further increase in pH. This indicates that the varying elution profiles of the two mammalian ANTs at pH 8.0 is due to a slight difference in their overall charges, as previously suspected.

Following chromatographic separation, the identities of the bovine heart PTP and ANT were confirmed by N-terminal analysis, as described in Section 2.2.3. The primary sequences were compared to those previously described by Aquila *et al.*

Figure 3.3.6: Preliminary separation of bovine heart mitochondrial ANT and PTP by Procion Red A column chromatography

The HTP-adsorbed fraction (from mitochondria pre-incubated with CAT) containing ANT and PTP was dialysed into 20mM Tris-HCl pH 8.0, 0.5% (w/v) Triton X-100, loaded onto a 5.0 x 1.0cm Procion Red A column equilibrated in the same buffer, and eluted with 0.1-3.0M NaCl. Fractions were collected and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: S, M_r standards; 1, unbound fraction; 2-6, salt-eluates (0.1, 0.25, 0.5, 1.0 and 3.0M NaCl).

Figure 3.3.6: Preliminary separation of bovine heart mitochondrial PTP and ANT by Procion Red A column chromatography

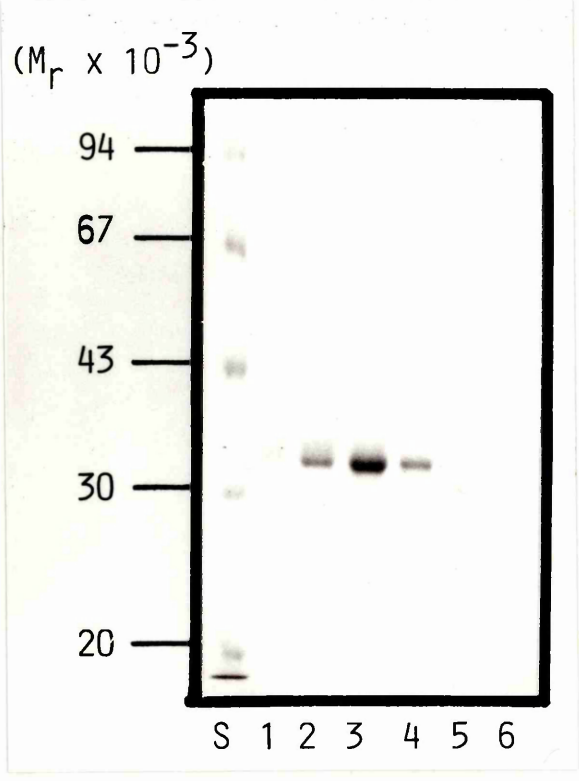
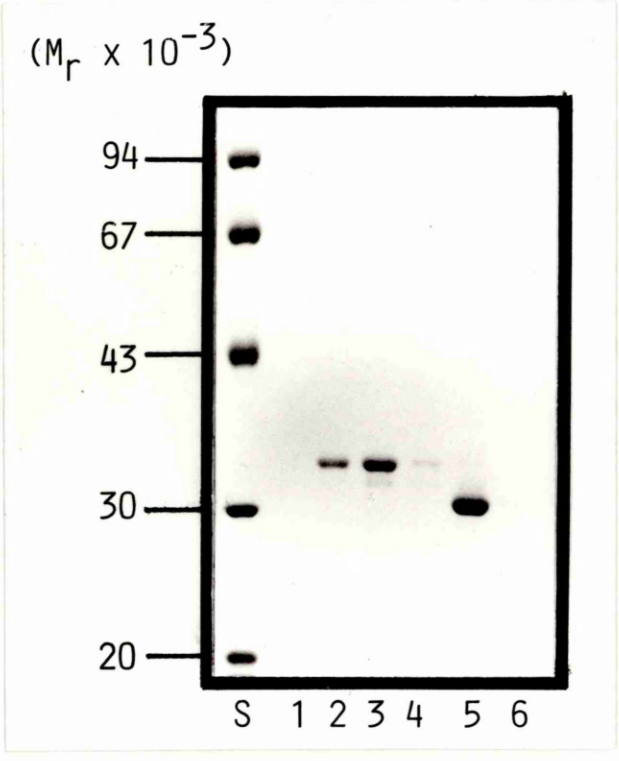


Figure 3.3.7: Separation of bovine heart mitochondrial ANT and PTP by Procion Red A column chromatography

The HTP-adsorbed extract (from mitochondria pre-incubated with CAT) containing ANT and PTP was dialysed into 20mM Tris-HCl pH 8.8, 0.5% (w/v) Triton X-100, loaded onto a 5.0 x 1.0cm Procion Red A column equilibrated in the same buffer, and eluted with 0.1-1.0M NaCl and 1% (w/v) SDS. Fractions were collected and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: S, M_r standards; 1, unbound fraction; 2-5, salt-eluates (0.1, 0.25, 0.5 and 1.0M NaCl); 6, 1% (w/v) SDS eluate.

(The co-isolate used in this experiment was extracted in the presence of 1mg/ml cardiolipin [see Section 3.3.4])

Figure 3.3.7: Separation of bovine heart mitochondrial PTP and ANT by Procion Red A column chromatography



(1982b) for ANT and Runswick *et al.* (1987) for PTP.

3.3.4 Procion Red A column chromatography represents an assay for native PTP conformation

During analysis of the HTP-adsorbed extracts from mammalian mitochondria pre-loaded with CAT by Procion Red A column chromatography, a discrepancy was noted in the elution profile of PTP. While some of the carrier was eluted with NaCl (optimally at 0.25M), a significant proportion remained bound to the column until treatment with SDS (see Figure 3.3.4). However, when cardiolipin (1mg/ml) was included in the extraction of the co-isolate (see Section 2.2.8), as in Figures 3.3.5 and 3.3.7, no PTP was detected in the SDS-eluate. This was re-examined by repeating the experiment illustrated in Figure 3.3.4, using rat liver mitochondria from the same preparation, but including cardiolipin (1mg/ml) in the extraction buffer. Analysis of the column fractions by SDS-PAGE clearly demonstrates the lack of significant quantities of PTP in the SDS-eluate (see Figure 3.3.8), with almost all the bound carrier being NaCl-eluted. The inclusion of cardiolipin had no observed effect on the subsequent elution profile of ANT at pH 7.0.

The above observations indicate that two distinct populations of PTP exist in the mammalian co-isolates, and both are capable of binding to Procion Red A. However, only one population can be eluted with NaCl, and the inclusion of cardiolipin in the purification procedure appears to greatly increase the proportion of the carrier in this conformation. As discussed previously (see Section 1.2.6), cardiolipin is thought to maintain PTP in its native conformation in reconstitution studies (Kadenbach *et al.*, 1982; Mende *et al.*, 1982; Cheneval *et al.*, 1983). It can thus be postulated that the fraction of PTP which is NaCl-eluted from the Procion Red A column exists in a native conformation, an important pre-requisite for physical analyses and crystallisation trials prior to X-ray diffraction studies.

This hypothesis was further tested by analysing denatured mammalian mitochondrial co-purified PTP and ANT by Procion Red A column chromatography. The HTP-adsorbed fraction from CAT-loaded rat liver mitochondria was heat-denatured at 80°C for 10min prior to loading onto a 2.0 x 1.0cm Procion Red A column. Analysis of the column fractions by SDS-PAGE is illustrated in Figure 3.3.9,

Figure 3.3.8: Analysis of rat liver mitochondrial (+CAT) HTP extract (+cardiolipin) by Procion Red A column chromatography

The HTP-adsorbed fraction containing ANT and PTP was prepared in the presence of 1mg/ml cardiolipin (see Section 2.2.8), dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100, loaded onto a 2.0 x 1.0cm Procion Red A column equilibrated in the same buffer, and eluted with 0.1-3.0M NaCl and 1% (w/v) SDS. Fractions were collected and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, rat liver HTP extract; 2, unbound fraction; 3-8, salt-eluates (0.1, 0.25, 0.5, 1.0, 2.0 and 3.0M NaCl); 9, 1% (w/v) SDS eluate; S, M_r standards.

Figure 3.3.8: Analysis of rat liver mitochondrial (+CAT) HTP extract (+cardiolipin) by Procion Red A column chromatography

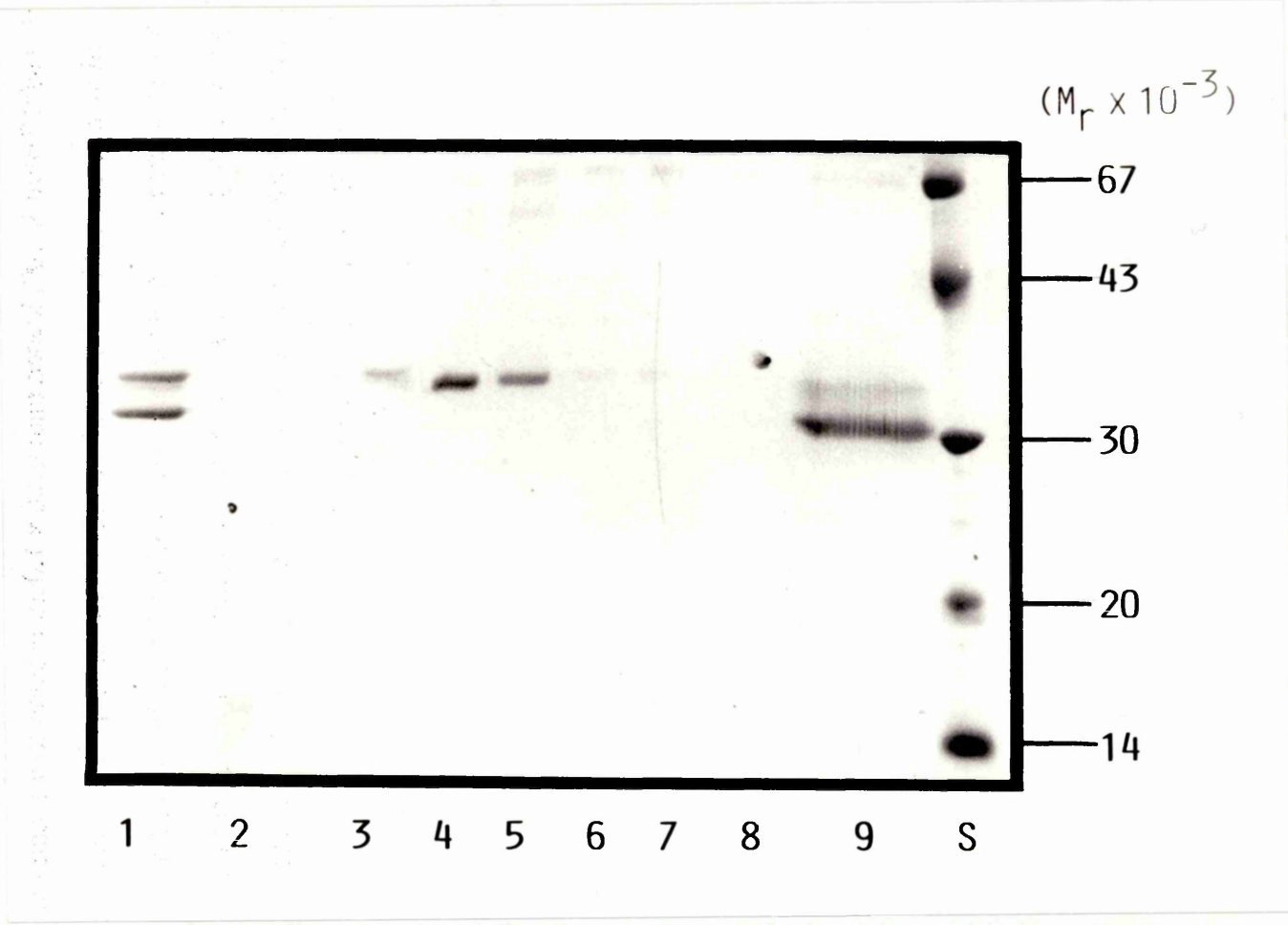
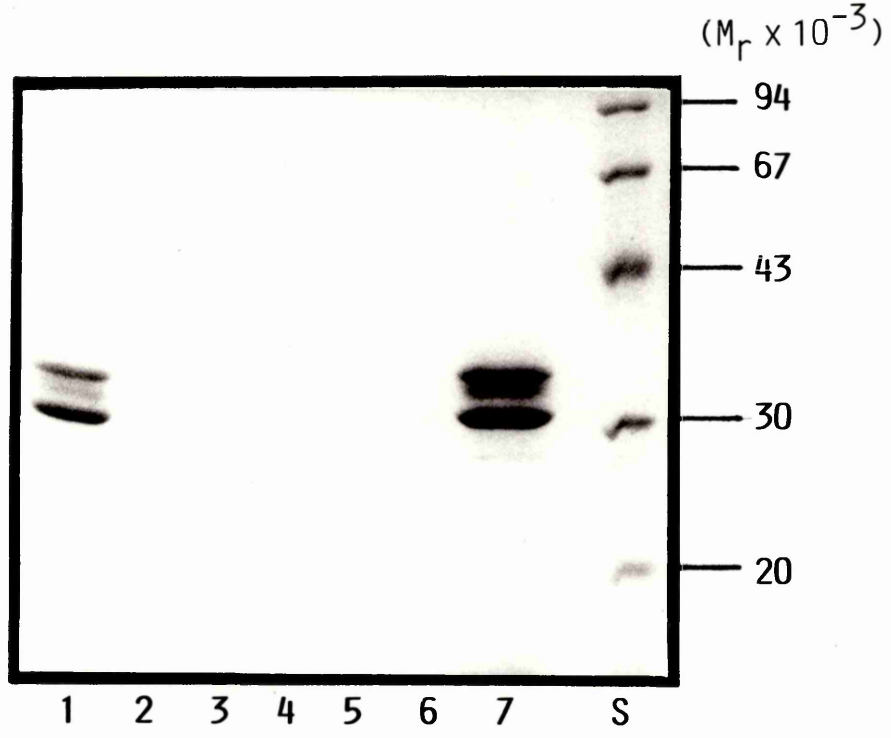


Figure 3.3.9: Analysis of denatured rat liver mitochondrial (+CAT) HTP extract by Procion Red A column chromatography

The HTP-adsorbed fraction containing ANT and PTP was dialysed into 20mM Tris-HCl pH 8.8, 0.5% (w/v) Triton X-100, heat-denatured at 80°C for 10min, loaded onto a 2.0 x 1.0cm Procion Red A column equilibrated in the same buffer, and eluted with 0.1-1.0M NaCl and 1% (w/v) SDS. Fractions were collected and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, rat liver HTP extract; 2, unbound fraction; 3-6, salt-eluates (0.1, 0.25, 0.5 and 1.0M NaCl); 7, 1% (w/v) SDS eluate; S, M_r standards.

Figure 3.3.9: Analysis of denatured rat liver mitochondrial (+CAT) HTP extract by Procion Red A column chromatography



and clearly demonstrates that denatured PTP binds to the Matrex gel but is eluted only upon treatment with SDS. This lends further support to the proposal that the Procion Red A column represents an assay for native PTP conformation. As this experiment was performed at pH 8.8, it was also possible to determine the effect of heat-denaturation on the elution profile of ANT. Figure 3.3.9 illustrates that ANT also remains bound to the column until treatment with SDS, indicating that the Matrex gel may represent an assay for both these carriers in their native states.

3.3.5 Preparation of PTP for analysis by Circular Dichroism

The preceding Sections (3.3.3 and 3.3.4) outline the methodology for the elution of a homogeneous population of native mammalian PTP from a Procion Red A column. Thus, important criteria for further analysis of the carrier have been satisified. However, the detergent used in the preparation of PTP, Triton X-100, is unsuitable for both circular dichroism (CD) studies and X-ray crystallography. The non-ionic detergents most commonly used for physical analysis and crystallographic studies on membrane proteins are lauryl dimethyl amine oxide (LDAO), *n*- octylglucoside and *n*-laurylmaltoside. However, with the exception of LDAO, the cost of these detergents limits their use in the large-scale preparation of a membrane carrier such as PTP. It was thus decided to employ the Procion Red A Matrex gel in an attempt to obtain purified native PTP solubilised in each of the above detergents in place of Triton X-100.

The concentrations of LDAO, *n*- octylglucoside and *n*- laurylmaltoside to be used for solubilising PTP were chosen after reference to the critical micelle concentration (CMC) values of the detergents. Although data were unavailable for LDAO, a concentration of 1% (w/v) was used on the basis of previous studies in our laboratory. For *n*- laurylmaltoside, a concentration of 0.5% (w/v) was chosen, easily fulfilling the criteria that a detergent should be used above its CMC value (Womack *et al.*, 1983) and at a detergent/protein weight ratio of at least 10 (Matson and Goheen, 1986). In contrast, *n*- octylglucoside has an extremely high CMC value (0.73% [w/v]), ~50-100x greater than those of *n*- laurylmaltoside (0.006% [w/v]) and Triton X-100 (0.015% [w/v]). A concentration of 1% (w/v) was chosen for this detergent. Although this exceeds the CMC value, potential solubility problems were envisaged since the detergent/protein weight ratio may, on some occasions, be less than 10. The cost of

this detergent limited its use at higher concentrations.

Initial attempts to switch PTP from solution in Triton X-100 to detergents suitable for analysis by CD were carried out using the rat liver carrier. Ultimately though, bovine heart PTP would be used to maximise protein yield. The HTP-adsorbed fraction (extracted in the presence of 1mg/ml cardiolipin) from CAT-loaded rat liver mitochondria was dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100, and loaded onto a 2.0 x 1.0cm Procion Red A column equilibrated in the same buffer. Subsequently, the column was washed firstly with 5 vol 20mM Tris-HCl pH 7.0 (to wash out the Triton X-100), then with 5 vol of the same buffer supplemented with 1% (w/v) LDAO. 5 vol 20mM Tris-HCl pH 7.0, 1% (w/v) LDAO, 0.25M NaCl was then applied to the column to elute the PTP. This experiment was also carried out using 0.5% (w/v) *n*- laurylmaltoside and 1% (w/v) *n*- octylglucoside in place of LDAO. Eluates were concentrated in Novacell stirred cells (see Section 2.2.2) and analysed by SDS-PAGE. In all three cases, no PTP was detected in the NaCl-eluted fractions. However, when the columns were washed with 1% (w/v) SDS, the carrier was eluted in each case.

It was presumed that, in the above experiments, precipitation of PTP had occurred in the absence of detergent when the columns were washed with 20mM Tris-HCl pH 7.0. It was thus decided to repeat the chromatographic analyses, omitting the detergent-free wash in each case. SDS-PAGE analysis of the fractions demonstrated quantitative recovery of PTP in the 0.25M NaCl-eluates using all three detergents. The transfer of PTP from solution in Triton X-100 to *n*- octylglucoside is illustrated in Figure 3.3.10.

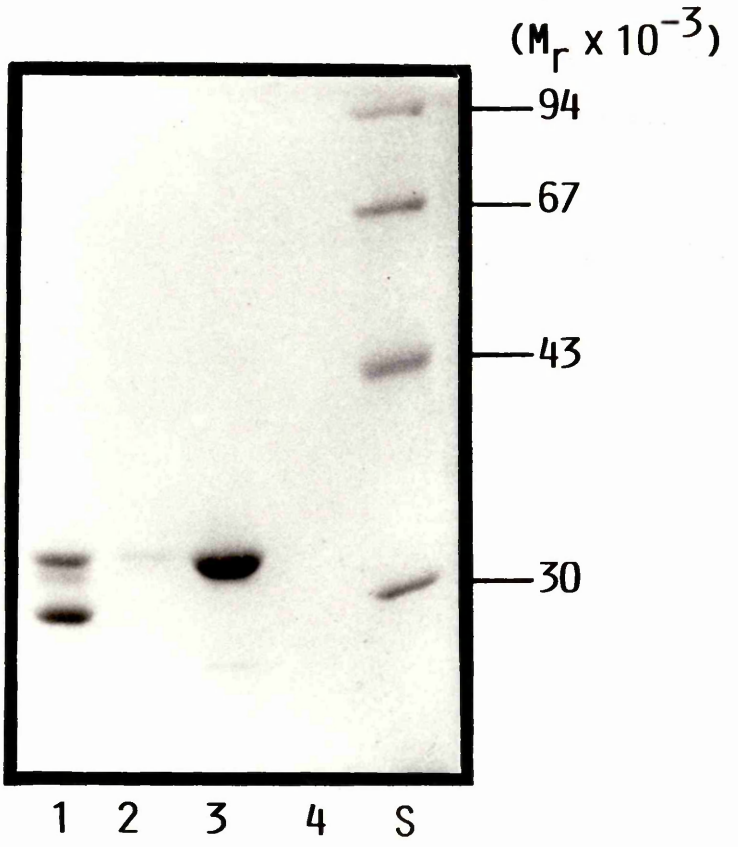
Subsequently, it was shown that bovine heart PTP can be transferred from solution in Triton X-100 to the other non-ionic detergents by application of the above methodology, except using a 5.0 x 1.0cm Procion Red A column. Bovine heart PTP was prepared in 0.5% (w/v) *n*- laurylmaltoside and 1% (w/v) *n*- octylglucoside for analysis by CD at the SERC-funded facility at Stirling University (see Section 2.2.10). Small samples of these preparations were also reserved for determination of protein concentration by amino acid analysis (see Section 2.2.1). It was thus possible to confirm the identity of the purified carrier as PTP from its amino acid content and subunit molecular weight. However, the protein concentrations obtained were

Figure 3.3.10: Transfer of rat liver mitochondrial PTP from solution in Triton X-100 to *n*- octylglucoside

The rat liver HTP extract containing PTP and ANT was dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100 (buffer A) and loaded onto a 2.0 x 1.0cm Procion Red A column equilibrated in the same buffer. The column was then re-equilibrated in 20mM Tris-HCl pH 7.0, 1.0% *n*- octylglucoside (buffer B) and the PTP eluted with 0.25M NaCl in buffer B. Fractions were collected and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, HTP extract; 2, unbound fraction; 3, 0.25M NaCl eluate; 4, 1.0M NaCl eluate; S, M_r standards.

(The co-isolate used in this experiment was extracted in the presence of 1mg/ml cardiolipin)

Figure 3.3.10: Transfer of rat liver PTP from solution in Triton X-100 to *n*- octylglucoside



considerably lower than expected (0.02-0.05mg/ml). This may be due to some denaturation of the Procion Red A-bound carrier during the detergent switch and/or the adhesion of mixed PTP-detergent micelles to the filter during protein concentration in Novacell stirred cells. In the case of *n*- octylglucoside, the detergent/protein weight ratio may be low enough to inhibit solubilisation of the PTP.

The CD spectra of bovine heart PTP in *n*- octylglucoside and *n*- laurylmaltoside are illustrated in Figure 3.3.11. The spectra show that there is very good correlation between the secondary structures of the carrier in both detergents. Computer analysis of the data in conjunction with the calculated protein levels revealed the values for the nature of the secondary structure in each case (see Figure 3.3.11). The extremely high α-helical contents (85-95%) are at least double the 41% value reported by Aquila (unpublished data) for ANT and UCP, and may be partly the result of discrepancies caused by the spectra being so noisy over the range 190-200nm. However, bovine heart PTP has at least 6 transmembrane domains and is also extremely resistant to proteolysis (Phelps, 1987), indicating that such high values may not be unreasonable. Considerable doubt must also be placed on Aquila's data which was both unpublished and reported when the technology associated with spectropolarimeters was severely restricted. In addition, limited data bases were available for the calculation of secondary structures.

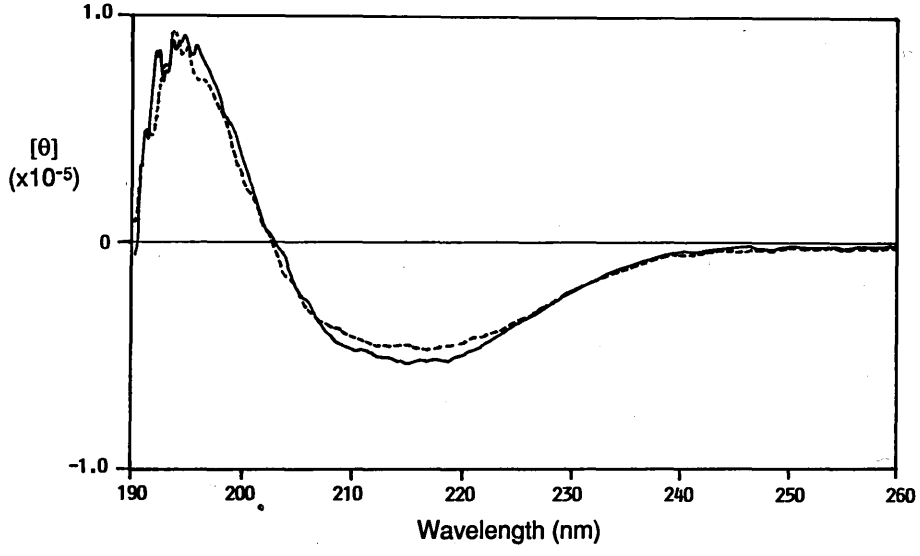
In *n*- octylglucoside, PTP was calculated to contain a small (5%) β-sheet structure, which may correspond to the putative 7th transmembrane domain (Aquila *et al.*, 1985) proposed for PTP, ANT and other related carriers (see Section 1.2.9). However, in *n*- laurylmaltoside, no evidence was obtained for the presence of β-pleated sheet structures.

The preparation of bovine heart PTP in 1% (w/v) *n*- octylglucoside was also employed to analyse the denaturation spectra of the purified carrier in this detergent. PTP was denatured by incubation with increasing concentrations (2.0-6.0M) of guanidine-HCl (GdnHCl), as described in Section 2.2.10, prior to secondary structure analysis by CD. The resulting spectra (see Figure 3.3.12) are illustrated over the wavelength range 210-260nm in order to concentrate on the main changes in secondary

Figure 3.3.11: CD spectra for bovine heart PTP

Bovine heart PTP was prepared in 1% (w/v) *n*- octylglucoside and 0.5% (w/v) *n*- laurylmaltoside, and CD-analysed using a spectropolarimeter linked to data processors.

Figure 3.3.11: CD spectra for bovine heart PTP

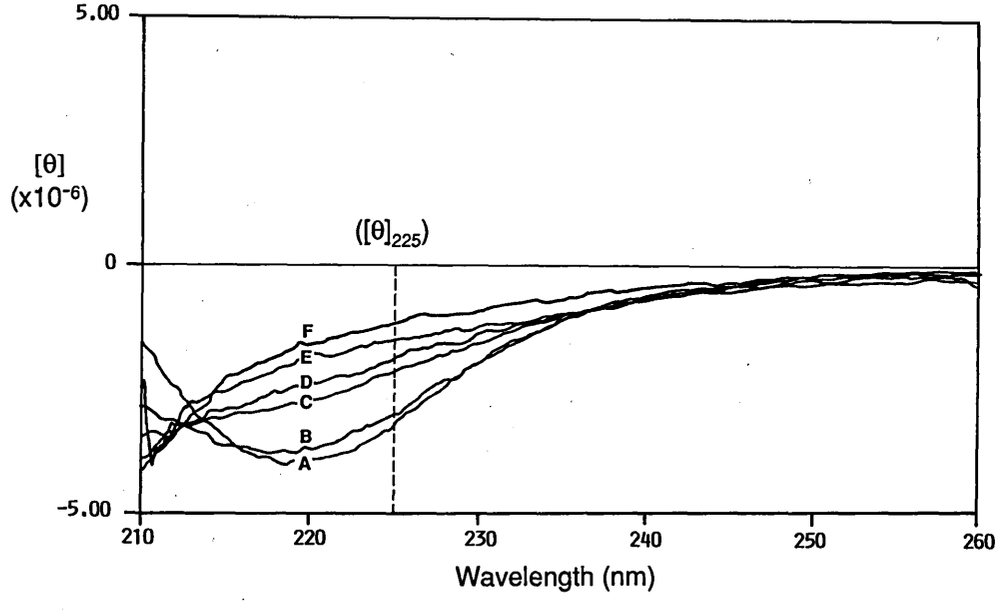


Secondary structure	α-helix (%)	β-sheet (%)	Other
——PTP (octylglucoside)	95 (±13)	5 (±13)	0 (±4 x 10^{-6})
-----PTP (laurylmaltoside)	85 (±18)	0 (±4 x 10^{-6})	15 (±18)

Figure 3.3.12: CD spectra for bovine heart PTP in the presence of GdnHCl

Bovine heart PTP was prepared in 1% (w/v) *n*- octylglucoside for analysis by CD. Samples of the purified protein were prepared in the presence and absence of varying concentrations of guanidine-HCl (GdnHCl), with the spectra being recorded 15min after the addition of this reagent.

Figure 3.3.12: CD spectra for bovine heart PTP in the presence of GdnHCl



A: PTP (control)
B: PTP (2M Gdn HCl)
C: PTP (2.5M Gdn HCl)
D: PTP (3M GdnHCl)
E: PTP (4M Gdn HCl)
F: PTP (6M Gdn HCl)

structure, which occur at 210-230nm. Figure 3.3.12 demonstrates that the largest such change at $[\theta]_{225}$ occurs between 2.0 and 2.5M GdnHCl, corresponding to the denaturation of secondary structure. Based upon this data, bovine heart PTP remains essentially in its native conformation at GdnHCl concentrations of up to approximately 2.0M. However, at concentrations above this level the carrier is rapidly denatured. This experiment was not repeated in an alternative detergent to determine the effect of this on the stability of PTP. In addition to this, no equivalent data were available for structurally related proteins for comparison with that obtained for PTP.

3.4 DISCUSSION

A variety of studies were carried out on the inner membrane carriers, PTP and ANT, from both rat liver and bovine heart mitochondria. PTP was purified to homogeneity from both mammalian sources, and monospecific high titre antiserum was raised against the rat liver carrier. In addition, it was found that, by pre-incubating freshly-prepared mitochondria with CAT, it was possible to stabilise the ADP/ATP carrier, thus facilitating the co-purification of the two inner membrane proteins (PTP and ANT) following adsorption chromatography on HTP. Further fractionation with Celite adsorbed out ANT.

The co-isolation procedure for PTP and ANT is almost identical to the reported methodology for purifying the ADP/ATP carrier to apparent homogeneity (Riccio *et al.*, 1975a,b), except that salt (0.5M NaCl or KCl) is included in the extraction buffer of the latter procedure. However, although the inclusion of 0.5M KCl in the extraction buffer of the co-isolate increased the proportion of ANT present, it was not possible to purify the translocase in this manner without significant PTP contamination. This result places an element of doubt on the observations of Riccio *et al.* (1975a,b), and subsequent reports on the purification and characterisation of ANT (Kramer *et al.*, 1977; Kramer and Klingenberg, 1977a,b; Kramer and Klingenberg, 1979). Significantly, none of the above reports includes an illustration of the analysis of ANT isolation by SDS-PAGE. It therefore seems likely that the results presented in these reports may have been influenced by the presence of contaminating PTP in the ANT preparations.

Various types of column were employed in an attempt to chromatographically

separate co-purified PTP and ANT. Initially, the co-isolate was applied to the FPLC columns, MonoQ (an anion exchanger) and MonoS (a cation exchanger). However, neither of these was found to be suitable for separation of the carriers. PTP did not bind to either column under the conditions tested, while ANT was found to bind only to the MonoQ column at pH 8.0. Although ANT remained in the unbound fraction over the pH range 7.0-7.5, it was not possible to identify the conditions at which the bound carrier could be eluted from the column by increasing the ionic strength (0-1.0M NaCl gradient). Such a binding pattern indicates that the adsorption of ANT to the MonoQ column is not solely electrostatic. No conditions were identified at which either carrier bound to the MonoS column.

In contrast to the FPLC columns, both PTP and ANT were observed to bind to the Matrex gel, Procion Red A, at pH 7.0. Although PTP was eluted with NaCl (optimally at 0.25M), elution of ANT resulted only after washing the column with SDS. However, when the pH was raised to 8.8, co-purified PTP and ANT were separated into homogeneous populations by differential elution from the Procion Red A column. PTP was eluted with 0.25M NaCl, ANT with 1.0M NaCl. Since this separation procedure works for both rat liver and bovine heart co-isolates, it is possible to purify large (1-3mg) quantities of both carriers from a single mitochondrial preparation. Following separation, the identities of the bovine heart carriers were confirmed by N-terminal protein sequence analysis.

The Procion Red A column was also found to represent an assay for native PTP conformation, an important pre-requisite for physical studies of the carrier and crystallisation trials. Heat-denatured PTP binds to Procion Red A, but can only be eluted with SDS. The inclusion of cardiolipin in the extraction process resulted in a greater proportion of the carrier being detected in the 0.25M NaCl-eluate. This was presumed to be due to the reported enhancement of PTP activity by this lipid (see Section 1.2.6). The use of this Matrex gel as an assay for native PTP conformation is particularly beneficial in view of the difficulty in reconstituting active PTP into artificial liposomes, and the low affinity of the carrier for its substrate, P_i. However, in future it will be necessary to assess the potential of Procion Red A as an assay for the native carrier by comparing the P_i-exchange activities of reconstituted PTP populations with

their elution profiles from the Matrex gel.

The conformations of the two PTP populations described above were not investigated. It is possible that PTP binds electrostatically to Procion Red A, but is eluted by increased ionic strength (0.25M NaCl) only if the carrier is in a homodimeric state, the proposed native conformation of Kramer and Klingenberg (1979) for ANT and related carriers. The complex molecular structure of Matrex gels would permit such a phenomenon. It would thus be reasonable to assume that Procion Red A also represents an assay for native ANT activity. Chromatographic analysis of the denatured PTP/ANT co-isolate at pH 8.8 indicated that this is the case. The conformation of purified ANT can be further checked by application to an HTP column, which would adsorb out any denatured carrier (Klingenberg *et al.*, 1974; Schleyer and Neupert, 1984).

Procion Red A column chromatography was also employed as a technique for transferring PTP from solution in Triton X-100 to non-ionic detergents suitable for physical analyses and crystallography trials. Although PTP was successfully transferred into LDAO, *n*- laurylmaltoside and *n*- octylglucoside, significant reductions in protein yields (~70-80%) were frequently observed during this process. This was thought to be due to some denaturation of the bound carrier during the detergent switch and/or attachment of mixed PTP-detergent micelles to the filter during protein concentration in Novacell stirred cells. Further research is required to minimise this protein loss, particularly in view of the relatively high protein concentrations required for crystallisation trials. Although the formation of crystals suitable for X-ray diffraction studies from membrane proteins is a difficult process, the techniques have been employed previously in our laboratory to analyse the B800-850 light harvesting complex from *Rps. acidophila* (strain 10050), and determine its oligomeric state (Papiz *et al.*, 1989). Ultimately, it is hoped that, by obtaining crystals of PTP suitable for X-ray diffraction studies, it will be possible to elucidate the high resolution three-dimensional structure of the protein. This in turn should lead to a greater understanding of the molecular mechanisms underlying the membrane insertion, intramembrane organisation and catalytic mechanisms of a family of related mitochondrial carriers (PTP, ANT, UCP, *etc.* ; see Section 1.2.9). In addition, the highly refined X-ray structure of PTP should reveal the protein's substrate-binding site,

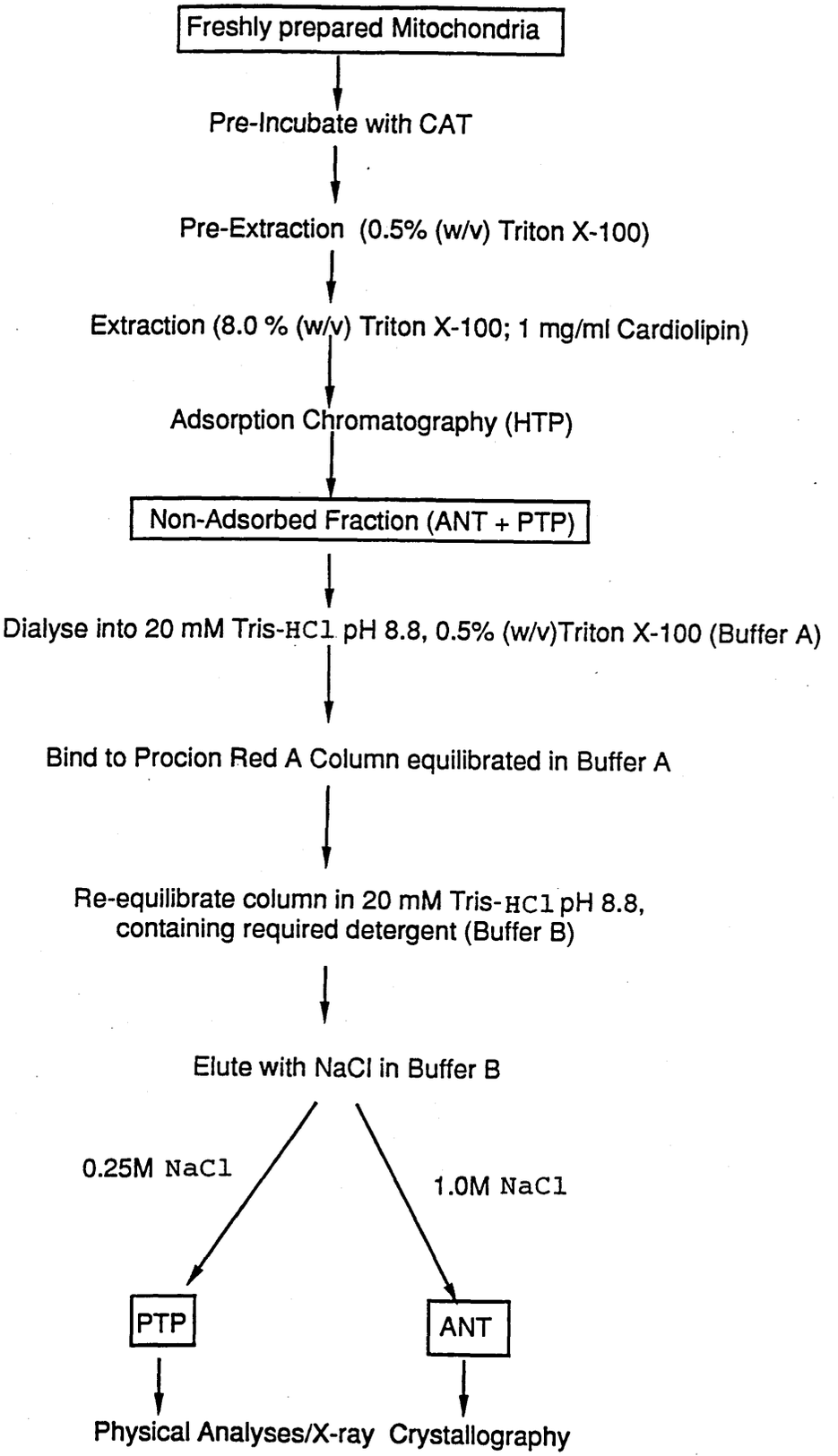
inviting comparison with the recently reported ligand-binding site of a bacterial phosphate transport protein (Luecke and Quiocho, 1990). Originally crystallised four years earlier (Kubena *et al.*, 1986), this phosphate-binding protein (PBP) is not, like the mammalian PTP, a transmembrane carrier, but instead is a periplasmic protein which acts as an initial high-affinity receptor for an osmotic active transport system for phosphate. This bacterial transport system involves the co-operation of a variety of proteins. It is likely that PBP and PTP possess structurally different substrate-binding sites since the bacterial protein, unlike the mammalian carrier, contains a very tight phosphate-binding site, which can also bind to and facilitate the transport of arsenate (Kubena *et al.*, 1986; Luecke and Quiocho, 1990).

Although the transfer of ANT from solution in Triton X-100 to alternative non-ionic detergents was not investigated, it is likely that the Procion Red A column could be employed for this carrier also. Figure 3.4.1 outlines a proposed scheme for the preparation of mammalian PTP and ANT for physical analyses and X-ray crystallography.

Bovine heart PTP was prepared in 0.5% (w/v) *n*- laurylmaltoside and 1.0% (w/v) *n*- octylglucoside for analysis by CD. The secondary structure of the carrier was extremely similar in each case, with α-helical content in the range 85-95%, suggesting that PTP was in a native conformation in both detergents. Although possibly rather high, such a value for α structure is not unrealistic in view of the 6-7 transmembrane domains possessed by PTP, and the protease-resistance of the carrier in the membrane (Phelps, 1987). CD analysis also indicated a slight structural difference in each detergent: the 5% β-sheet structure present in *n*- octylglucoside, which may correspond to the putative 7th transmembrane domain, was not detected in *n*- laurylmaltoside. The low level of this secondary structure observed in the former detergent may be below the level of significance.

Another possible reason for the apparently high α-helical content of PTP is the procedure adopted to deduce the secondary structure of CD-analysed proteins. This CONTIN procedure (Provencher and Glockner, 1981) is based on reference CD spectra derived from 16 actual globular proteins of known secondary structure (from

Figure 3.4.1: Preparation of mammalian PTP and ANT for physical analyses and X-ray crystallography



X-ray diffraction studies). When used to analyse the CD spectra of other globular proteins, the reference values in the CONTIN data are appropriate. However, this may not be the case for non-globular and membrane-spanning proteins. Chen *et al.* (1974) report that the mean residue weight CD signal due to a helix is actually a function of the helix length, increasing to a limit as the helix lengthens. Thus, for proteins with very high helical content or long stretches of helix, the CONTIN reference values may not be appropriate and may overestimate the % helical content. This may be the case for a membrane-spanning protein such as PTP.

By incubating bovine heart PTP in *n*- octylglucoside with increasing concentrations of GdnHCl (2.0-6.0M), it was also possible to obtain the denaturation spectra for the carrier in this detergent. CD analyses indicated that the largest change in the spectrum occurred between 2.0 and 2.5M GdnHCl, corresponding to the rapid loss of secondary structure. However, no comparison with structurally related proteins was possible as no such data have been reported in the literature. In future, it is hoped to repeat the CD analyses of native and denatured PTP in different detergents, and compare the data with that obtained previously, and also with equivalent analyses of the bovine heart ADP/ATP carrier, prepared as described in Section 3.3.3. In this manner, it will be possible to assess the stability of ANT and PTP in a variety of detergents and determine which is the most suitable for crystallisation trials on the carriers.

CHAPTER 4: ISOLATION AND CHARACTERISATION OF THE YEAST MITOCHONDRIAL PHOSPHATE AND ADENINE NUCLEOTIDE CARRIERS

4.1 INTRODUCTION

In recent years, much biochemical and genetic research has been carried out using yeast, in particular strains of the baker's yeast *S.cerevisiae*. As a result, classical genetics have become highly developed in this organism, making it a suitable model for both advanced molecular genetic analyses and further biochemical research. In our laboratory, the use of *S.cerevisiae* as a biochemical model has been facilitated by the availability of large quantities of baker's yeast (see Section 2.1.4).

Recent work in our laboratory has identified a 30,000-M_r polypeptide in *S.cerevisiae* whole cell and mitochondrial extracts which cross-reacts strongly with antibodies raised against rat liver PTP (Gibb, 1985). It has been further demonstrated that the cytosolic precursor of this putative yeast PTP lacks a cleavable presequence (Hodgson *et al.*, 1988; see Section 4.2). Since Wohlrab's group were attempting to clone and sequence the gene encoding yeast PTP at this stage (Phelps *et al.*, 1991), it was decided to develop and optimise a procedure for purifying this carrier from yeast mitochondria. This was carried out by taking advantage of the aforementioned cross-reactivity of anti-rat liver PTP serum with the equivalent yeast carrier; and by utilising the established procedure for the preparation of mammalian PTP (Gibb *et al.*, 1986). Further protein-chemical analyses were performed to establish a method for co-purifying PTP and the closely associated ANT from yeast mitochondria, and to carry out preliminary characterisation of the carriers.

4.2 BIOSYNTHESIS OF YEAST PTP

The biosynthesis of the 30,000-M_r putative yeast PTP identified by Gibb (1985) was investigated by immune replica analysis of CCCP-inhibited yeast cells. In this study, cell extracts containing accumulated cytosolic precursors were prepared following growth of the yeast petite (rho^-) mutant *S.cerevisiae* D273-10B-1 in the presence of the respiratory uncoupler CCCP, as described in Section 2.2.15 (Reid and

Schatz, 1982). Cell extracts were subjected to analysis by SDS-PAGE followed by Western blotting with antiserum raised against bovine heart pyruvate dehydrogenase multienzyme complex (PDC; see Figure 4.2.1), and anti-rat liver PTP serum (see Figure 4.2.2). Figure 4.2.1 clearly demonstrates the presence of higher-M_r precursors of subunits of yeast PDC (E1β and E2; no cross-reaction of yeast E1α, E3 or X with bovine anti-PDC serum was observed). Accumulation of pre-E2, with an M_r value 3-4,000 greater than its mature counterpart, is particularly apparent. In contrast, the presence of a higher-M_r precursor to the putative yeast PTP is not detected (see Figure 4.2.2) suggesting that pre-PTP exhibits a similar mobility to the mature carrier.

These results are borne out by densitometric scanning of the immune blots illustrated in Figures 4.2.1 and 4.2.2, with the data being presented graphically (see Figure 4.2.3). In the presence of 1-5μM CCCP, an increase in the levels of pre-PDC E2 per cell is observed, corresponding to decreasing levels of its mature equivalent. The apparent reduction in precursor accumulation at CCCP concentrations >5μM reflects significant inhibition of growth of *S.cerevisiae* D273-10B-1 at these levels of the uncoupler. In the case of PTP, constant levels of the 30,000-M_r polypeptide per cell irrespective of CCCP concentration indicates the absence of a higher-M_r cytosolic precursor of this protein. A decline in the levels of the 30,000-M_r putative yeast PTP per cell upon increasing CCCP concentrations (as observed for PDC E2) would have indicated the presence of an undetectable higher-M_r pre-PTP.

The results in this section indicate that yeast PTP, unlike its mammalian counterparts (Runswick *et al.*, 1987; Ferreira *et al.*, 1989), is not initially synthesised with a proteolytically-cleavable N-terminal extension. In common with ANT from various sources, this protein may contain an internal sequence responsible for mitochondrial targeting (see Section 1.1.6.2).

Figure 4.2.1: Accumulation of PDC subunit precursors in yeast cells inhibited by CCCP

As described in Section 2.2.15, cell extracts were prepared from *S.cerevisiae* D-273-10B-1 grown on YPG in the presence of increasing concentrations of CCCP (0, 1, 2, 5, 10 and 20 μM as illustrated). Extracts were subjected to SDS-PAGE prior to immune replica analysis with anti-bovine heart PDC serum. Lanes: S, M_r standards (^{125}I-radiolabelled).

Figure 4.2.1: Accumulation of PDC subunit precursors in yeast cells inhibited with CCCP

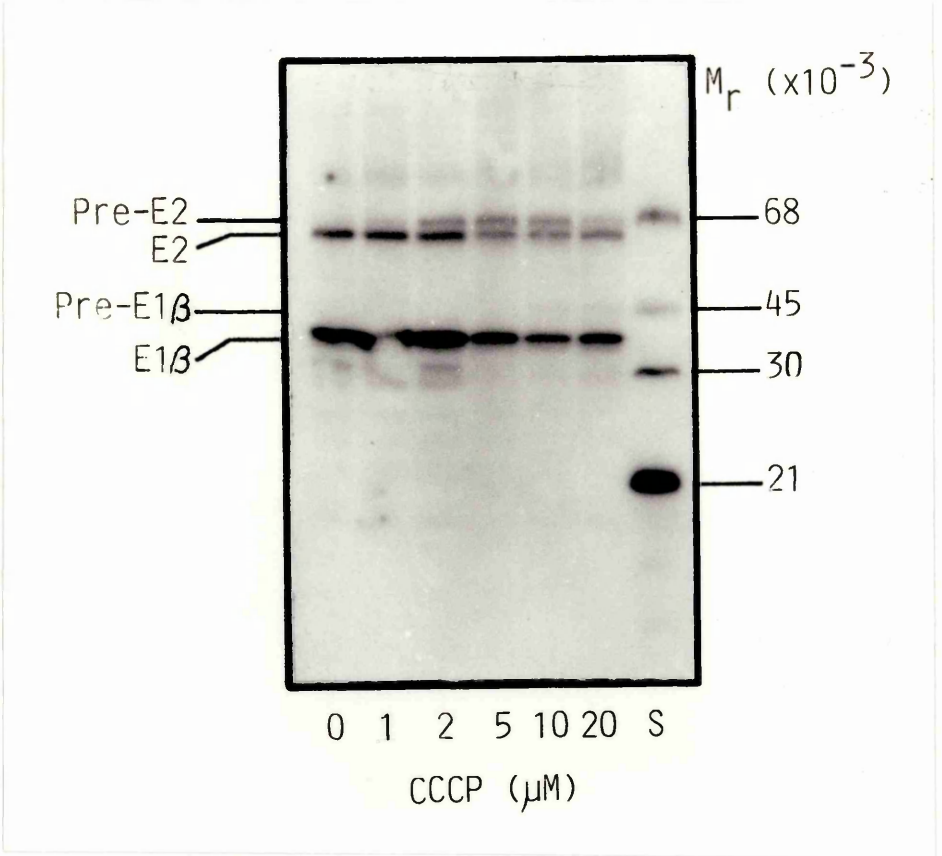


Figure 4.2.2: Accumulation of PTP precursors in yeast cells inhibited by CCCP

As described in Section 2.2.15, cell extracts were prepared from *S.cerevisiae* D273-10B-1 grown in the presence of increasing concentrations of CCCP (0, 1, 2, 5, 10 and 20 μM in lanes 1, 2, 3, 4, 5, and 6 respectively). Extracts were subjected to SDS-PAGE prior to immune replica analysis with anti-rat liver PTP serum. Lanes: 7, purified rat liver PTP; 8, M_r standards (^{125}I-radiolabelled).

The apparent low M_r value bands in lanes 2 and 3 represent contaminating ^{125}I-radiolabelled protein A and not immunogenic polypeptides.

Figure 4.2.2: Accumulation of PTP precursors in yeast cells inhibited with CCCP

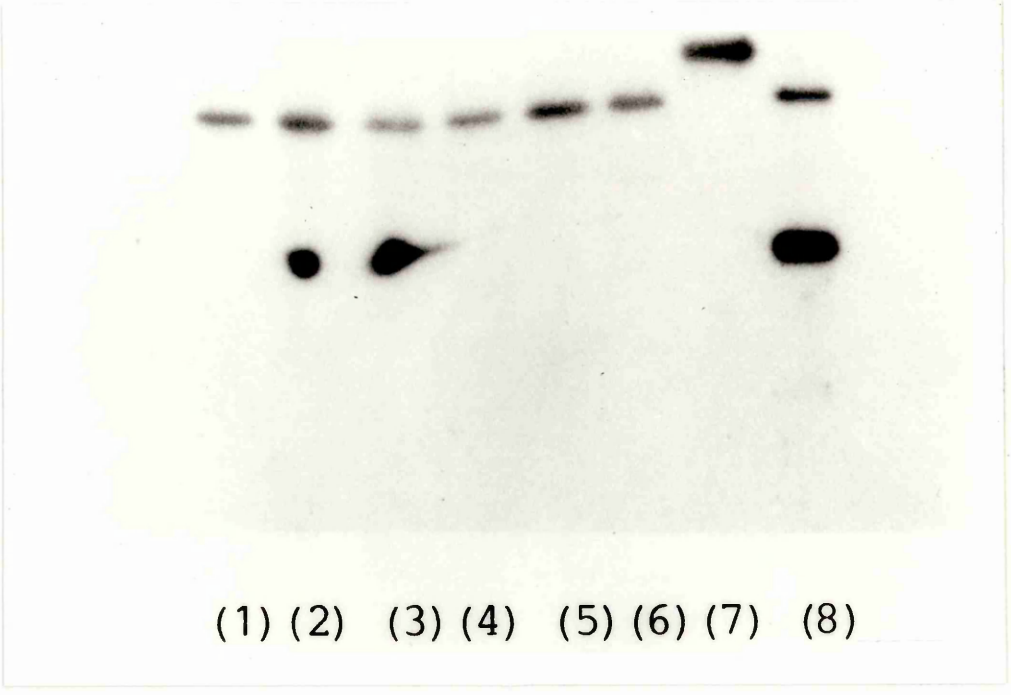
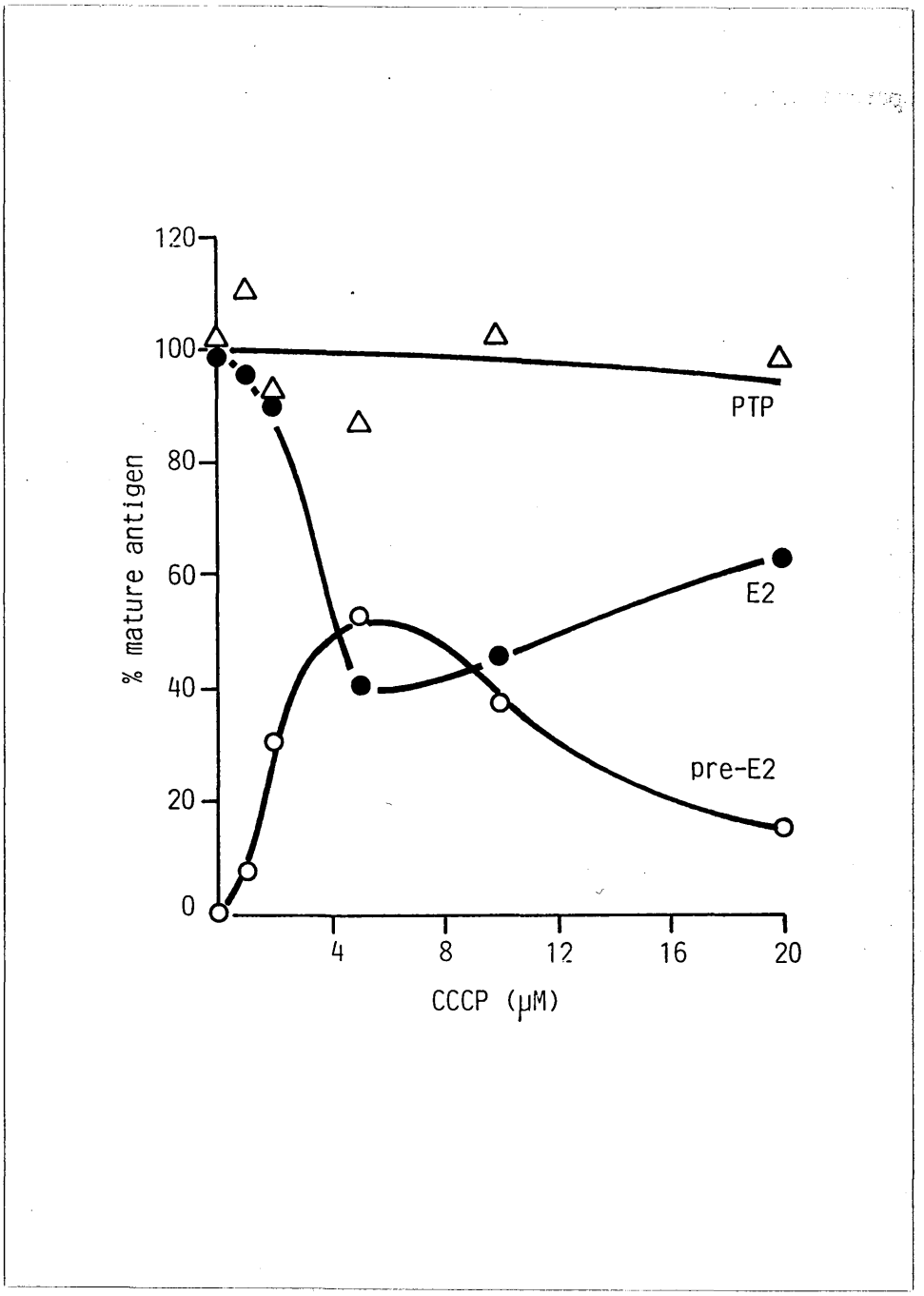


Figure 4.2.3: Densitometric scan of precursor accumulation in CCCP-inhibited yeast cells

The Western blots illustrated in Figures 4.2.1 and 4.2.2 were further analysed by scanning densitometry, as described in Section 2.2.6(b). The data are illustrated graphically as percent mature antigen versus CCCP concentration (μM).

Figure 4.2.3: Densitometric scan of precursor accumulation in CCCP-inhibited yeast cells



4.3 PURIFICATION OF YEAST PTP AND ANT

4.3.1 Purification of yeast PTP

The phosphate carrier was isolated from yeast (*S. cerevisiae*) mitochondria basically by employing the purification scheme previously described for the equivalent mammalian protein (see Section 2.2.8). Mitochondria were prepared from yeast tablets supplied by DCL Ltd. (see Section 2.1.4), as described in Section 2.2.7(c). The organelles were pre-extracted with a 0.5% (w/v) Triton X-100 buffer, and the resultant pellet extracted with 8% (w/v) Triton X-100 prior to fractionation with hydroxylapatite (HTP) and Celite. Fractions from the various purification steps were analysed by SDS-PAGE (see Figure 4.3.1), revealing an apparently homogeneous 30,000-M_r polypeptide following adsorption chromatography on Celite. As with the corresponding mammalian preparation, the critical step in the isolation of the putative yeast PTP is HTP fractionation (see Figure 4.3.1). Virtually all proteins present in the 8% (w/v) Triton X-100 extract are adsorbed out by the column matrix, with only PTP and, on occasions, a few other proteins of similar M_r values remaining in the unbound fraction. Further treatment with Celite adsorbs out these contaminating proteins. However, as Figure 4.3.1 illustrates, it is possible, with careful preparation, to obtain a homogeneous preparation of the 30,000-M_r yeast protein following HTP extraction.

Preliminary characterisation of the putative yeast PTP was carried out immunologically in order to ensure that it corresponds to the 30,000-M_r protein identified by Gibb (1985), which cross-reacts strongly with anti-rat liver PTP serum. In addition to this, antiserum was raised against the purified yeast protein by immunising a New Zealand White rabbit as described in Section 2.2.6(a). This antiserum was also characterised by employing it for immune replica analysis of the various steps in purification of the putative yeast PTP. Immunological characterisation of the yeast protein and its antiserum is illustrated in Figure 4.3.2. Fractions from the yeast PTP purification scheme were subjected to SDS-PAGE prior to Western blot analysis using antisera raised against both the rat liver and yeast carriers. The monospecificity of the anti-yeast PTP serum is clearly demonstrated by Figure 4.3.2(A), as no cross-reactivity with any other mitochondrial proteins is apparent.

Figure 4.3.1: Purification of PTP from yeast (*S.cerevisiae*) mitochondria

Mitochondria were prepared as described in Section 2.2.7(c), and PTP was purified as outlined in Section 4.3.1. Samples were acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, yeast mitochondria; 2, 0.5% (w/v) Triton X-100 pre-extracted pellet; 3, 8% (w/v) Triton X-100 extract; 4, HTP-adsorbed extract; 5, Celite-adsorbed extract; S, M_r standards.

Figure 4.3.1: Purification of PTP from yeast mitochondria

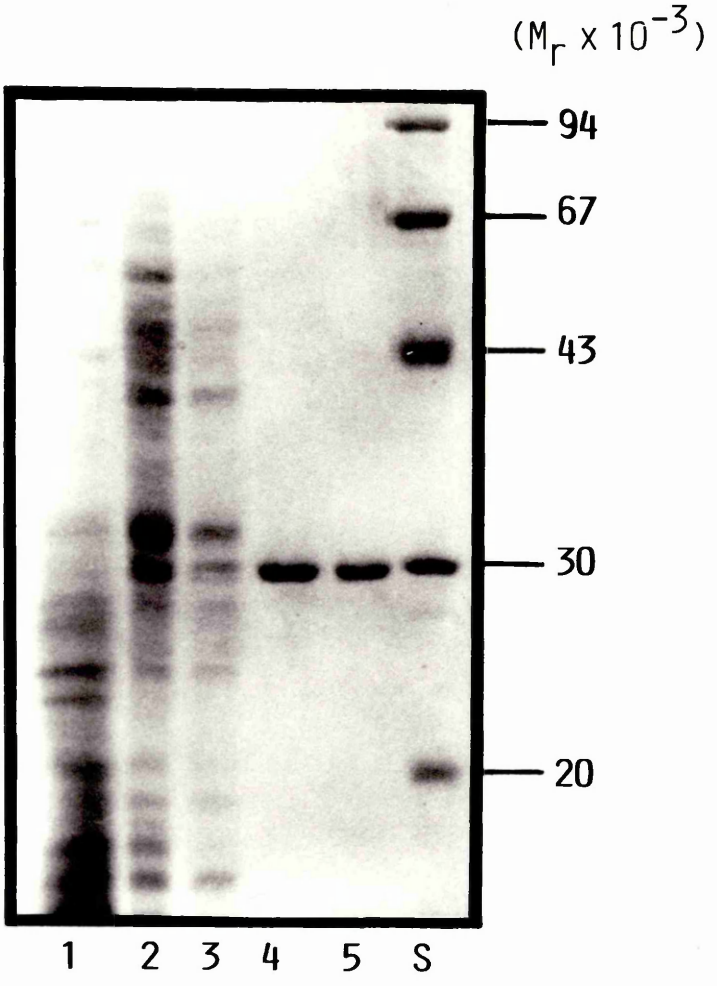


Figure 4.3.2: Western blot analysis of PTP purification from yeast (*S.cerevisiae*) mitochondria

Mitochondria were prepared as described in Section 2.2.7(c), and PTP was purified as outlined in Section 4.3.1. Samples were acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was transferred to nitrocellulose paper and subjected to immune replica analysis with anti-yeast PTP serum (A) and anti-rat liver PTP serum (B). Lanes: 1, yeast mitochondria; 2, 0.5% (w/v) Triton X-100 pre-extracted pellet; 3, 8% (w/v) Triton X-100 extract; 4, HTP-adsorbed extract; 5, Celite-adsorbed extract; S, M_r standards (^{125}I-radiolabelled).

Figure 4.3.2: Western blot analysis of PTP purification from yeast mitochondria

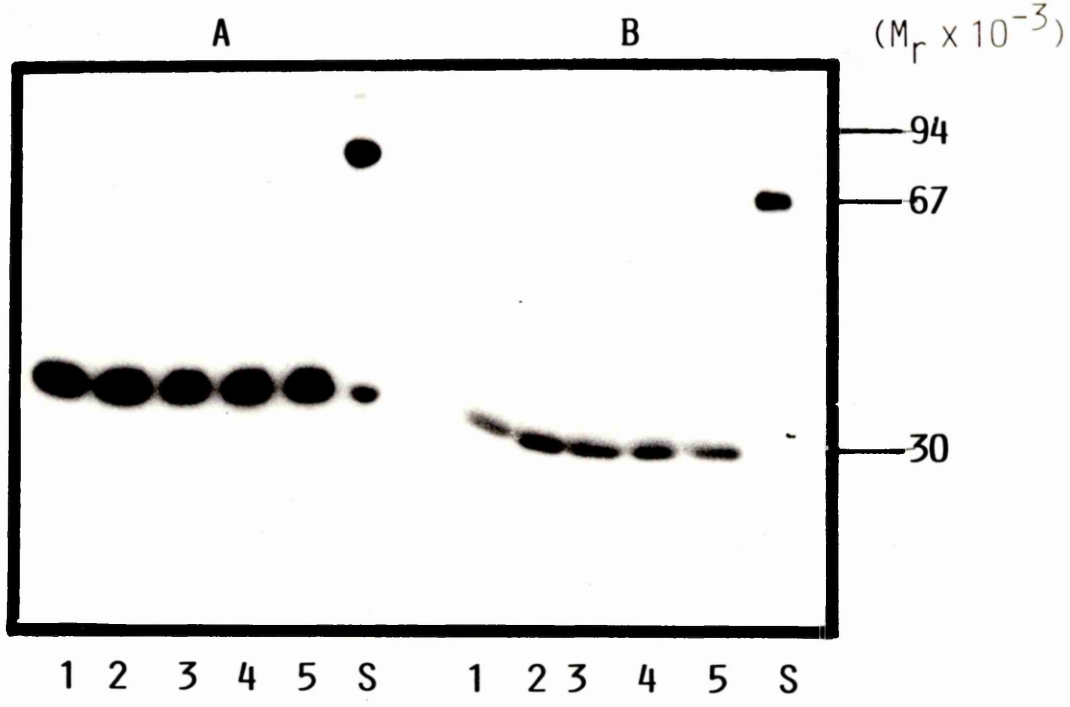


Figure 4.3.2(B) illustrates the strong and unique cross-reactivity of the yeast mitochondrial 30,000-M_r protein with monospecific, high titre anti-rat liver PTP serum, indicating that it corresponds to the immunogenic polypeptide identified by Gibb (1985). Further Western blot analysis of the anti-yeast PTP serum was carried out by assessing its cross-reactivity with equivalent amounts (0.1, 0.2 and 0.5μg) of the rat liver and yeast carriers. As Figure 4.3.3 illustrates, the antiserum shows significant cross-reactivity with rat liver PTP, though less strongly than with the corresponding yeast protein.

The purification scheme for the putative yeast PTP and the immunological characteristics of this protein strongly resemble those of the equivalent mammalian carrier. This suggests that the isolated protein corresponds to the yeast mitochondrial phosphate carrier.

4.3.2 Co-purification of yeast PTP and ANT

The purification schemes for the mammalian primary mitochondrial exchange carriers, PTP and the closely related ADP/ATP translocase, contain many similarities, with HTP fractionation representing the critical step in both (see Sections 1.2.4 and 1.2.5). However, unless ANT is first stabilised by one of its specific inhibitors (ATR, CAT or BKA), the carrier is denatured upon extraction and is adsorbed out by HTP (Klingenberg *et al.*, 1974). The previous chapter (Section 3.2.2) describes the co-purification of PTP and ANT from rat liver and bovine heart mitochondria in the HTP-adsorbed extract, following the inclusion of CAT in the preparations.

A similar procedure was adopted in order to obtain a yeast PTP/ANT co-isolate. Freshly-prepared yeast mitochondria were pre-incubated with 50-100μM CAT for 30min at 4°C prior to being subjected to a standard PTP preparation, as described in the preceding Section (4.3.1). Analysis by SDS-PAGE (Figure 4.3.4) reveals the presence of both PTP (M_r 30,000) and a 34,000-M_r CAT-stabilised polypeptide following chromatography on HTP. The 34,000-M_r protein, assumed to be yeast ANT, is adsorbed out by Celite. This agrees with the behaviour of the co-isolated mammalian ADP/ATP carrier (see Section 3.2.2), and with previous observations by Wohlrab

Figure 4.3.3: Western blot analysis of purified PTP from rat liver and yeast (*S.cerevisiae*) mitochondria

Varying amounts of rat liver and yeast PTP were acetone precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was transferred to nitrocellulose paper and subjected to immune replica analysis with anti-yeast PTP serum. Lanes: 1-3, 0.1, 0.2 and 0.5μg rat liver PTP respectively; 4-6, 0.1, 0.2 and 0.5μg yeast PTP respectively; S, M_r standards (^{125}I-radiolabelled).

Figure 4.3.3: Western blot analysis of purified PTP from rat liver and yeast mitochondria

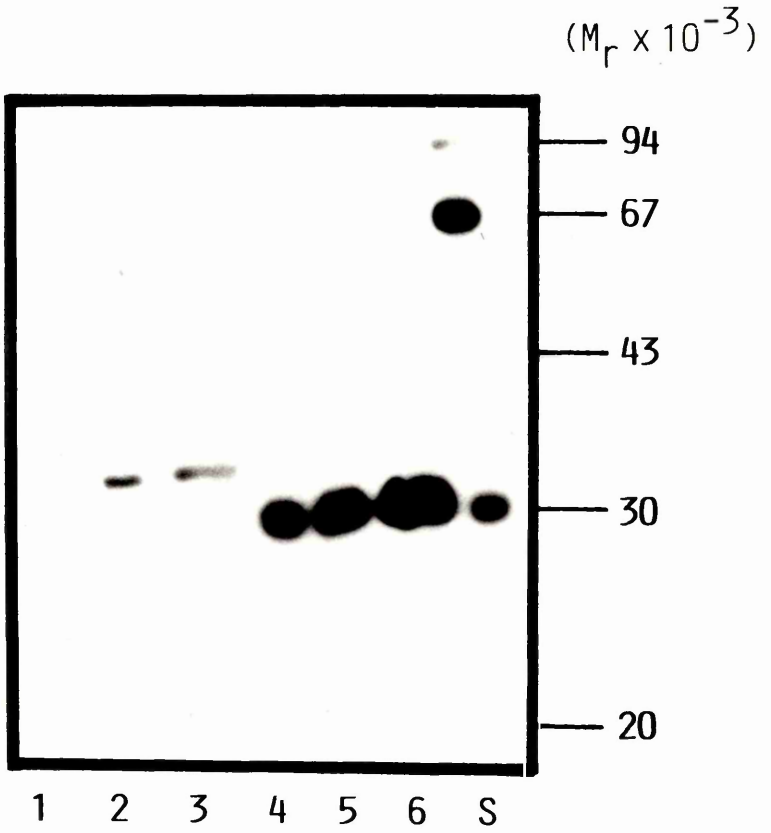
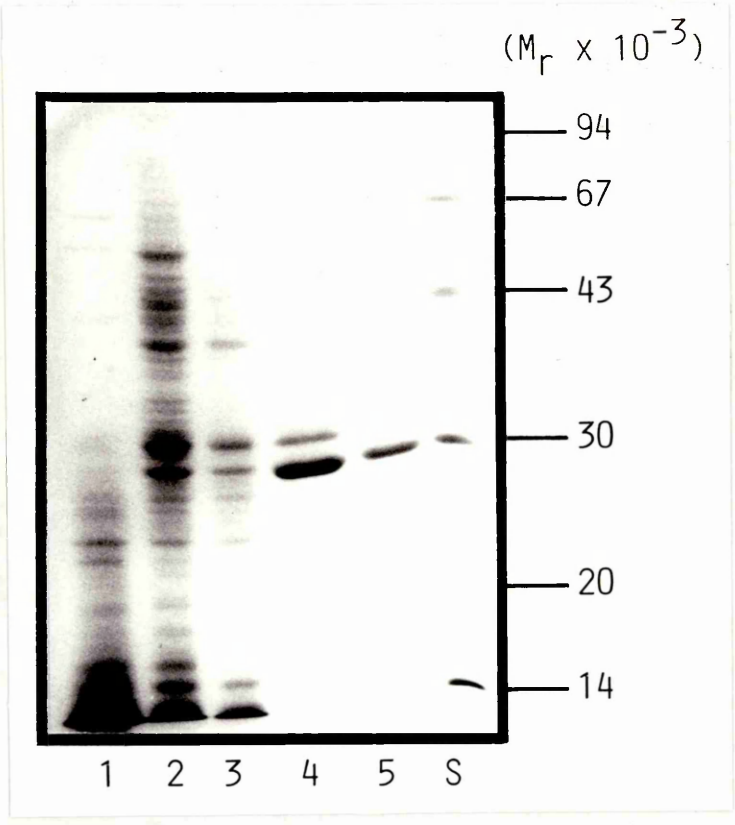


Figure 4.3.4: Purification of PTP (and ANT) from yeast (*S.cerevisiae*) mitochondria pre-incubated with CAT

Mitochondria were prepared as described in Section 2.2.7(c), and the proteins were purified as outlined in Section 4.3.2. Samples were acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, yeast mitochondria; 2, 0.5% (w/v) Triton X-100 pre-extracted pellet; 3, 8% (w/v) Triton X-100 extract; 4, HTP-adsorbed extract; 5, Celite-adsorbed extract; S, M_r standards.

Figure 4.3.4: Purification of PTP (and ANT) from yeast mitochondria pre-incubated with CAT



(1980) and Wohlrab and Flowers (1982) who detected contaminating ANT in their PTP preparations. Figure 4.3.5 illustrates the effect of CAT on the isolation of PTP from yeast mitochondria. In this experiment, a single batch of fresh mitochondria was used, with half being pre-incubated with CAT (100μM) as described above, and half being treated as normal.

4.4 ANALYSIS OF YEAST PTP AND ANT BY COLUMN CHROMATOGRAPHY

4.4.1 Introduction

The above section (4.3.2) outlines the methology for the co-purification of PTP and the closely related ADP/ATP carrier from yeast (*S.cerevisiae*) mitochondria. In the previous chapter (Section 3.3), the analysis of a similarly prepared mammalian co-isolate by various chromatographic techniques is described. Accordingly, it was decided to carry out similar analyses of the HTP-adsorbed extracts from CAT-loaded yeast mitochondria in order to obtain preliminary characterisation of the co-purified proteins. It was also hoped to develop a chromatographic procedure for efficiently separating the carriers, as the basis for obtaining optimal yields of both from a single mitochondrial preparation.

Unfortunately, insufficient time was available to repeat the full range of chromatographic analyses of the mammalian PTP/ANT co-isolate outlined in Chapter 3. In view of the encouraging results obtained when rat liver PTP/ANT was subjected to chromatography using the Matrex gel, Procion Red A (see Section 3.3.3), it was decided to concentrate on this technique for the analysis of the yeast mitochondrial PTP/ANT co-isolate.

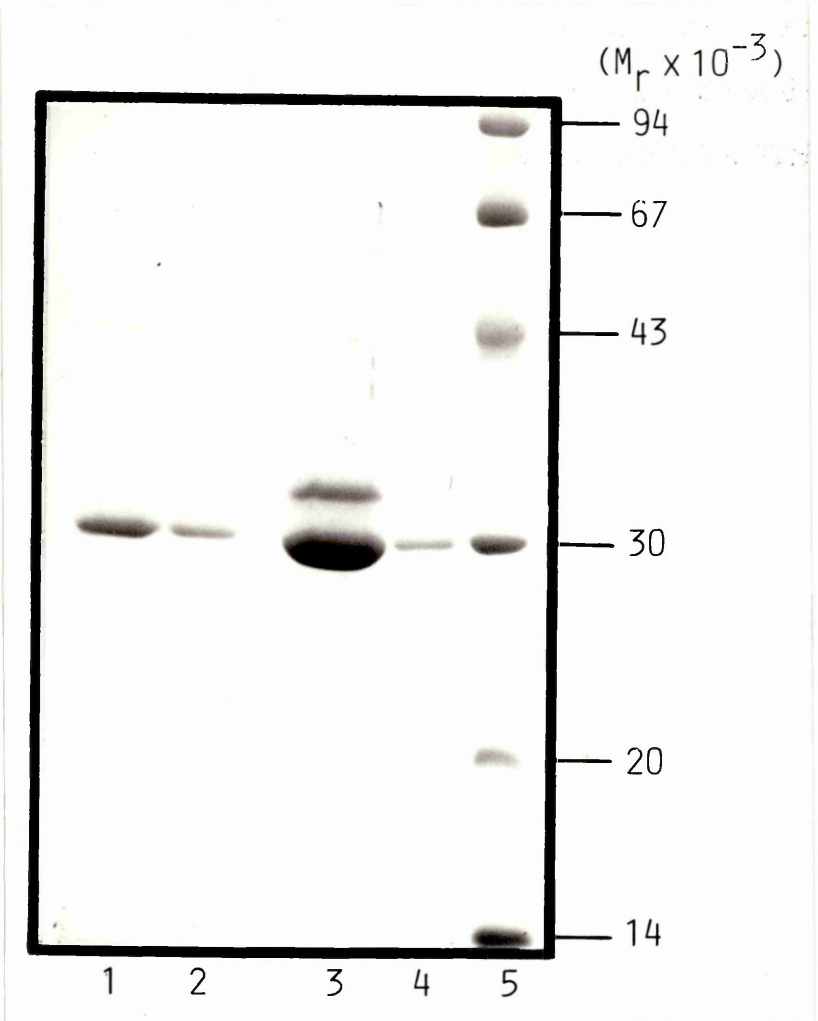
4.4.2 Analysis of yeast PTP and ANT by Procion Red A column chromatography

The HTP-adsorbed extract isolated from yeast mitochondria pre-loaded with CAT was analysed using columns poured with the Matrex gel, Procion Red A. Co-purified PTP and ANT was dialysed into 20mM NaCl pH 7.0, 0.5% (w/v) Triton X-100, and loaded onto a 2.0 x 1.0cm column which was equilibrated in the same

Figure 4.3.5: Effect of CAT on the purification of PTP from yeast mitochondria

PTP was purified from yeast (*S.cerevisiae*) mitochondria pre-incubated with the ANT inhibitor carboxyatractyloside (CAT; see Section 4.3.2), and also from mitochondria not subjected to CAT-pretreatment. The HTP and Celite-adsorbed extracts were acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1 and 2, HTP and Celite extracts respectively; 3 and 4, HTP and Celite extracts (mitochondria pre-incubated with CAT); S, M_r standards.

Figure 4.3.5: Effect of CAT on the purification of PTP from yeast mitochondria



buffer. Elution was carried out using a salt gradient (0.1-1.0M NaCl) and 1% (w/v) SDS. Unbound and eluted fractions were concentrated by exposure to poly (ethylene glycol) as described in Section 2.2.2, and prepared for analysis by SDS-PAGE (see Figure 4.4.1).

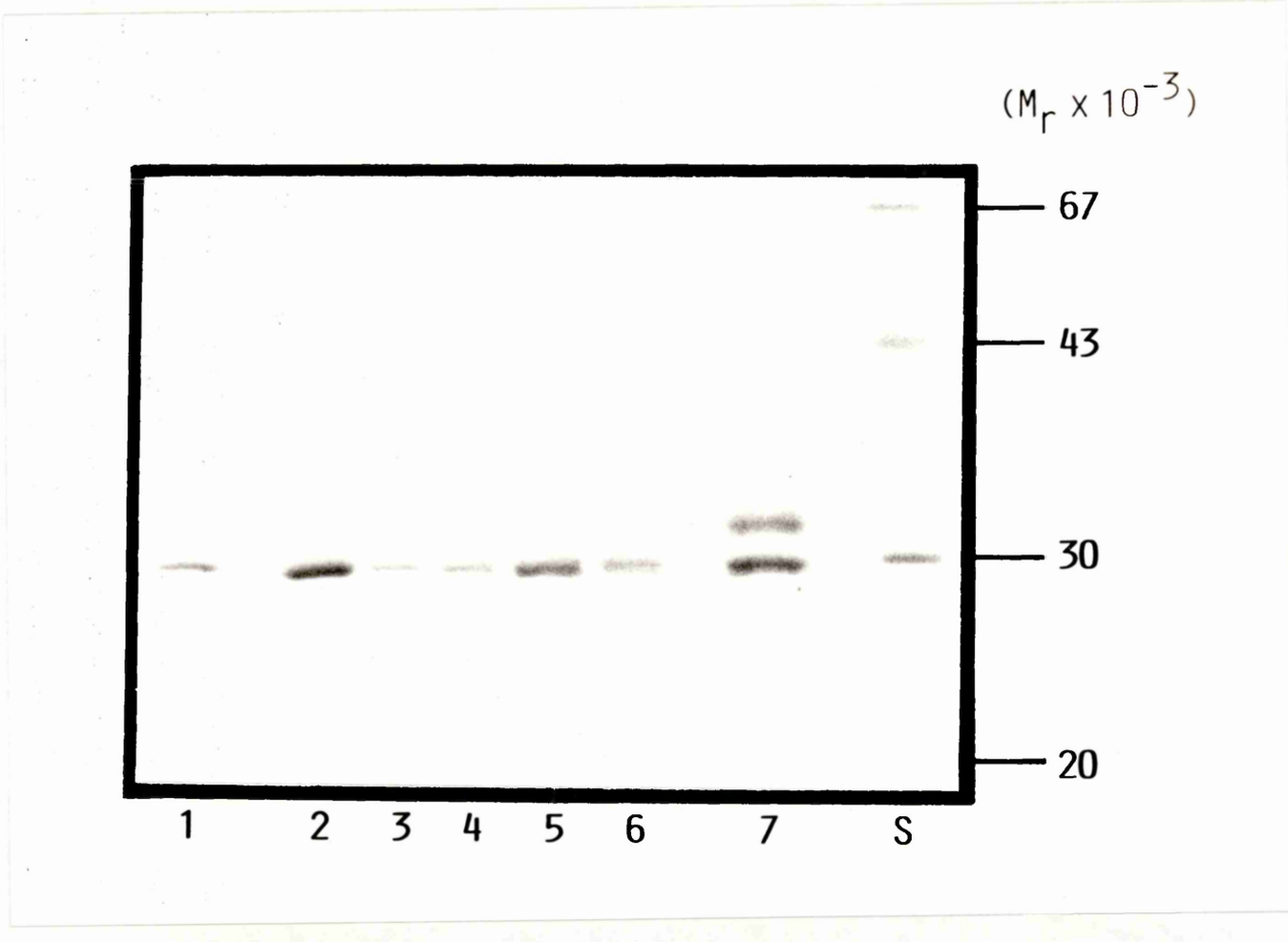
The binding and elution profiles of the yeast carriers at pH 7.0 are very similar to those of their mammalian counterparts (see Figure 3.3.4). As Figure 4.4.1 illustrates, PTP is eluted at 0.1-1.0M NaCl (0.1-0.5M for mammalian PTP), with optimal release at 0.5M NaCl (0.25M NaCl for mammalian PTP). In common with the equivalent mammalian carrier, a significant proportion of the loaded PTP remains bound to the column until treatment with SDS and may represent denatured protein. Yeast ANT, like its mammalian equivalent, readily binds to Procion Red A at pH 7.0, remains bound during treatment with salt, and is dislodged only upon elution with SDS, resulting in denaturation of the carrier.

Whereas mammalian PTP readily binds to Procion Red A, Figure 4.4.1 clearly demonstrates the existence of a third population of the 30,000-M_r polypeptide preparation (in addition to the NaCl- and SDS-eluted fractions) which elutes with the unbound fraction. It is possible that this is due to saturation of the protein-binding sites on the Procion Red A column. However, this is unlikely in view of the reported binding capacity of Matrex gels (1-3mg protein/ml) and the amount of protein used in this experiment (200μg). It thus appears that purified yeast PTP may exist in three distinct conformations. Alternatively, it is possible that the unbound fraction represents a distinct mitochondrial protein with an apparent M_r value virtually identical to that of the putative phosphate transporter (~30,000). If this is the case, the most likely candidate for the contaminant is the outer membrane pore-forming protein, porin (M_r 29,000). This protein has been previously purified from yeast (Mihara *et al.*, 1982) and subsequently characterised (Gasser and Schatz, 1983). Its mode of synthesis and membrane insertion has also been investigated *in vitro* (Mihara *et al.*, 1982; Gasser and Schatz, 1983). Interestingly, porin is also a common contaminant in mammalian PTP preparations (T. Lever, personal communication) and has been implicated as the mitochondrial DCCD-reactive protein, previously thought to be PTP (see Section 1.2.7).

Figure 4.4.1: Analysis of yeast mitochondrial (+CAT) HTP extract by Procion Red A column chromatography

The HTP-adsorbed fraction containing ANT and PTP was dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100, loaded onto a 2.0 x 1.0cm Procion Red A column equilibrated in the same buffer, and eluted with 0.1-1.0M NaCl and 1% (w/v) SDS. Fractions were collected and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, yeast HTP extract; 2, unbound fraction; 3-6, salt-eluates (0.1, 0.25, 0.5 and 1.0M NaCl); 7, 1% (w/v) SDS eluate; S, M_r standards.

Figure 4.4.1: Analysis of yeast mitochondrial (+CAT) HTP extract by Procion Red A column chromatography



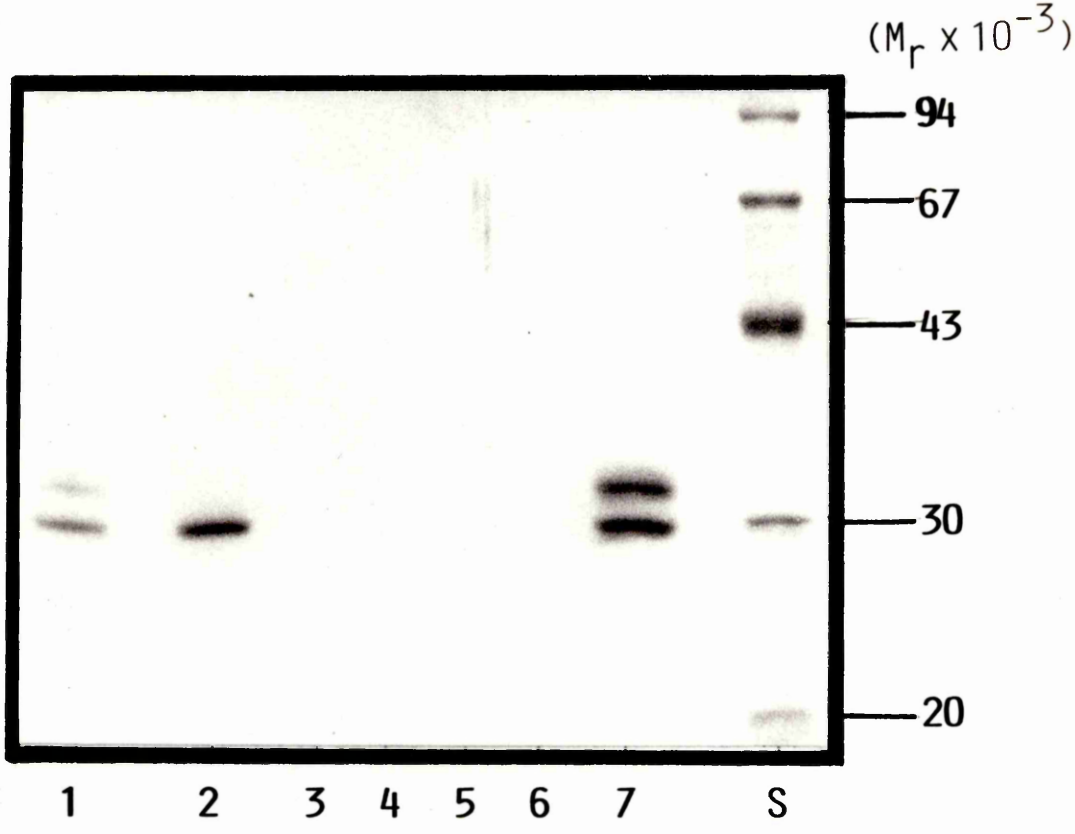
The above results indicate that the binding to Procion Red A of the NaCl-eluted fraction of the 30,000-M_r polypeptide preparation, possibly representative of yeast PTP, is electrostatic, since an increase in ionic strength leads to elution of the carrier. However, the mode of binding of yeast ANT to the column is unclear, and may involve electrostatic interactions, hydrophobic interactions and/or affinity binding. Previous results indicating that mammalian ANT binds electrostatically to Procion Red A led to the separation of the mammalian carriers (see Section 3.3.3). Consequently, it was decided to repeat the above chromatographic analysis of yeast mitochondrial co-purified PTP and ANT at pH 6.0 (using KP_i as the buffer) and 8.0 (Tris-HCl). However, when the unbound and salt-eluted fractions were analysed by SDS-PAGE, the results obtained were identical to those illustrated in Figure 4.4.1 at both pH values. Thus, under the conditions tested, it was not possible to separate co-isolated yeast PTP and ANT; nor was the mode of binding of ANT to the column established. Further Procion Red A analysis is required to clarify these points: elution with higher NaCl concentrations, substrates/substrate analogues; further investigation of the pH-dependence of binding (mammalian ANT elutes with 1.0M NaCl at pH 8.8 [see Section 3.3.3]).

In the previous chapter it is postulated that Procion Red A column chromatography represents an assay for native mammalian PTP conformation (see Section 3.3.4). In order to ascertain if this also applies to the equivalent yeast carrier, chromatographic analysis was carried out on the denatured yeast co-isolate. The HTP-adsorbed fraction from CAT-loaded yeast mitochondria was heat-denatured at 80°C for 10min prior to loading onto a 2.0 x 1.0cm Procion Red A column. Elution was carried out with a salt gradient (0.1-1.0M NaCl) and 1% (w/v) SDS as before. Analysis of the column fractions by SDS-PAGE is illustrated in Figure 4.4.2, and demonstrates that, upon denaturation, the 30,000-M_r polypeptide population which is normally NaCl-eluted appears to bind to the Matrex gel as before, but is dislodged only upon treatment with SDS. This lends credence to the proposal that only native PTP can be NaCl-eluted from the Procion Red A column. Unfortunately, insufficient time was available to further investigate this possibility by chromatographically analysing the yeast PTP/ANT co-isolate prepared in the presence of cardiolipin. Figure 4.4.2 also

Figure 4.4.2: Analysis of denatured yeast mitochondrial (+CAT) HTP extract by Procion Red A column chromatography

The HTP-adsorbed fraction containing ANT and PTP was dialysed into 20mM Tris-HCl pH 7.0, 0.5% (w/v) Triton X-100, heat-denatured at 80°C for 10min, loaded onto a 2.0 x 1.0cm Procion Red A column equilibrated in the same buffer, and eluted with 0.1-1.0M NaCl and 1% (w/v) SDS. Fractions were collected and acetone-precipitated prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, yeast HTP extract; 2, unbound fraction; 3-6, salt-eluates (0.1, 0.25, 0.5 and 1.0M NaCl); 7, 1% (w/v) SDS eluate; S, M_r standards.

Figure 4.4.2: Analysis of denatured yeast mitochondrial (+CAT) HTP extract by Procion Red A column chromatography



demonstrates the continued presence of an unbound 30,000-M_r polypeptide population, possibly representing contaminating porin.

4.5 N-TERMINAL AMINO ACID ANALYSIS OF PUTATIVE YEAST PTP

The 30,000-M_r putative phosphate carrier was purified to homogeneity from yeast mitochondria as outlined in Section 4.3.1. The absence of contaminating polypeptides in the preparation was confirmed by SDS-PAGE analysis, although the presence of another protein with a near-identical M_r value, probably porin, could not be ruled out (see Section 4.4.2). The purified protein was subjected to N-terminal analysis at the SERC-funded amino acid sequencing facility at the University of Aberdeen (see Section 2.2.3). Eighteen of the first 20 residues at the N-terminus were resolved, with sixteen of these being confirmed by a second analysis (see Figure 4.5.1).

The primary structure of the putative yeast PTP was compared with those of the equivalent bovine heart and rat liver carriers (Runswick *et al.*, 1987; Ferreira *et al.*, 1989; see Figure 4.5.1). Although there is no apparent homology between the yeast carrier and its mammalian counterparts at the N-terminal region, this is perhaps not surprising since the most highly conserved domains among the tripartite family of mitochondrial carriers are the proposed α-helical segments and the regions of the hydrophilic domains that connect them to the helices (Runswick *et al.*, 1987). The N- and C-termini are among the most poorly conserved domains.

Figure 4.5.1 also illustrates that the 30,000-M_r yeast mitochondrial protein does not correspond to the ADP/ATP carrier, as its N-terminus is clearly different from that of yeast ANT (Adrian *et al.*, 1986). The amino acid sequence also differs from those proteins encoded by the two further ANT genes identified in yeast (*AAC2* and *AAC3* ; Lawson and Douglas, 1988). As Section 4.3.2 outlines, the 34,000-M_r CAT-stabilised protein co-isolated with PTP from yeast mitochondria is proposed to represent ANT. Accordingly, it had been hoped to confirm its identity by N-terminal analysis. However, this was not possible as separation of the co-purified carriers was not achieved under the conditions tested (see Section 4.4.2). The co-isolate was prepared

Figure 4.5.1: N-terminal amino acid sequences of mitochondrial inner membrane proteins

Putative yeast PTP (as outlined in Section 4.5)

1 20

SPPVYSDI(S)(R)NINDLLN--F

(), unconfirmed residue; -, undetected residue

Bovine heart PTP (Runswick *et al.*, 1987)

1 20

AVEEQYSCDYGSGRFFILCG

Rat liver PTP (Ferreira *et al.*, 1989)

1 20

AVEGYSCEFGSMKYYALCGF

Yeast ANT (Adrian *et al.*, 1986)

1 20

MSHTETQTQQSHFGVDFLMG

Yeast import receptor (Murakami *et al.*, 1990)

1 20

MYVSAAPAIPQYSVSDYMKF

Yeast PTP (Phelps *et al.*, 1991)

1 20

MSVSAAPAIPQYSVSDYMKF

for amino acid analysis in an attempt to elucidate the N-terminii of both carriers. However, when the sample was subjected to the continuous SDS-PAGE system employed prior to Edman degradation, no resolution of the two carriers was observed and only the 30,000-M_r polypeptide was ultimately sequenced. This contrasts with the behaviour of the co-purified carriers on the discontinuous SDS-PAGE system employed in our laboratory, which results in clear resolution of the proteins (see Figures 4.3.4 and 4.3.5).

Figure 4.5.1 also illustrates the N-terminal amino acid sequences of a putative yeast import receptor (Murakami *et al.*, 1990) and phosphate carrier (Phelps *et al.*, 1991). These proteins differ in the N-terminal regions by one residue only (residue 2: tyrosine in the import receptor; serine in PTP), and further analysis of their primary structures reveals that there are only seven further residue discrepancies. Clearly, both these groups have cloned and sequenced the same gene. However, there is controversy over whether it encodes a phosphate carrier, as suggested by Wohlrab's group (Phelps *et al.*, 1991), or a receptor for protein import into yeast mitochondria, as proposed by Blobel's group (Pain *et al.*, 1990; Murakami *et al.*, 1990). Both groups agree that the encoded protein has an M_r value of 32,800 and shows approximately 40% homology with the primary structures of rat liver and bovine heart PTP (Runswick *et al.*, 1987; Ferreira *et al.*, 1989). There is also general agreement that the protein contains the tripartite structure common to PTP, ANT, UCP and other members of this family of structurally related mitochondrial membrane proteins which are thought to evolve from a single gene (see Section 1.2.9). The observed homology with mammalian PTPs is the basis for the identification of this protein as the equivalent yeast carrier by Wohlrab's group. Phelps *et al.* (1991) also claim that the protein is identical to a reconstitutively active PTP which has been reported as isolated from yeast mitochondria by Guerin *et al.* (1991, in press). In contrast, Blobel's group have used anti-idiotype antibodies to characterise the 32,800-M_r integral membrane protein as an import receptor (Pain *et al.*, 1990). These antibodies were prepared using a chemically synthesised signal peptide of a mitochondrial precursor protein, subsequently demonstrated to be the 32,800-M_r protein. It is claimed, from immunoelectron microscopy and subfractionation of the organelles, that this protein is located in contact sites between

the inner and outer mitochondrial membranes. The role of these sites in protein import has been discussed previously (see Section 1.1.6.4).

The N-terminal data illustrated in Figure 4.5.1 suggests that the protein discussed above, which may represent yeast PTP, is not identical to the putative phosphate carrier isolated in this study. However, it may not be possible to carry out Edman degradation of yeast PTP as a result of the N-terminus being masked (A. Phelps, personal communication). If this is the case, it is still possible that the two proteins are identical and that the sequence obtained (Figure 4.5.1) may represent the N-terminal of a contaminant in the yeast PTP preparation, probably porin (see Section 4.4.2). N-terminal analysis of the Procion Red A unbound and NaCl-eluted 30,000-M_r polypeptide fractions was subsequently carried out to investigate this possibility. As illustrated in Figure 4.5.2, it was not possible to obtain any sequence data for the salt-eluted protein, while the N-terminal amino acid sequence obtained previously (Figure 4.5.1) was shown to be that of the unbound polypeptide fraction. Figure 4.5.2 also illustrates the N-terminal protein sequence of *S.cerevisiae* porin from the corresponding cDNA clone (Kleene *et al.*, 1987). With the exception of the terminal methionine residue, which is missing from the sequence of the Procion Red A unbound polypeptide , it is apparent that these two proteins (porin and the unbound 30,000-M_r protein fraction) are identical.

The above data indicate that the protein present in the Procion Red A NaCl-eluate has a masked N-terminus and may thus represent yeast PTP (A. Phelps, personal communication), while the unbound fraction has been positively identified as corresponding to contaminating porin in the PTP preparation as previously suspected (see Section 4.4.2).

4.6 DISCUSSION

The biosynthesis of the putative yeast mitochondrial phosphate carrier was investigated by accumulating cytosolic precursors in yeast cells (*S.cerevisiae* D-273-10B-1) grown in the presence of CCCP. Analysis of the cell extracts by SDS-PAGE and immune blotting indicated that yeast PTP, in contrast to the equivalent bovine heart and rat liver carriers (Runswick *et al.*, 1987; Ferreira *et al.*, 1989), is not

Figure 4.5.2: N-terminal amino acid sequences of yeast mitochondrial 30,000-M_r proteins

Procion Red A NaCl-eluate: N-terminal blocked

Procion Red A unbound fraction

1 20

SPPVYSDI(S)(R)NINDLLN--F

(), unconfirmed residue; -, undetected residue

S.cerevisiae porin (Kleene *et al.*, 1987)

1 20

MSPPVYSDISRNINDLLNKD

initially synthesised as a higher-M_r precursor. However, these results agree with the observations of Phelps *et al.* (1991), who have cloned and sequenced the gene encoding yeast PTP and noted the absence of an N-terminal-extended signal sequence.

A variety of protein-chemical studies were subsequently carried out on the isolation and preliminary characterisation of the inner membrane carriers, PTP and ANT, from yeast (*S.cerevisiae*) mitochondria. By employing the purification scheme for the equivalent carrier from mammalian mitochondria, yeast PTP was purified to apparent homogeneity. The putative yeast carrier has an apparent M_r value of ~30,000 from SDS-PAGE. Antiserum was raised against the purified yeast protein and was shown to cross-react strongly with rat liver PTP. In addition, anti-rat liver PTP serum was employed to demonstrate that the 30,000-M_r polypeptide is identical to the putative yeast phosphate carrier identified immunologically by Gibb (1985). The PTP isolated in this study is thus characterised on the bases of its purification procedure and imunological properties in relation to the corresponding rat liver carrier.

The procedure previously utilised to co-purify PTP and the closely related ADP/ATP carrier from mammalian mitochondria was repeated to obtain a co-isolate of the equivalent yeast carriers. It was found that, by pre-incubating freshly-prepared yeast mitochondria with CAT, it was possible to stabilise a 34,000-M_r protein which subsequently co-purified with PTP following adsorption chromatography on HTP. Further fractionation with Celite adsorbed out this additional protein. On the basis of its stabilisation by CAT, the 34,000-M_r protein is presumed to represent yeast ANT. This proposal is further supported by its purification characteristics, which are identical to those of the mammalian ADP/ATP carrier.

In the previous chapter (Sections 3.3.3-3.3.5), Procion Red A columns were used to separate co-purified mammalian PTP and ANT, assay for native PTP conformation, and prepare native PTP in detergents other than Triton X-100 for analysis by CD. Accordingly, this Matrex gel was employed for chromatographic analysis and further characterisation of the yeast mitochondrial co-isolate. At pH 7.0, the binding and elution characteristics of the yeast PTP/ANT co-isolate were broadly similar to those of the equivalent mammalian proteins. Both carriers were observed to

bind to the Procion Red A column, with PTP eluting optimally at 0.5M NaCl. Elution of ANT resulted only after washing of the column with SDS. A significant proportion of the loaded PTP behaves in a similar manner to ANT, remaining bound to the column until treatment with SDS. This observation was also noted for mammalian PTP, and was explained by the presence of denatured carrier which electrostatically binds to Procion Red A, but cannot be dislodged by increased ionic strength due to its conformation. This may also be the case for the equivalent yeast carrier.

In contrast to the mammalian analyses, a proportion of the putative yeast PTP was also observed in the unbound fraction from the Procion Red A column. In view of the relatively high binding capacity (1-3mg/ml) of Matrex gels, this is unlikely to be due to saturation of the Procion Red A column. It thus appeared possible that a third PTP population, with a distinct conformation, was present in the yeast mitochondrial co-isolate. It is also feasible that the 30,000-M_r yeast mitochondrial isolate represents two subunits of a multimeric phosphate transport system, as exists in bacteria. However, it is more likely that the unbound fraction represents a distinct polypeptide with the same apparent M_r value as yeast PTP, the most likely candidate being porin (see Section 4.4.2). In order to investigate this possibility, the sequence obtained for the unbound Procion Red A polypeptide fraction (see Section 4.5) was compared to the published primary structure of yeast porin (Kleene *et al.*, 1987). The two proteins were shown to be identical.

In future, it may be beneficial to attempt chromatographic separation of the two ~30,000-M_r yeast mitochondrial polypeptides by application to mersalyl-ultrogel, which can be used to remove contaminating porin from mammalian PTP preparations (T. Lever, personal communication). All fractions prepared would subsequently be subjected to N-terminal amino acid analysis. The efficient separation of PTP and porin by one or both of the above chromatographic techniques (mersalyl-ultrogel, Procion Red A) may also be the basis for establishing which of these carriers is the mitochondrial DCCD-reactive protein (see Section 1.2.7). Mersalyl-ultrogel chromatography of the yeast 30,000-M_r fraction holds added interest since Phelps *et al.* (1991) have reported that yeast PTP lacks the NEM-reactive Cys^{42} presumed to be responsible for binding to the chromatography matrix.

Chromatographic analysis of the yeast mitochondrial co-isolate was repeated in order to assess the pH-dependence of the carriers' binding and elution profiles with respect to Procion Red A. In this manner, it was hoped to separate the two proteins into homogeneous populations. However, varying the the pH over the range 6.0-8.0 had no effect on the binding or elution properties of either PTP or ANT. This had also been the case for the corresponding rat liver carriers, although a small fraction of bound bovine heart ANT was eluted with 1.0M NaCl at pH 8.0. Therefore, although it was not possible to chromatographically separate the co-purified carriers under the conditions tested, the binding and elution properties of the isolated proteins with Procion Red A over the pH range 6.0-8.0 further support the proposal that they represent the yeast mitochondrial phosphate and adenine nucleotide carriers. Unfortunately, insufficient time was available to repeat the Procion Red A analysis of the yeast PTP/ANT co-isolate at pH 8.8, the level at which separation of the equivalent mammalian carriers was achieved.

The Procion Red A column was previously demonstrated to represent a potential assay for the native conformation of mammalian PTP, and possibly for the ADP/ATP carrier also (see Sections 3.3.4 and 3.4). The possibility that this also applies to yeast PTP was investigated by chromatographically analysing heat-denatured yeast PTP/ANT. Under these conditions, the yeast PTP fraction which, when native, elutes optimally with 0.5M NaCl, was observed to remain bound to the column until treatment with SDS. As this was also the case for mammalian PTP, evidence is thus provided that Procion Red A column chromatography may also represent an assay for native yeast PTP conformation. However, it will be necessary to assess the effect of the inclusion of cardiolipin in the extraction process of the yeast co-isolate. If this results in a greater proportion of PTP being detected in the 0.5M NaCl-eluate following Procion Red A column chromatography, it would strongly support the suggestion that the Matrex gel is assaying for native carrier. Further to this, it will be necessary to compare the P_i-exchange activities of reconstituted yeast PTP populations with their Procion Red A elution profiles. The presence of an unbound 30,000-M_r polypeptide fraction following chromatography was unaffected by heat-denaturation.

The SERC-funded amino acid sequencing facility at the University of Aberdeen

was employed to determine the N-terminal sequence of the putative yeast PTP. No homology was observed with the N-terminii of the equivalent mammalian phosphate carriers. This was as expected, since the N- and C-terminii represent poorly-conserved domains within the family of mitochondrial inner membrane carriers with related tripartite structures (Runswick *et al.*, 1987). Primary structure analysis also demonstrated that the purified 30,000-M_r yeast mitochondrial protein does not correspond to the ADP/ATP carrier. It had been hoped to confirm the identity of ANT by determining the N-terminal sequence of the CAT-inhibited 34,000-M_r protein co-purified from yeast mitochondria with PTP. However, chromatographic separation of the co-isolate was not achieved under the conditions tested, and it was not possible to resolve the carriers using the continuous SDS-PAGE system employed prior to Edman degradation. Separation of the carriers, and subsequent N-terminal analysis of the CAT-stabilised protein thus represents the major step for characterising yeast ANT.

Determination of the N-terminal sequence of yeast PTP initially indicated that the protein was distinct from that encoded by the gene cloned and sequenced by Murakami *et al.* (1990) and Phelps *et al.* (1991). This transmembrane inner mitochondrial membrane protein is claimed to represent either a phosphate carrier (Phelps *et al.*, 1991) or a protein import receptor (Murakami *et al.*, 1991). However, if the N-terminal of yeast PTP is masked (A. Phelps, personal communication), it is possible that the proteins are identical and that the sequence obtained represents the N-terminus of contaminating porin. N-terminal protein sequence analysis of the Procion Red A unbound and salt-eluted polypeptide fractions indicated that this is the case (see Section 4.5).

The evidence presented by each of the above groups in relation to the yeast PTP and/or import receptor gene is summarised in Section 4.5. Wohlrab's group have demonstrated that the 32,800-M_r protein lacks the NEM-sensitive Cys^{42} present in mammalian PTP's; this being replaced by a Thr residue. The reconstitutively active yeast PTP purified by Guerin *et al.* (1991, in press) is also insensitive to NEM, lending some support to Wohlrab's group's claim that they have isolated the gene encoding this carrier (Phelps *et al.*, 1991). The NEM-sensitivity of the putative PTP isolated in this study was not investigated, and this clearly represents an important area of future

research. Phelps *et al.* (1991) have also established that the precursor to the 32,800-M_r protein lacks the N-terminal presequence present in the bovine heart and rat liver PTP's (Runswick *et al.*, 1987; Ferreira *et al.*, 1989). This also appears to be the case for the putative PTP isolated in this study (Hodgson *et al.*, 1988; see Section 4.2).

The 40% identity of the yeast 32,800-M_r protein with rat liver and bovine heart PTPs is strong evidence that it represents the equivalent yeast carrier. Alternatively, this homology may simply represent the conservation of amino acids intimately involved in the formation of the tripartite structure possessed by this family of mitochondrial proteins. It is quite feasible that an import receptor could be a member of this family. A yeast RNA-splicing protein has also been reported to possess the tripartite structure common to PTP, ANT, UCP and the malate/2-oxoglutarate carrier (Gargouri, 1988).

The evidence presented by Blobel's group that the 32,800-M_r protein represents an import receptor is based on a relatively new technique with a simple yet elegant principle. Initially, a first antibody is raised that recognises a ligand in a fashion that mimics recognition by the physiological receptor. The combining site of this receptor-mimicing antibody thus has structural features similar to those of the receptor's own ligand-binding site. Subsequently, a second set of antibodies is raised against the combining site of the first antibodies, and are similar in structure to the regions of the ligand which bind to the physiological receptor. These anti-idiotype antibodies mimic the original ligand in binding to its receptor, with the obvious advantage that affinities should be high enough to isolate that receptor. This technique has been successfully employed to identify the cell-surface receptors for hormones, neurotransmitters, neuropeptides and growth factors (Kohler *et al.*, 1989).

By first producing an antiserum against a synthetic version of the signal sequence of cytochrome oxidase subunit IV, and then raising anti-idiotype antibodies to the original antiserum, Pain *et al.* (1990) identified the 32,800-M_r integral membrane protein as a strong candidate for a yeast mitochondrial import receptor. The gene encoding this protein was subsequently cloned, characterised and sequenced (Murakami *et al.*, 1990). However, there are considerable doubts over the reliability of receptor anti-idiotypes, particularly in relation to alleged import receptors (Meyer, 1990, "Mimics-or gimmicks"). The use of anti-idiotype antibodies or synthetic signal

sequences to isolate import receptors has consistently resulted in the identification of previously characterised transport proteins (Meyer, 1990; Schnell *et al.*, 1991). These findings have created the notion of bifunctional translocators within the membranes of intracellular organelles. It would thus appear reasonable to suggest that the 32,800-M_r mitochondrial protein characterised by Blobel's and Wohlrab's groups, possibly identical to that purified in this study, can transport both phosphate and protein precursors. Further support for this concept comes from recent evidence that a single mitochondrial protein can have two distinct functions: the *Neurospora* enhancing protein of the matrix-processing peptidase complex is also a component of the cytochrome reductase complex in the inner membrane (Schulte *et al.*, 1989).

In summary, further characterisation studies, in particular reconstitution experiments, are required to establish if the 32,800-M_r yeast mitochondrial inner membrane protein represents a phosphate transporter, an import receptor, or both. The same applies to the 30,000-M_r putative yeast PTP isolated in this study. If these proteins are different, it is possible that they represent individual subunits of a complex multimeric yeast phosphate transport system as exists in bacteria. The more likely scenario that the two proteins are identical also requires further investigation. It would be beneficial to proteolytically digest the putative yeast PTP isolated in this study and obtain some internal sequence for comparison with the primary structure of the protein encoded by the gene cloned by Murakami *et al.* (1990) and Phelps *et al.* (1991).

CHAPTER 5: REGULATION OF THE YEAST PYRUVATE DEHYDROGENASE COMPLEX

5.1 INTRODUCTION

The mitochondrial pyruvate dehydrogenase multienzyme complex (PDC) occupies a key position in the metabolism of the eukaryotic cell, catalysing the essentially irreversible conversion of pyruvate to acetyl CoA. Activity of PDC from eukaryotic sources, including mammalian, avian, and plant tissues, and *Neurospora crassa* is regulated by a phosphorylation-dephosphorylation cycle (see Reed, 1974, Denton *et al.*, 1975 and Wieland, 1983 for reviews). Phosphorylation by PDC kinase inactivates the complex, while dephosphorylation by PDC phosphatase results in its reactivation. The phosphorylation sites in mammalian PDC are located on three serine residues in the α subunit of the pyruvate dehydrogenase (E1) component of the complex (Yeaman *et al.*, 1978; Sugden *et al.*, 1979; Mullinax *et al.*, 1985).

Attempts thus far to demonstrate PDC kinase activity in yeast have been unsuccessful (Kresze and Ronft, 1981a). However, the first indirect evidence that yeast PDC may be controlled *in vivo* by a phosphorylation-dephosphorylation mechanism was presented by Uhlinger *et al.* (1986). In this paper, highly purified PDC from *S.cerevisiae* was phosphorylated and inactivated with a purified heterologous (bovine kidney) PDC kinase, in the presence of [γ^{32}P]ATP. The protein-bound radioactivity was localised in the E1α subunit of the complex. Furthermore, dephosphorylation and reactivation of the phosphorylated, inactive complex was demonstrated in the presence PDC phosphatase from bovine heart. Tryptic digestion of the ^{32}P-labelled complex yielded a single phosphopeptide, which was purified to homogeneity and subjected to amino acid sequencing. The resulting sequence was found to be very similar to that of a tryptic phosphopeptide derived from the E1α subunit of bovine kidney and heart PDC.

It was decided to determine the presence or absence of PDC kinase activity in yeast by *in situ* studies. Freshly prepared, intact yeast mitochondria were incubated with [γ^{32}P]ATP, and analysed by SDS-PAGE and autoradiography. Prior to this, inactivation and radiolabelling studies were carried out on purified yeast PDC in the

presence and absence of a heterologous kinase.

5.2 INACTIVATION AND RADIOLABELLING STUDIES ON PURIFIED PDC

Prior to employing *in situ* studies on yeast mitochondria, a series of experiments were carried out on purified PDC from bovine heart and yeast sources. The complex was purified from bovine heart as outlined in Section 2.2.11(a), while yeast PDC was prepared from *S.cerevisiae* supplied by DCL Ltd. (see Section 2.1.4) as described in Section 2.2.11(b). In both cases, PDC activity was checked at each purification stage as outlined in Section 2.2.12(a). Following preparation, the mitochondrial complexes were analysed by SDS-PAGE to determine the degree of purity. As Figure 5.2.1 clearly illustrates, both the bovine heart and yeast PDCs were purified to near homogeneity. Very few contaminants are apparent in both preparations upon Coomassie blue-staining, although an 80,000-M_r polypeptide of unknown origin is clearly visualised in the yeast PDC isolate. This contaminant may represent the E1 subunit of OGDC. Furthermore, the E2 (subunit M_r value 58,000) and E3 (56,000) subunits of the yeast complex are poorly resolved on this gel.

For the purposes of subsequent inactivation and radiolabelling studies on the yeast complex, a purified heterologous PDC kinase was required. Bovine heart PDC was thus separated into its individual components by FPLC, employing the Superose and MonoQ columns as described in Section 2.2.13. SDS-PAGE analysis of the resulting column fractions is illustrated in Figure 5.2.2. The dissociation of the whole complex into E2/X and E1/E3 fractions is clearly demonstrated (Lanes 2 and 3), as is the separation of the E1/E3 fraction into individual components (Lanes 4 and 5). This technique also serves as a purification scheme for PDC kinase, which is differentially eluted from the MonoQ column upon application of a salt (NaCl) gradient . Although kinase activity was checked at all stages of purification as outlined in Section 2.2.12(b), purified PDC kinase was not detected by Coomassie blue-staining of the SDS-polyacrylamide gel (see Figure 5.2.2). In contrast, a small amount of residual E3 in the preparation is detectable in this manner.

A variety of studies were carried out using the purified preparations of bovine

Figure 5.2.1: Purification of PDC from bovine heart and yeast sources

PDC was prepared from bovine heart as described in Section 2.2.11(a), and from yeast (*S.cerevisiae*) as described in Section 2.2.11(b). Samples were mixed with Laemmli sample buffer prior to resolution on a 10% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: S, M_r standards; 1, bovine heart PDC (subunit M_r values: E2, 72,000; E3, 55,000; X, 51,000; E1α, 41,000; E1β, 36,000); 2, yeast PDC (subunit M_r values: E2, 58,000; E3, 56,000; E1α, 45,000; E1β, 35,000).

Figure 5.2.1: Purification of PDC from bovine heart and yeast sources

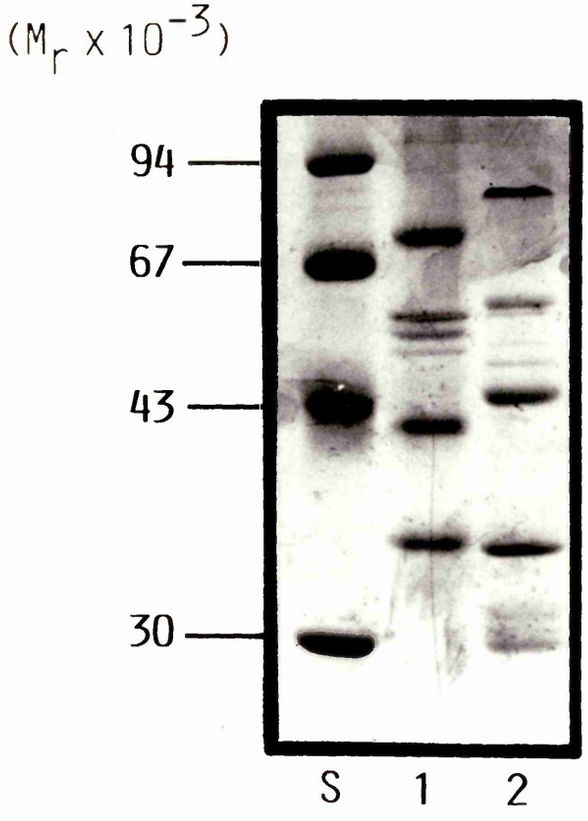
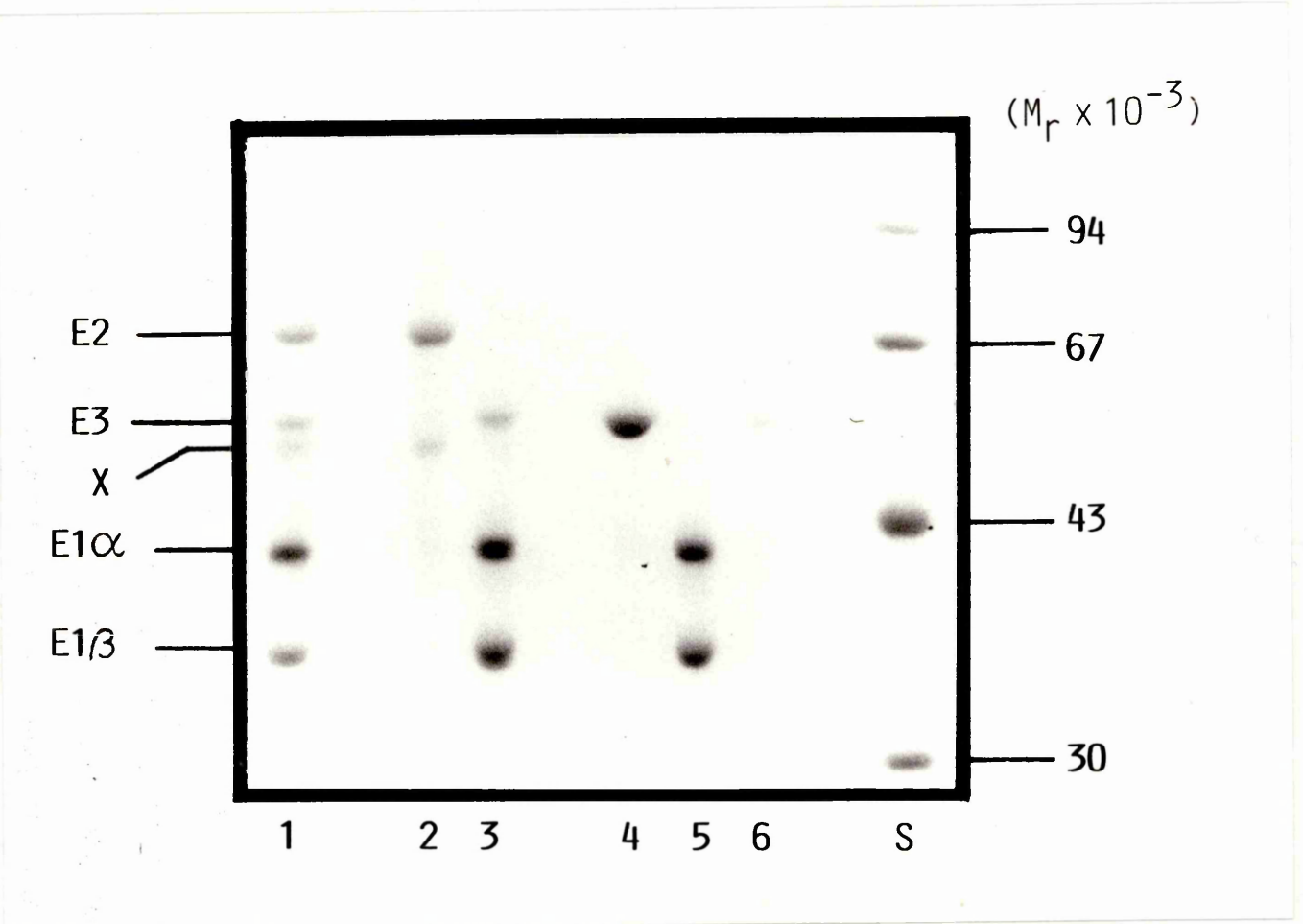


Figure 5.2.2: Separation of PDC components by FPLC

PDC was separated into its individual components by FPLC, as described in Section 2.2.13. The complex was first dissociated into E1/E3 and the E2/X core assembly using a Superose 12 column. A MonoQ column was then used to separate E3, E1 and the associated kinase. All fractions were mixed with Laemmli sample buffer prior to resolution on a 10% (w/v) SDS-polyacrylamide gel which was Coomassie blue-stained. Lanes: 1, intact bovine PDC; 2 and 3, Superose 12 fractions (E2/X and E1/E3 respectively); 4-6, NaCl-eluates from MonoQ column (4, E3; 5, E1; 6, kinase and residual E3); S, M_r standards.

Figure 5.2.2: Separation of PDC components by FPLC



heart PDC, yeast PDC and bovine kinase. The purified complexes were assayed for PDC kinase activity as outlined in Section 2.2.12(b). In addition to this, the effect of a heterologous (bovine heart) kinase on the activity of yeast PDC was assessed. Kinase activity was assayed in each case by measuring the rate of inactivation of the complex in the presence of 0.2mM ATP at 30°C (Hucho *et al.*, 1972), and also by estimation of the rate of incorporation of ^{32}P-phosphoryl groups from [γ^{32}P]ATP into the E1α subunit of PDC (Stepp *et al.*, 1983). In the case of bovine heart PDC, both rapid inactivation of the complex and incorporation of ^{32}P-phosphoryl groups were observed, presumably due to intrinsic kinase activity. The phosphorylation and inactivation of the yeast complex in the presence of a heterologous kinase were also demonstrated, confirming the results of Uhlinger *et al.* (1986), as discussed in Section 5.1. However, in the absence of the bovine heart kinase, no reduction in yeast PDC activity was observed; nor was there any indication of ^{32}P-phosphoryl incorporation into the yeast PDC E1α subunit. These results agree with previous observations by Kresze and Ronft (1981a) and Uhlinger *et al.* (1986), who detected no intrinsic kinase activity at any stage during purification of yeast PDC.

There exist a variety of possible reasons for the inability to detect PDC kinase activity in yeast:-

1) yeast is unique among eukaryotic organisms, in that PDC is not regulated by a phosphorylation-dephosphorylation mechanism on the E1α subunit;

2) the kinase is labile;

3) the kinase is loosely bound to the complex and dissociates during purification;

4) the level of control exhibited by the yeast kinase is not as critical as for mammalian PDC. If this is so, the kinase may be present at virtually undetectable levels;

5) regulation of yeast PDC is dependent on growth conditions. If this is the case, the kinase may be inducible.

5.3 RADIOLABELLING STUDIES ON INTACT MITOCHONDRIA

5.3.1 [γ^{32}P]ATP-labelling of rat liver mitochondria

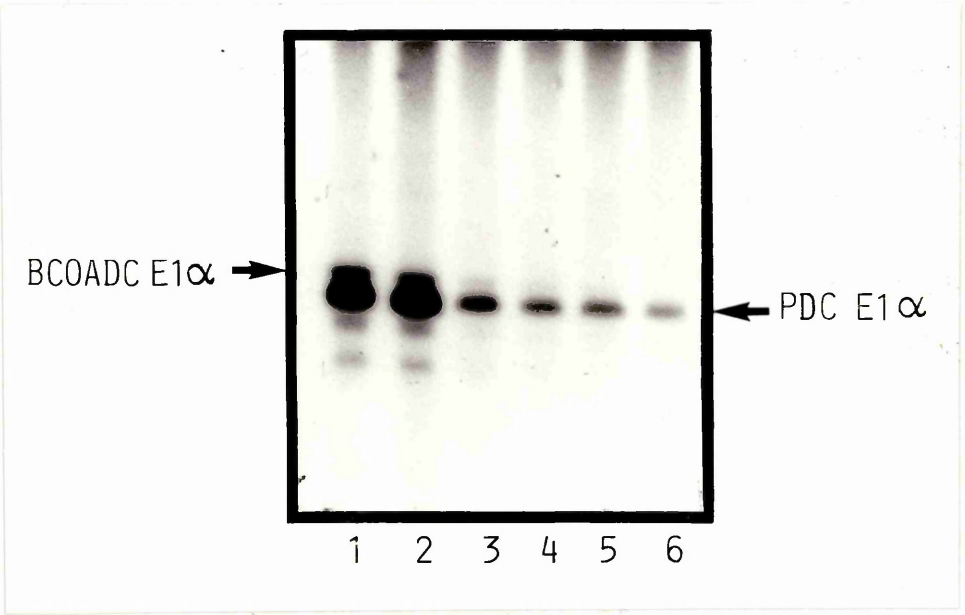
Prior to the application of radiolabelling experiments on yeast mitochondria, similar *in situ* studies were initially carried out on the equivalent organelles from a mammalian source. In addition to serving as a control for the subsequent [γ^{32}P]ATP-labelling of yeast mitochondria, these experiments would facilitate the optimisation of conditions for the radiolabelling of mitochondrial proteins. Two reversibly phosphorylated proteins are known to exist in mammalian mitochondria: the E1α subunits of the multienzyme complexes PDC and BCOADC (Reed, 1974; Denton *et al.*, 1975; Reed and Yeaman, 1987). Both complexes contain an intrinsic kinase which phosphorylates the corresponding E1α subunit and inactivates the complex.

Radiolabelling experiments were carried out on freshly prepared, intact rat liver mitochondria suspended in isolation buffer (see Section 2.2.7[a]) to a final assay volume of 250µl. Mitochondria (500µg protein/assay) were labelled in the presence of 10µCi [γ^{32}P]ATP/assay. The following reagents were also included in the assay samples to the denoted final concentrations: ATP (2µM); 25mM NaF (to inhibit phosphatase activity); protease inhibitors (0.5mM PMSF, 0.5mM benzamidine-HCl, 0.3µM leupeptin). The samples were incubated at 37°C for 15min, following which the mitochondria were pelleted (10,000 xg for 5min) and solubilised in Laemmli sample buffer prior to analysis by SDS-PAGE and autoradiography. Figure 5.3.1 illustrates the ATP-dependent ^{32}P-phosphorylation of rat liver mitochondrial proteins. In this experiment, increasing amounts of the mammalian kinase inhibitor dichloroacetate were also included. The two heavily labelled polypeptides in Lanes 1 and 2 are presumed to represent the E1α subunits of PDC and BCOADC (M_r values 41,000 and 46,000 respectively). This is confirmed by dichloroacetate inhibition of the kinase activities: as

Figure 5.3.1: PDC kinase and BCOADC kinase activities in rat liver mitochondria

Rat liver mitochondria (500μg/assay) were incubated with [γ^{32}P]ATP (10μCi/assay) as described in Section 5.3.1. The mammalian kinase inhibitor dichloroacetate was included to inhibit radiolabelling of the PDC and BCOADC E1α subunits. Mitochondria were pelleted and solubilised in Laemmli sample buffer prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was processed for autoradiography. Lanes: 1 and 2, [γ^{32}P]ATP-labelled rat liver mitochondria; 3-6, in the presence of dichloroacetate (1, 2, 5 and 10mM respectively).

Figure 5.3.1: PDC kinase and BCOADC kinase activities in rat liver mitochondria



the concentration of this reagent increases from 1-10mM (Lanes 3-6), there is a significant reduction in the level of phosphorylation of the 41,000- and 46,000-M_r polypeptides.

Of additional interest in this autoradiograph is the presence of two phosphoproteins of unknown origin with M_r values lower than the E1α subunits. The radiolabelling of these polypeptides is also inhibited by dichloroacetate and they may represent degradation products of the PDC and/or BCOADC E1α subunits. However, although preliminary experiments indicated that at least one of these phosphopeptides may be immunologically distinct from both PDC and BCOADC, this line of research was not pursued further.

5.3.2 [γ^{32}P]ATP-labelling of yeast mitochondria

S.cerevisiae D273-10B were grown aerobically in yeast growth media supplemented with various carbon sources (as outlined in Section 2.2.14) to an absorbance at 600nm of 1.0-2.0, representing the late logarithmic phase. Yeast mitochondria were subsequently prepared as outlined in Section 2.2.7(c). Radiolabelling experiments were carried out on freshly prepared, intact mitochondria in isolation buffer (see Section 2.2.7[c]) to a final assay volume of 250µl. Yeast mitochondria (500µg protein/assay) were incubated at 37°C for 15min in the presence of [γ^{32}P]ATP (10µCi/assay) prior to being pelleted (10,000 xg for 5min), solubilised in Laemmli sample buffer and analysed by SDS-PAGE and autoradiography. Also included in the assay samples was 25mM NaF to inhibit phosphatase activity. Furthermore, the following variations in growth and assay conditions were included in order to optimise the phosphorylation of yeast mitochondrial proteins:-

1) the concentration of unlabelled ATP was varied (2 and 20µM final);

2) protease inhibitors (0.5mM PMSF, 0.5mM benzamidine-HCl, 0.3µM leupeptin) were included in some assay samples;

3) some assay samples were supplemented with oligomycin (0.1mg/ml) and CCCP

(10mM). It was hoped that by uncoupling the mitochondria with CCCP and inhibiting ATPase activity with oligomycin, it would be possible to facilitate the entry of exogenous ATP and prevent its subsequent breakdown;

4) prior to mitochondrial preparation, yeast were grown in media supplemented with a variety of carbon sources, both fermentable and non-fermentable: YPG (galactose), YPD (glucose), YPEG (ethanol/glycerol), YPDG (glycerol/glucose), YPS (succinate), YPL (lactate). This was to investigate the possibility that the kinase is inducible under certain growth conditions;

5) in order to further investigate the possible inducibility of yeast PDC kinase, yeast were grown aerobically in YPG, followed by 1h of anoxic growth prior to mitochondrial preparation.

6) mitochondria isolated from yeast supplied by DCL Ltd. were also assayed for kinase activity;

Figure 5.3.2 illustrates the [γ^{32}P]ATP-radiolabelling of yeast mitochondria under the conditions described above, with the additional inclusion of rat liver mitochondria as a control. The presence of a 45,000-M_r phosphoprotein is apparent, notably in Lanes 37-40 (yeast grown aerobically in YPG; mitochondria assayed in the absence of oligomycin and CCCP) . This polypeptide may correspond to the E1α subunit of yeast PDC. Phosphorylation of this polypeptide is greater in the presence of 2μM, rather than 20μM ATP. There are no apparent beneficial effects of the protease inhibitors, although they were included in future experiments as a precaution.

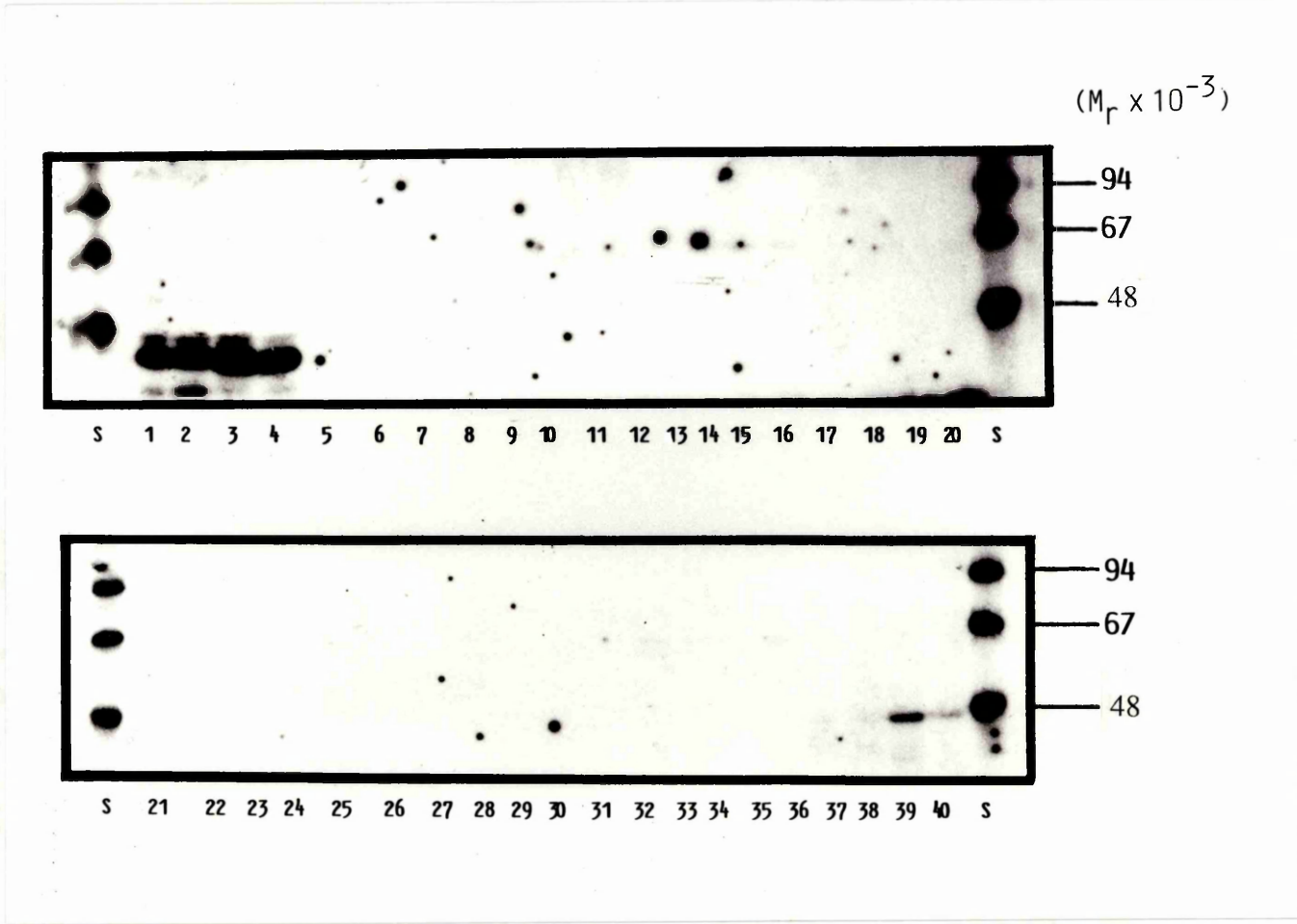
Optimisation of yeast mitochondrial protein phosphorylation was thus observed following growth in medium supplemented with galactose, a fermentable substrate. Additional experiments confirmed that sugar substrates were generally superior to non-fermentable carbon sources for the subsequent radiolabelling of mitochondria, with the apparent exception of glucose-supplemented growth medium. However, in some experiments, the 45,000-M_r phosphoprotein also labelled well following growth in lactate-supplemented medium (YPL).

A series of radiolabelling experiments were carried out on freshly isolated,

Figure 5.3.2: [γ^{32}P]ATP-labelling of rat liver and yeast mitochondria

Mitochondria (500μg/assay) were prepared from rat liver (lanes 1-4) and yeast (*S.cerevisiae* D273 10B) grown in YPG (lanes 5-8 and 37-40), YPD (lanes 9-12), YPEG (lanes 13-16), YPDG (lanes 17-20), YPS (lanes 21-24), YPL (lanes 25-28), YPG (with 1h anoxic growth prior to harvesting; lanes 29-32), and also from yeast supplied by DCL (lanes 33-36). [γ^{32}P]ATP (10μCi/assay) was included, as was unlabelled ATP to a final concentration of 20μM (lanes 1, 2, 5, 6, 9, 10, 13, 14, 17, 18, 21, 22, 25, 26, 29, 30, 33, 34, 37, 38) or 2μM (lanes 3, 4, 7, 8, 11, 12, 15, 16, 19, 20, 23, 24, 27, 28, 31, 32, 35, 36, 39, 40). Other variables were included: protease inhibitors (0.5mM PMSF, 0.5mM benzamidine-HCl, 0.3μM leupeptin; lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40); 0.1mg/ml oligomycin and 10mM CCCP (lanes 1-36). Mitochondria were pelleted and solubilised in Laemmli sample buffer prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was processed for autoradiography. S, M_r standards (^{125}I-radiolabelled).

Figure 5.3.2: [γ^{32}P]ATP-labelling of rat liver and yeast mitochondria



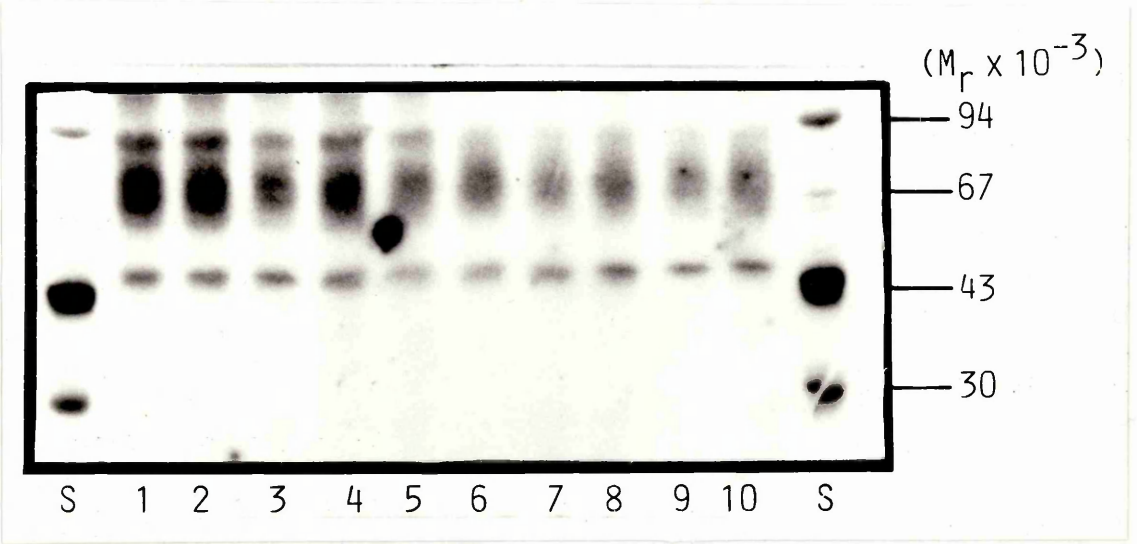
intact yeast mitochondria (500μg/assay) following aerobic growth of *S.cerevisiae* in YPG to the late logarithmic phase. On the basis of the results described above and illustrated in Figure 5.3.2, prepared samples of 250μl mitochondrial suspension/assay were incubated in the presence of 10μCi [γ^{32}P]ATP/assay under the following conditions: ATP (2μM final concentration); NaF (25mM); protease inhibitors (0.5mM PMSF, 0.5mM benzamidine-HCl, 0.3μM leupeptin). In addition to this, the mammalian PDC kinase inhibitor dichloroacetate (1,2,5,10mM final concentrations) was included in some of the assay samples, and mitochondria isolated from a further strain of yeast (*S.cerevisiae* S288C) were assayed for kinase activity in an identical manner to *S.cerevisiae* D273-10B mitochondria. As before, the radiolabelled mitochondria were pelleted (10,000 xg for 5min), solubilised in Laemmli sample buffer and analysed by SDS-PAGE and autoradiography. Figure 5.3.3 illustrates the [γ^{32}P]ATP-radiolabelling of yeast mitochondria under the conditions described above. The presence of a 45,000-M_r phosphoprotein, which may correspond to PDC E1α, is apparent in the samples originating from both strains of yeast. However, in each case, a reduction in ^{32}P-phosphorylation of the putative PDC E1α in the presence of increasing concentrations of dichloroacetate, which would correspond to the inhibition of kinase activity, is not observed.

In order to positively identify the 45,000-M_r yeast mitochondrial phosphoprotein as the E1α component of PDC, immune precipitation studies were carried out on the radiolabelled organelles. Yeast mitochondria (100 and 500μg/assay) were prepared from both strains of *S.cerevisiae* (D273-10B and S288C) grown to late logarithmic phase in YPG, and incubated in the presence of 10μCi [γ^{32}P]ATP/assay. Assay conditions were as described above. Samples were subsequently subjected to analysis by immune precipitation as outlined in Section 2.2.6(c) using anti-yeast PDC E1 serum (10 and 50μl/assay), and non-immune rabbit serum (NRS; 50μl/assay) as a

Figure 5.3.3: Incubation of yeast mitochondria with [γ^{32}P]ATP

Yeast (*S.cerevisiae* D273-10B and S288C) mitochondria (500μg/assay) were incubated with [γ^{32}P]ATP (10μCi/assay) as described in Section 5.3.2. The mammalian kinase inhibitor dichloroacetate was included to ascertain its effect on the putative radiolabelling of yeast PDC E1α. Mitochondria were pelleted and solubilised in Laemmli sample buffer prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was processed for autoradiography. Lanes: 1, [γ^{32}P]ATP-labelled D273-10B mitochondria; 2-5, in the presence of dichloroacetate (1, 2, 5 and 10mM respectively); 6-10, identical to lanes 1-5 except using S288C mitochondria; S, M_r standards (^{125}I-radiolabelled).

Figure 5.3.3: Incubation of yeast mitochondria with [γ^{32}P]ATP



negative control. A sample of *S.cerevisiae* D273-10B mitochondria (500μg/assay) incubated with 10μCi [γ^{32}P]ATP but not subjected to immune precipitation served as a positive control. Following dissociation, the immune precipitates were analysed by SDS-PAGE and fluorography. Figure 5.3.4 illustrates the immune precipitation of phosphoproteins from [γ^{32}P]ATP-labelled yeast mitochondria under the conditions described above. It is apparent that, in mitochondria from both strains of *S.cerevisiae*, the 45,000-M_r ^{32}P-phosphoprotein is immune precipitated with either 10 or 50μl yeast PDC E1 antiserum. This applies to both the 100 and 500μl assay samples of yeast mitochondrial protein. These results strongly suggest that the 45,000-M_r polypeptide which is phosphorylated under the conditions adopted in these experiments corresponds to the E1α subunit of yeast PDC, thus indicating the presence of PDC kinase activity in yeast mitochondria.

5.4 DISCUSSION

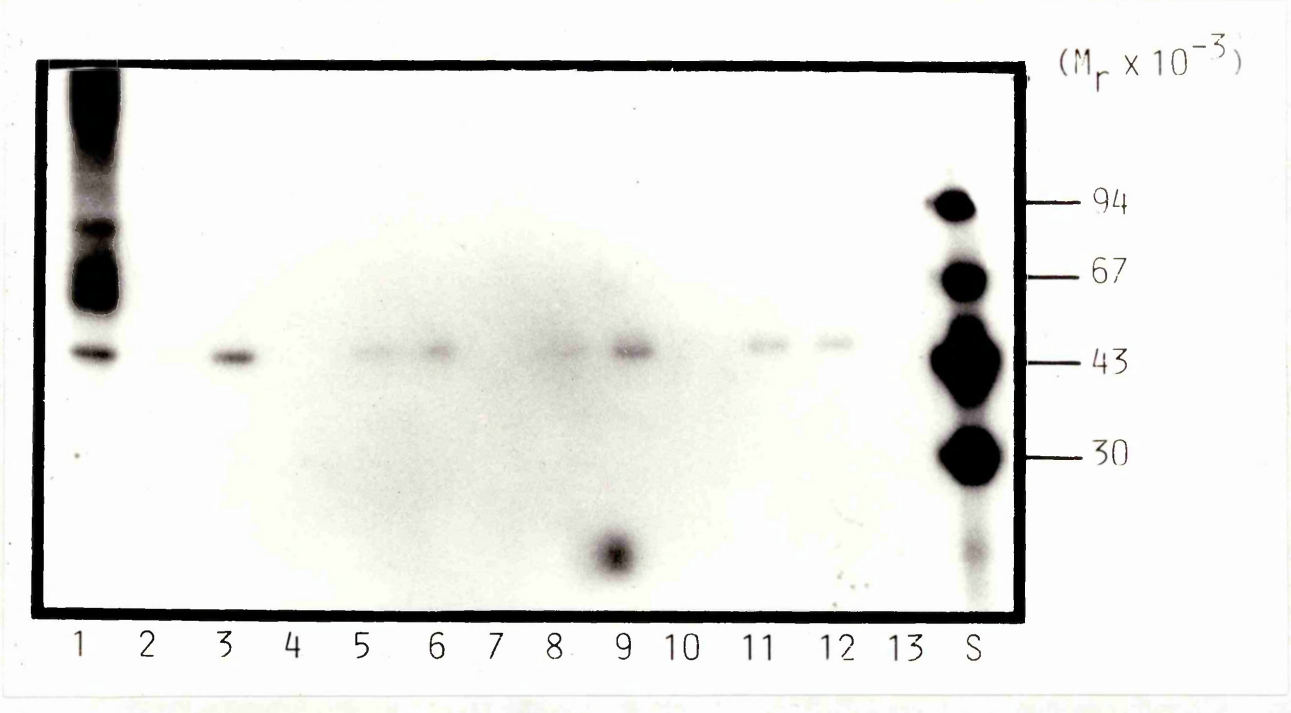
A series of experiments was carried out to investigate an apparent anomaly in the regulation of the yeast PDC multienzyme complex. Whereas PDC activity in all other eukaryotic organisms investigated thus far is controlled by a phosphorylation-dephosphorylation cycle on the α subunit of the pyruvate dehydrogenase (E1) component of the complex, no such mechanism had yet been detected in yeast.

Initially, inactivation and radiolabelling studies were carried out on highly purified yeast PDC prepared from *S.cerevisiae* supplied by DCL Ltd. The complex was subjected to PDC kinase activity assays in the presence and absence of a heterologous kinase dissociated from bovine heart PDC during separation of the complex by FPLC. Highly purified bovine heart PDC was treated in an identical manner as a control. Although inactivation and phosphorylation of the mammalian complex was demonstrated under the assay conditions, no intrinsic kinase activity was detected in yeast PDC, agreeing with the previous observations of Kresze and Ronft

Figure 5.3.4: Immune precipitation of phosphoproteins from [γ^{32}P]ATP-labelled yeast mitochondria

Yeast (*S.cerevisiae* D273-10B and S288C) mitochondria were incubated with [γ^{32}P]ATP (10μCi/assay) and subjected to immune precipitation with yeast PDC E1 antiserum and non-immune rabbit serum (NRS), as described in Section 2.2.6(c). Immune precipitates were dissociated by boiling in Laemmli sample buffer prior to resolution on a 12.5% (w/v) SDS-polyacrylamide gel which was processed for fluorography. Lanes: 1, [γ^{32}P]ATP-labelled D273-10B mitochondria (500μg); 2-4, [γ^{32}P]ATP-labelled D273-10B mitochondria (500μg) immune precipitated with yeast PDC E1 antiserum (10 and 50μl) and NRS (50μl) respectively; 5-7, identical to lanes 2-4, except using 100μg D273-10B mitochondria; 8-13, identical to lanes 2-7, except using S288C mitochondria; S, M_r standards (^{125}I-radiolabelled).

Figure 5.3.4: Immune precipitation of phosphoproteins from [γ^{32}P]ATP-labelled yeast mitochondria



(1981a) and Uhlinger *et al.* (1986). However, the yeast complex was ^{32}P-phosphorylated and inactivated in the presence of mammalian kinase, a phenomenon previously detected by Uhlinger *et al.* (1986).

Initially, *in situ* radiolabelling studies were carried out on freshly prepared, intact rat liver mitochondria in the presence of [γ^{32}P]ATP. Phosphorylation of the PDC and BCOADC E1α subunits was demonstrated under these conditions. The presence of PDC and BCOADC kinase activities was confirmed by the reduction of radiolabelling observed in the presence of the mammalian kinase inhibitor dichloroacetate. Two additional phosphoproteins, with lower M_r values than the E1α subunits of the complexes, were detected during the [γ^{32}P]ATP radiolabelling of rat liver mitochondria. The origin of these polypeptides is unknown, although at least one appears to be immunologically distinct from PDC and BCOADC. Further research is clearly required to identify and characterise these phosphoproteins.

Similar *in situ* [γ^{32}P]ATP-radiolabelling studies were subsequently carried out on intact yeast mitochondria, freshly prepared from *S.cerevisiae* D273-10B subjected to a variety of growth conditions. The assay contents were also manipulated in several ways in order to establish the optimal conditions for phosphorylation of yeast mitochondrial proteins. Following aerobic growth in galactose-supplemented growth medium (YPG), a 45,000-M_r phosphoprotein was observed in yeast mitochondria, possibly corresponding to the E1α subunit of PDC. This polypeptide was also detected in mitochondria prepared from a further strain of yeast, *S.cerevisiae* S288C. However, in mitochondria from both yeast strains, no significant reduction in the radiolabelling of this ^{32}P-phosphoprotein was observed in the presence of the mammalian PDC kinase inhibitor dichloroacetate. This suggests that the yeast mitochondrial kinase which phosphorylates the 45,000-M_r polypeptide is resistant, or possibly less susceptible, to dichloroacetate inhibition.

The strongest evidence thus far for the presence of PDC kinase activity in yeast was obtained from immune precipitation experiments on [γ^{32}P]ATP-radiolabelled yeast

mitochondria. In mitochondria prepared from both strains of yeast, the 45,000-M_r phosphoprotein was immmunologically identified as the E1α component of yeast PDC. It therefore seems likely that PDC activity in yeast, in common with all other eukaryotic organisms thus investigated, is regulated by a phosphorylation-dephosphorylation mechanism on the E1α subunit of the complex. However, as indicated in this and previous studies (Kresze and Ronft, 1981a; Uhlinger *et al.*, 1986), PDC kinase is not co-purified with the complex. In contrast, the mammalian kinase is tightly bound to PDC, is co-purified with the complex, and can be dissociated by FPLC (see Section 2.2.13). The results obtained in this study thus indicate that yeast PDC kinase may be loosely bound to the complex and is dissociated during PDC purification. However, preliminary experiments have failed to detect PDC kinase activity in crude yeast mitochondrial extracts, suggesting that this regulatory enzyme may be labile. Alternatively, this may be due to the presence of a kinase- (or phosphatase-) regulating protein factor in yeast mitochondria.

As discussed above, optimal radiolabelling of the yeast PDC E1α occurred following aerobic growth in medium supplemented with galactose, a fermentable carbon source. In general, growth on fermentable carbon sources resulted in better phosphorylation of the E1α component than growth on non-fermentable carbon sources, suggesting that the kinase is inducible. However, this observation may also be due to different phosphorylation states of the E1α subunit prior to assaying for kinase activity, following growth in each type of substrate. In a fermentable carbon source, PDC is likely to be very active with the E1α subunit in an non-phosphorylated state. Therefore, considerable scope exists for phosphorylation under the assay conditions. In contrast, PDC is likely to be inactive during growth on a non-fermentable carbon source. Thus, the E1α subunit is already heavily phosphorylated prior to application of the mitochondrial kinase assay. The poor radiolabelling of the E1α subunit following growth in glucose-supplemented medium is probably due to catabolite repression. If this is the case, growth on this substrate would result in depressed levels of all

mitochondrial enzymes including PDC (Dawes and Lindsay, personal communication).

Phosphorylated E1α was also observed following growth in YPL. This may be due to the conversion of lactate to pyruvate (by lactate dehydrogenase) prior to entry into the citric acid cycle. PDC would thus require to be active during growth on this substrate.

CHAPTER 6: GENERAL DISCUSSION & FUTURE DIRECTION

6.1 PTP AND ANT

A series of protein-chemical studies was carried out on the mitochondrial phosphate and adenine nucleotide transport proteins (PTP and ANT) from both mammalian and yeast sources. PTP was purified to apparent homogeneity from rat liver, bovine heart and yeast mitochondria, with the inclusion of carboxyatractyloside (CAT) resulting in the co-isolation of ANT in each case.

The mammalian PTP/ANT co-isolate was subjected to rigorous chromatographic analysis in order to separate the carriers into homogeneous populations. It was ultimately observed that following application of the co-purified proteins to the Matrex gel Procion Red A, the carriers were differentially eluted by a salt gradient (0-1.0M NaCl) at pH 8.8. At this stage, the identities of bovine heart PTP and ANT were confirmed by N-terminal protein sequence analysis.

Considerable evidence was also presented that Procion Red A column chromatography represents an assay for native mammalian PTP conformation, an important pre-requisite for further analysis of the carrier. However, as indicated in Section 3.4, it will be necessary to confirm this observation by conducting reconstitution experiments and comparing the phosphate flux of different PTP populations with their Procion Red A elution profiles.

It was further proposed in Section 3.4 that Procion Red A also represents an assay for native mammalian ANT conformation, on the basis that the Matrex gel is selectively eluting carriers in the homodimeric state. This hypothesis also requires further research in the form of reconstitution experiments (as described above), and comparison of the elution profiles of ANT populations from hydroxylapatite (HTP) and Procion Red A columns. As outlined previously (Section 3.4), HTP adsorbs out denatured ANT (Klingenberg *et al.*, 1974; Schleyer and Neupert, 1984).

The Matrex gel Procion Red A was further employed as a technique for transferring mammalian PTP from solution in Triton X-100 to alternative non-ionic detergents more suitable for physical analyses of the carrier and crystallography trials. It is proposed that this methodology would function equally well for ANT; however, it

will be necessary to confirm this experimentally. Further work will also be required to identify and rectify the significant reductions in protein yield observed during the detergent replacement process. This is particularly important in view of the protein levels required for crystallisation trials. The conditions for the detergent switch may have to be altered if it results in denaturation of the bound carrier; *e.g.* an increase in the wash volumes may be required before and/or after the switch. Alternatively, if mixed PTP-detergent micelles are attaching to the filter during protein concentration in the Novacell stirred cells, it may be necessary to employ an alternative protein concentration technique.

In future, it is hoped to carry out further CD analyses of mammalian PTP in alternative detergents, both in the native state and following exposure to the denaturing agent GdnHCl. The spectra and predicted secondary structures will be compared to those obtained previously (see Section 3.3.5). The CD spectra for mammalian native and denatured ANT will also be obtained in a range of detergents. In this way, it will be possible to assess the relative stability of each of the carriers in various detergents and determine which are the most suitable for crystallisation trials. Similarly, the effects of freezing, exposure to heat, and time since purification on the stability of PTP and ANT in each detergent will be assessed by CD analysis.

In addition to the above, further physical analysis of the proteins will be carried out by differential scanning microcalorimetry (DSC). An added advantage of this technique is that it can be applied to samples prepared in Triton X-100. DSC analysis permits measurement of the thermal transitions occurring in aqueous solution during the unfolding of individual domains of proteins. As with CD, the stability of PTP and ANT in various detergents will be determined.

Ultimately it is hoped to obtain crystals of bovine heart PTP and/or ANT suitable for X-ray diffraction studies and determine the high resolution three-dimensional structure of at least one of the carriers. It may then be possible to elucidate the molecular mechanisms underlying the membrane insertion, intramembrane organisation, and catalytic mechanisms of the 'tripartite' family of mitochondrial carriers.

Furthermore, it is hoped to carry out the physical analyses and X-ray crystallography studies described above on PTP and ANT isolated from yeast

mitochondria. However, several criteria require to be fulfilled and various problems solved before equivalent studies can be undertaken with the yeast carriers. There may be problems with crystallography studies on yeast PTP and ANT since the levels of mitochondria and subsequently purified protein obtained from yeast are significantly lower than from bovine heart. No such restrictions are envisaged prior to the physical analyses.

As outlined in Chapter 4, the 30,000-M_r protein isolated from yeast mitochondria using the mammalian PTP purification scheme was identified tentatively as yeast PTP on the basis of its immunological characteristics. Similarly, the co-purified CAT-stabilised 34,000-M_r polypeptide was identified as yeast ANT on the basis of its complexing with the specific inhibitor. However, although the identity of the ADP/ATP carrier can be confidently assumed on this basis, further characterisation studies are required to positively identify the putative yeast PTP isolated in this study. These studies are necessitated by the results obtained from the chromatographic analysis of the yeast mitochondrial co-isolate, and recently published literature (Pain *et al.*, 1990; Murakami *et al.*, 1990; Phelps *et al.*, 1991).

In contrast to mammalian PTP and ANT, application of the yeast mitochondrial co-isolate to the Matrex gel Procion Red A did not result in the separation of the carriers into homogeneous populations under the conditions tested. However, it is hoped that this will be achieved by repeating the chromatographic analysis at a higher pH. As stated above, the mammalian carriers were differentially eluted by a salt gradient (0-1.0M NaCl) at pH 8.8. Separation of the yeast carriers would facilitate positive identification of ANT by N-terminal amino acid analysis.

The potential of Procion Red A analysis as an assay for native yeast PTP and ANT is suggested. However, as is the case for the mammalian carriers, this hypothesis requires further research in the form of reconstitution studies (for PTP and ANT) and comparison of elution profiles from Procion Red A and HTP (for ANT). An interesting observation during chromatographic analyses of the yeast co-isolate was the presence of an unbound protein population following application to Procion Red A. This indicates that the 30,000-M_r polypeptide isolate represents two proteins with near-identical M_r values. As outlined in Sections 4.4-4.6, the most likely explanation is

that the NaCl-eluate corresponds to yeast PTP while the unbound fraction represents contamination by the outer membrane protein porin.

Recently, Wohlrab's group have cloned and sequenced the gene encoding yeast PTP (Phelps *et al.*, 1991). However, Blobel's group have isolated the same gene and determined that it encodes a mitochondrial protein import receptor (Pain *et al.*, 1990; Murakami *et al.*, 1990). The *in vivo* role of the protein encoded by this gene is clearly a matter of considerable debate although, as discussed in Section 4.6, it may function as a transporter for both proteins and phosphate. Whatever its function, the protein was initially thought to be distinct from that isolated in this study, as indicated by comparing the N-terminal amino acid sequences of both. However, since the N-terminal of yeast PTP is masked (A. Phelps, personal communication), the protein sequence obtained in this study may be that of the contaminant porin. Subsequent N-terminal amino acid analyses of the unbound and NaCl-eluted Procion Red A 30,000-M_r fractions strongly suggested that this is the case. Whereas the N-terminal of the salt-eluted polypeptide was found to be blocked, the contaminating unbound protein was positively identified as porin.

It thus appears likely that the putative yeast PTP isolated in this study is identical to that encoded by the gene cloned by Murakami *et al.* (1990) and Phelps *et al.* (1991). This postulation is based upon chromatographic analysis of the proposed PTP/ANT/porin yeast mitochondrial isolate as well as the putative yeast PTP's purification properties and immunological characteristics in relation to the equivalent mammalian carrier. Furthermore, the N-terminal of both putative yeast PTPs is blocked. The absence of an N-terminal-extended signal sequence in both proteins lends further support to the above proposal. Guerin *et al.* (1991, in press) have also isolated a reconstitutively active yeast mitochondrial PTP. Wohlrab's group claim that this represents the protein encoded by the gene they have cloned (Phelps *et al.*, 1991) on the basis that both lack the NEM-reactive Cys^{42} present in mammalian PTPs.

There is clearly a requirement to elucidate the functional role of the various proteins implicated in phosphate transport in yeast mitochondria: the protein encoded by the gene cloned by Blobel's and Wohlrab's groups (Murakami *et al.*, 1990; Phelps *et al.*, 1991); Guerin's reconstitutively active PTP (1991, in press); the 30,000-M_r protein isolated in this study. This research will involve detailed structural and functional studies, and reconstitution experiments on each of the proteins involved. It will also be

necessary to obtain internal amino acid sequences from the putative yeast PTPs isolated by Guerin *et al.* (1991, in press) and in this study to determine if they are identical to the protein encoded by the PTP and/or import receptor gene previously cloned.

6.2 YEAST PDC

A series of experiments were carried out to investigate an apparent anomaly related to the control of PDC activity in yeast. Whereas activity of this multienzyme complex in all other eukaryotic systems studied thus far is regulated by a phosphorylation-dephosphorylation mechanism on the E1α subunit, no PDC kinase activity has ever been detected in yeast cells.

Initially, the absence of intrinsic kinase activity in purified yeast PDC was confirmed by inactivation and radiolabelling studies, supporting previous observations by Kresze and Ronft (1981a) and Uhlinger *et al.* (1986). A novel *in situ* approach was thus adopted to determine the presence or absence of PDC kinase in yeast cells. Freshly prepared, intact mitochondria from two strains of *S.cerevisiae* were subjected to radiolabelling studies with [γ^{32}P]ATP. As outlined in Chapter 5, a 45,000-M_r phosphoprotein was identified which may correspond to the E1α subunit of PDC, indicating the presence of kinase activity in yeast mitochondria. Immune precipitation studies with anti-yeast PDC E1 serum strongly supported this conclusion. The strong labelling of this phosphoprotein observed following growth in galactose-supplemented medium could be caused by the PDC being in an active, non-phosphorylated state prior to assaying. Alternatively, the kinase may be inducible. Radiolabelling of the putative PDC E1α was poor following growth in media supplemented with non-fermentable carbon substrates (glycerol and succinate) and glucose, which may result in catabolite repression. Phosphorylation of E1α was also observed following growth in YPL. This may be due to the conversion of lactate to pyruvate prior to entry to the citric acid cycle. Under these conditions, PDC would be active and E1α non-phosphorylated prior to assaying.

Although the results obtained from these *in situ* experiments offer strong support to the presence of PDC kinase activity in yeast cells, they truly represent a pilot study leading ultimately to the elucidation of the regulatory mechanism for the yeast

complex. Future experiments will determine the phosphorylation site (or sites) on the E1α subunit, and also lead to the identification, purification and characterisation of the kinase.

The following experimental approach will be adopted to identify the *in situ* phosphorylation site(s) on yeast PDC E1α. In the process, it will also be possible to determine if this is identical to the single site identified *in vitro* by Uhlinger *et al.* (1986) following phosphorylation of purified yeast PDC with a heterologous kinase and subsequent sequencing of the E1α tryptic phosphopeptide:-

1) Purified yeast PDC in the presence of a heterologous kinase, and intact yeast mitochondria will be radiolabelled with [γ^{32}P]ATP;

2) Following autoradiography, the phosphorylated E1α bands will be removed from the gel and subjected to tryptic digestion;

3) In each case, the phosphopeptide(s) will be characterised by high voltage electrophoresis or high pressure liquid chromatography (HPLC);

4) The phosphorylation site(s) in each case will be identified by amino acid sequencing.

It will thus be possible to compare the phosphorylation sites *in situ* and *in vitro*, as well as determining the number of these sites *in situ* .

The possibility that the phosphorylation sites are identical *in situ* and *in vitro* will be further investigated by preparing yeast mitochondrial extracts which can be tested for their viability as crude preparations of PDC kinase. If this is the case, it may be possible to determine if the two kinases (purified bovine heart and crude yeast) compete for the same site on yeast PDC E1α.

The preparation of crude mitochondrial extracts with yeast PDC kinase activity also represents the starting point for purification of the kinase. Separation of the protein components in the extracts will be achieved by FPLC and/or HPLC, with the peaks being analysed for PDC kinase activity, and by SDS-PAGE (to determine the level of purity). However, preliminary experiments have failed to detect kinase activity in these crude preparations. This may be due to the presence of a kinase- (or phosphatase-) regulating protein factor in yeast mitochondria.

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