IMPORT OF PROTEINS

INTO

MAMMALIAN MITOCHONDRIA

by

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

 March, 1986

MITOCHONDRIAL BIOGENESIS

DOCTOR OF PHILOSOPHY (1986)
(Biochemistry)

McMASTER UNIVERSITY
Hamilton, Ontario

TITLE: Import of Proteins into Mammalian Mitochondria

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NUMBER OF PAGES: xiv, 203

ABSTRACT

Most mitochondrial proteins are encoded in the nuclear genome and imported into mitochondria after their synthesis on cytoplasmic ribosomes. Two rat liver mitochondrial proteins, malate dehydrogenase and the adenine nucleotide carrier, were selected to study the mechanism of importing nuclear coded mitochondrial proteins into the organelle.

Rat liver proteins were synthesized in vitro and the nascent forms of the two proteins were immunoprecipitated by monospecific antibodies against each protein. Size determination by sodium dodecyl sulphate-polyacrylamide gel electrophoresis indicated that each precursor protein is about 1,500-2,000 daltons larger than its mature monomeric form. In contrast to secretory proteins, both proteins are exclusively synthesized on membrane free polysomes. Molecular mass sieving experiments indicated that both precursor proteins exist as aggregates or complexes after their synthesis. These aggregates or complexes may play a role in transfering the two precursor proteins to the mitochondrial outer membrane.

The import of both precursor proteins into mitochondria was studied with an in vitro system, consisting of the translation mixture and mitochondria isolated from Chinese hamster ovary cells. The precursor of malate dehydrogenase, synthesized in vitro, could be

imported into isolated mitochondria in the absence of protein synthesis. The import was accompanied with the conversion of the larger precursor to the mature monomeric size. The newly processed precursor, in mitochondria, was insensitive to externally added protease, suggesting that the protein had been translocated into at least the inter-membrane space, if not the matrix. The proteolytic cleavage was arrested by the chelator, o-phenanthroline, indicating the protease responsible for the conversion requires metallic cations as a cofactor. Time course studies showed that import and processing of the precursor was completed within 10 to 30 min at 30°C and the precursor was converted to the mature size by a single cleavage. Import was found to depend on the electrochemical potential across the inner membrane, as shown by the inhibitory effect of the uncoupler, carbonyl cyanide m-chlorophenylhydrazone.

The same in vitro import system was used to investigate the import of the precursor of the rat liver adenine nucleotide carrier. Although processing of the precursor to its mature size was not observed, there were indications that the precursor was imported into mitochondria: (1) a time-dependent transfer of the precursor from the incubation solution to mitochondria was observed, and the process was greatly reduced in the presence of an uncoupler. (2) Unlike the precursor in the incubation solution, the precursor immunoprecipitated from the mitochondrial fraction was protease insensitive. The reasons for the lack of processing of the imported precursor in the in vitro system is not known. However, the results suggest that proteolytic processing of the precursor is not an obligatory step in its import.

The success in establishing an *in vitro* system in which the processing of the precursor of rat liver malate dehydrogenase occurred, allowed probing of the details of the import process. In the presence of an uncoupler, binding, but not processing, of the precursor to mitochondria was observed. Further experiments showed that the binding process fulfils most of the criteria for a genuine ligand-receptor interaction. It is temperature insensitive and completed very rapidly at 0°C. The binding process is not inhibited by the presence of an uncoupler. The bound precursor is protease sensitive, indicating that its putative receptor is located on the mitochondrial outer membrane. An apparent saturation of the binding sites was observed with a fixed amount of mitochondria and excess amount of *in vitro* synthesized proteins. Most importantly, it was observed that processing of bound precursor was independent of the volume of reaction mixture, suggesting that bound precursor was directly processed to its mature size. In contrast, processing of unbound precursor was dependent on reaction volume. These results suggest that binding of the precursor of malate dehydrogenase to the mitochondrial outer membrane is an intermediate step in the import process.

Mitochondrial malate dehydrogenase is a NAD^+-dependent dehydrogenase. The enzyme has been purified to homogeneity from different sources by the affinity column, 5'-AMP-Sepharose. However, the precursor of rat liver malate dehydrogenase synthesized *in vitro* did not bind to the affinity column, indicating the absence of a NAD^+-binding

site on the precursor protein. After conversion to the mature size by isolated mitochondria, the processed protein bound to the affinity column and was eluted with the endogenous enzyme by 40 uM NADH. These results strongly indicate that processing of the precursor to its mature size is followed by conformational changes resulting in the formation of a functional NAD^+-binding site. This conclusion is supported by studies with antisera prepared against the sodium dodecyl sulphate denatured enzyme, which are conformationally specific. The antiserum recognized only the sodium dodecyl sulphate denatured mature protein and the *in vitro* processed precursor protein, but not the native functional enzyme. However, the antiserum could immunoprecipitate the precursor protein with or without prior denaturation. The above results suggest that the *in vitro* imported precursor protein has been converted into a functional enzyme, and indicate that the *in vitro* import system reflects the process that occurs *in vivo*.

ACKNOWLEDGEMENTS

I am deeply indebt to the following people for their contribution to this investigation and the preparation of this thesis:

Dr. K.B. Freeman for his invaluable guidance and critical evaluations throughout my research, and his patience in the preparation of this thesis.

Drs. S.T. Bayley and G.E. Gerber for their advice and discussions.

Ms. H. Patel for her excellent technical assistance.

Ms. L. Aziz, Mr. T. Ho, Drs. J.R. Mason and R.W. Yatscoff for their assistance and suggestion.

I also wish to acknowledge the financial support of the Province of Ontario, the Medical Research Council of Canada, and McMaster University.

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ABBREVIATIONS

AMP	adenosine monophosphate
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
CAT	carboxyatractyloside
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CHO	Chinese hamster ovary
cpm	count.minute^{-1}
Da	dalton
DNA	deoxyribonucleate
EDTA	ethylene-diamine-tetraacetic acid
EGTA	ethylene-glycol-tetraacetic acid
GTP	guanosine triphosphate
HCl	hydrochloric acid
Hepes	4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid
HTP	hydroxylapatite
KCl	potassium chloride
kDa	kilodalton
kg	kilogram
mCi	millicurie
MEM	minimal essential medium
$MgCl_2$	magnesium chloride
mM	millimolar

mmol	millimole
mRNA	messenger ribonucleate
MSH	2-mercaptoethanol
mtDNA	mitochondrial deoxyribonucleate
NaCl	sodium chloride
NAD^+	nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide
NEM	N-ethyl-maleimide
pMDH	precursor of malate dehydrogenase
RER	rough endoplasmic reticulum
SDS	sodium dodecyl sulphate
SRP	signal recognition particle
TCA	trichloroacetic acid
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleate
u	μ-micro
w/v	weight per volume
v/v	volume per volume

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INTRODUCTION

1.1 THE MAIN THEME

The discovery of deoxyribonucleic acid in mitochondria (Nass and Nass, 1963a,b) created a new phase in the study of mitochondrial biogenesis. What was the origin of the mitochondrial genome? What information is stored in this heritable genetic material? Is there any communication between the two physically separated genomes, nuclear DNA and mitochondrial DNA (mtDNA), within an eukaryotic cell? Solutions to these questions would not only increase our knowledge on the molecular aspect of cell formation, but also, would shine light on the origin of the eukaryotic cell.

Two major hypotheses were put forth to explain the original source of mitochondrial genome. Raff and Mahler (1972, 1975) proposed that the genome could have arisen as an episome from nuclear DNA and be subsequently packaged into a membranous vesicle. Alternatively, the genome could have originated from a bacterium which was incorporated into a proto-eukaryotic cell and established a symbiotic relationship with the host. This hypothesis was popularized by Margulis (1970, 1975).

The striking similarity between the bacterial and mitochondrial protein synthesizing systems supports the endosymbiotic hypothesis. Both systems use formyl-methionine, instead of methionine, in the initiation of

translation (Smith and Marcker, 1968) and the peptide bond formation is sensitive to chloramphenicol (Kalf, 1963; Kroon, 1963). However, the prokaryotic origin of mtDNA was challenged by the facts that mitochondrial genomes have unique genetic codes (eg. Barrell et al., 1979) that require, in the case of vertebrates, only 22 tRNAs for decoding (Anderson et al., 1981) and the lack of homology between many, but not all, mitochondrial and bacterial rRNAs sequences (Kuntzel and Kochel, 1981; Gray et al., 1984). The issue is further complicated by the finding of the transfer of genetic material between mitochondrial and nuclear genomes (Farrelly and Butow, 1983; Wright and Cummings, 1983). At present, the endosymbiotic hypothesis is still the favoured among the two hypotheses mentioned previously.

Development of new biochemical techniques, including DNA sequencing methods and recombinant DNA technology have helped enormously in the unravelling of the organization of mammalian mitochondrial genomes. Complete DNA sequences of mouse, bovine and human mtDNA are now available (Anderson et al., 1981, 1982; Bibb et al., 1981). All three circular genomes are about 1×10^7 daltons in size, and are highly compact in their organization. No intervening sequence has been identified in the structural gene coding regions. The majority of the genome codes for rRNAs and tRNAs, with only 6% of the coding region for mRNAs. The transcriptional products include the 12S and 16S rRNAs, 22 tRNAs and mRNAs for cytochrome c oxidase subunits I, II and III, ATPase subunit 6 and cytochrome b (Anderson et al., 1981). There are also coding regions called unidentified reading frames, because their protein products were not known

(Anderson et al., 1981). During the last few years, proteins encoded by some of these unidentified reading frames have been identified. For instance, in humans, six of the unidentified reading frames code for the components of the respiratory chain NADH dehydrogenase (Chomyn et al., 1985).

The size of the mitochondrial genomes of Saccharomyces cerevisiae and Neurospora crassa are about 5 to 6 times larger than that of human (Anderson et al., 1981). The larger size is not because of an increase in coding capacity for proteins of the mitochondrial inner membrane, but is due to the presence of intervening sequences within some genes and of AT rich sequences between genes (see review by Dujon, 1983). In yeast, more than 20 unidentified reading frames have been identified, most of them are located in introns (Bonitz et al., 1980). One of the unidentified reading frames was found to code for subunit 8 of ATPase (Macreadie et al., 1983; Velours et al., 1985). Similarly, six unidentified reading frames in Neurospora crassa code for subunits of NADH:ubiquinone reductase (Ise et al., 1985). Some of the unidentified reading frames located in introns code for proteins involved in the splicing of mitochondrial mRNAs (Lazowska et al., 1980; Guiso et al., 1984). The mitochondrial genome of Neurospora has two unique features. It has a very high content of AT rich palindromes, up to 5-10% of the total genome, which may be involved in RNA processing (Yin et al., 1981; Burke and RajBhandary, 1982). Another unique feature is the presence of a sequence that is very similar to the coding sequence of subunit 9 of ATPase (Boogaart et al., 1982). The protein is coded in the nuclear genome and translated on cytoplasmic

ribosomes in Neurospora (Jäckl and Sebald, 1975).

In its structural aspect, the mitochondrion is a highly compartmentalized organelle, divided into four compartments by two membranes. The four sub-mitochondrial compartments are the outer membrane, the inter-membrane space, the inner membrane and the matrix. Each compartment has its own unique protein composition for carrying out specific functions. For instance, proteins of the electron transport chain and the H^+-ATPase complexes which are essential for the synthesis of ATP are located in the mitochondrial inner membrane. Enzymes for the tricarboxylic acid cycle, fatty acid oxidation and for the transcription of mtDNA and translation of mitochondrial mRNAs are located in the matrix. Overall there are more than three hundred proteins localized in the various compartments of a mitochondrion. However, as mentioned previously, only about thirteen polypeptides are encoded in the mitochondrial genome. This implies that over 90% of mitochondrial proteins, including those essential for mitochondrial replication, are coded in the nuclear genome, synthesized on cytoplasmic ribosomes and imported into mitochondria. How do these mitochondrial polypeptides reach their functional locations in the mitochondrion? The molecular mechanisms by which these polypeptides are transfered to and localized in their specific compartments of a mitochondrion will be the main theme of this thesis.

1.2 MECHANISMS FOR IMPORTING PROTEINS INTO SPECIFIC ORGANELLES

In a eukaryotic cell, the size of the nuclear genome is about 2 x 10^6 times larger than the mitochondrial genome. Proteins encoded in the nuclear genome are synthesized on cytoplasmic ribosomes and incoporated into various organelles, the plasma membranes, or remain in the cytoplasm. Some proteins are secreted extra-cellularly. Each organelle has its own unique protein composition and can be identified by specific marker enzymes. This suggests that the processes by which these proteins are segregated and localized in their respective organelles have to be very specific. During the past years, an effort has been made to elucidate the molecular mechanisms of these processes.

Conceptually, the localization of a polypeptide into a specific organelle can be divided into three concatenated steps: (1) translation of the mRNA of the polypeptide on cytoplasmic ribosomes; (2) transport to, and translocation of the polypeptide into or across the membrane that delineates a specific organelle; (3) transformation of the translocated polypeptide into a functional protein. The last step likely involves conformational changes of the polypeptide and/or assembly with other polypeptides to form a functional multimeric enzyme.

The key issue in the mechanism of protein localization is, however, the specific uptake of cytoplasmically synthesized polypeptides into a particular organelle. Each organelle is delineated by membranes with unique proteins and phospholipids, such as the mitochondrial outer

membrane, the porous nuclear membrane, and the membrane of endoplasmic reticulum. These membranes are the gateway for entry into specific cellular compartments. Thus, translocation of polypeptides into or across these specific membranes is the most likely point in the localization process where specificity is conferred, i.e. only polypeptides destined for a specific organelle are translocated across the organellar membrane(s). However, how are polypeptides transported to their specific sites of translocation? How is the specificity in translocation achieved? What initiates the translocation process? Resolving these questions is essential in the understanding of the mechanism of localizing proteins into a specific organelle.

1.2.1 Protein Localization with Co-translational Translocation

Research on secretory proteins has given considerable insight into how specificity in translocating polypeptides across an organellar membrane can be achieved. Secretory proteins are synthesized on polysomes that are bound to the membranes of the RER (Siekevitz and Palade, 1960), segregated into the lumen of the organelle (Redman et al., 1966), and eventually secreted through secretory vesicles which arise from the Golgi apparatus (Jamieson and Palade, 1967a,b).

More interestingly, Redman and Sabatini (1966) showed that incomplete nascent polypeptides of secretory proteins, synthesized in the presence of puromycin, can also be segregated into the lumen of the RER. Furthemore, *in vitro* experiments using dog pancreas microsomes

showed that translocation would not occur if the nascent polypeptides on ribosomes have more than 100 amino acid residues (Blobel and Dobberstein, 1975a,b). These results led to the conclusion that the translocation of secretory proteins across the RER membranes is tightly coupled to the elongation step of their synthesis. This mechanism is refered as the co-translational translocation or vectorial translation.

The Signal Hypothesis

What is the mechanism that allows specific translocation of secretory proteins into the RER lumen? How is the translocation step coupled to the synthesis of these proteins? To answer these questions, Blobel and Sabatini (1971a,b) proposed the signal hypothesis. It was later revised by Blobel and Dobberstein (1975a,b). There are two essential features in the hypothesis: (1) the presence of a unique peptide at the amino terminus of the nascent secretory protein, which distinguishes it from other proteins in the cytoplasm. This peptide is essential in binding the polypeptide growing on a free ribosome to the membrane of the RER, thus committing the polypeptide to the secretory pathway. In essence, this peptide is the signal that confers the specificity to the translocation process. The signal peptide is usually not required for the normal functioning of the mature protein and is removed after the polypeptide has entered the lumen of RER. (2) The translocation process is a receptor-mediated event. It was postulated that there is specific receptor on the RER membrane which recognized the signal peptide. The interaction of the signal peptide with its receptor

would lead to the formation of a hydrophilic channel for the growing polypeptide to enter the lumen of the RER.

The signal hypothesis, at present, has been widely accepted with compelling evidence. The N-terminal signal peptide was first identified on the light chain of immunoglobulins (Milstein et al., 1972) and has subsequently been found on all eukaryotic secretory proteins so far examined (see review by Watson, 1984), with the possible exception of ovalbumin, whose signal resides in an internal sequence of the amino-half of the protein (Braell and Lodish, 1982). These peptides are usually 15 to 30 amino acid residues in length, and can compete with one another for sequestration into microsomal vesicles. However, no sequence homology is found among the known peptides (Inouye et al., 1977; Steiner et al., 1980). From the comparison of the known signal peptides, four common physical features are found and may play a role in the binding of the growing polypeptide to the RER membranes: (1) the presence of one or two charged amino acids in the initial segment of the peptide (Inouye, 1982; Vlasuk, 1983). (2) The central region of the peptide is always occupied by a stretch of at least nine hydrophobic amino acids (von Heijne, 1981,1982; Emr & Silhavy, 1983; Finkelstein, 1983). (3) There is usually an alpha helix in the central hydrophobic segment and a beta turn near the cleavage site (Austen, 1979). (4) The cleavage site is usually flanked on both sides by a small uncharged amino acid (Inouye and Halegona, 1979; von Heijne, 1983,1984).

Walter et al. (1981a,b,c) and Meyer et al. (1982) provided

conclusive evidence for the hypothesis that the translocation process is a receptor mediated event. Walter and Blobel (1980) purified a membrane associated protein complex from an extract of RER membranes. This complex, the signal recognition particle (SRP), is about 250,000 daltons in size and consists of six distinct polypeptides and a 7S RNA molecule (Walter and Blobel, 1982). In vitro experiments showed that the SRP binds specifically to ribosomes synthesizing secretory proteins (Walter et al., 1981a), and arrests the translation of the mRNAs of these proteins in the absence of microsomal membranes (Walter and Blobel, 1981b). However, with the addition of microsomal vesicles, it mediates the binding of the SRP-ribosomal complex to the receptor on the membranes with the resumption of protein synthesis and translocation of the polypeptides into microsomal vesicles (Walter and Blobel, 1981c). Using a SRP-affinity column, Meyer et al. (1982) isolated a 72,000 daltons integral membrane protein from an extract of microsomal membranes. This protein, named the SRP receptor or the docking protein, interacts specifically with the SRP and releases the elongation arrest caused by the particle. The release is followed by the translocation of the growing polypeptide across the microsomal membrane.

The yet unresolved question in the mechanism of vectorial transfer is how the interaction of the SRP-ribosomal complex with the docking protein results in the translocation of the polypetide across the RER membranes. Is the interaction self-sufficient for the translocation process as postulated by von Heijne (1981) or does it require other RER membrane proteins, such as ribophorins I and II

(Kreibich et al., 1978a,b; 1980), as proposed in the original signal hypothesis (Blobel and Sabatini, 1971a,b)?

The Post-Translational Sorting Mechanisms

Many proteins, other than secretory proteins, are also synthesized on membrane-bound polysomes and co-translationally translocated into or across the RER membranes. These include proteins destined for plasma membranes (Rothman and Lodish, 1977; Dobberstein et al., 1979; Ploegh et al., 1979), lysosomes (Hasilik et al., 1980), and RER membranes (Swank and Paigen, 1973). However, the fact that the RER is an initial common pathway for the transport of precursor proteins to several cellular compartments necessitates the presence of additional signals for sorting these proteins into their final locations after the translocation process.

Studies on the biosynthesis of the heavy chain of immunoglobulins showed how transmembrane proteins synthesized on bound polysomes can be anchored in the RER membranes and be transfered subsequently to the plasma membranes via the Golgi apparatus. This protein has secreted and membrane-bound forms (Melcher and Cone, 1973). Both are encoded by the same structural gene. However, because of a difference in mRNA splicing, the polypeptide of the membrane bound form has an additional segment of hydrophobic amino acids at its carboxyl terminus (Alt et al., 1980; Early et al., 1980; Kehry et al., 1980; Roger et al., 1980). This segment serves as a halt signal for the

transfer of the polypeptide across the membrane. A similar hydrophobic transmembrane domain has also been identified in the primary sequence of the well studied G protein of vesicular stomatitis virus (Chatis and Morrison, 1970; Ghosh, 1980; Rose and Gallione, 1981; Rose and Bergman, 1982,1983). Recently, Guan and Rose (1984) showed that a hybrid protein of growth hormone with this hydrophobic peptide resulted in the anchorage of the protein in the Golgi apparatus.

The G protein of vesicular stomatitis virus traverses the plasma membrane only once. However, many membrane proteins, such as the erythrocyte anion transport protein (Drickamer, 1976), span the plasma membrane several times. It was postulated that the transmembrane peptides of these proteins, due to their hydrophobic nature, are co-translationally inserted into the RER membranes (Blobel, 1980; Sabatini et al., 1982).

For lysosomal enzymes, evidence has indicated that the post-translational addition of mannose-6-phosphate to the oligosaccharide chain of the nascent polypeptides is essential in their proper localization. Lysosomal enzymes lacking the recognition signal, mannose-6-phosphate, are functional but are misdirected out of cells (Hickman et al., 1974; Kaplan et al., 1977; Fischer et al., 1980). Furthermore, extra-cellular lysosomal enzymes can be internalized by human fibroblasts and Chinese hamster ovary cells through receptors that recognize the 6-phosphomannosyl residue (Rome et al., 1979; Hasilik and Neufeld, 1980a,b). The internalized lysosomal enzymes are channelled to

the Golgi complex via clarithin coated vesicles (Willingham et al., 1981a,b) and subsequently into lysosomes via the Golgi-endoplasmic reticulum-lysosomal region (Novikoff, 1980). Thus, different mechanisms are used to segregate precursor proteins in the RER to various cellular compartments.

The Transfer of Proteins from the RER to Plasma Membrane

After localization in the RER, newly synthesized secretory and plasma membrane proteins are transported, via the Golgi apparatus, to the plasma membrane (see reviews by Sabatini et al., 1982; Silhavy et al., 1983). Vesicles are used to transport these proteins from the RER to the Golgi apparatus, and from the Golgi to the plasma membranes (Palade, 1975). Little is known about the mechanism that regulates this intra-cellular protein transportation, and whether the tranport vesicles are clarithin coated remains controversial (Rothman et al., 1980; Wehland et al., 1982).

Initially, it was hypothesized that secretory proteins are non-selectively transported to the cell surface at the same rate (Sturgess et al., 1978). However, recent evidence indicates that some secretory proteins are shuttled between the organelles at different rates and are not secreted extra-cellularly with the same kinetics (Ledford and Davis 1983; Lodish et al., 1983; Fries et al., 1984). Furthermore, Fitting and Kabatt (1982) showed that two viral membrane glycoproteins are transported to the cell surface at different rates.

These results strongly suggested that the transfer of secretory and membrane proteins from RER to the plasma membrane is a specific, probably receptor-mediated, process.

Many secretory proteins and plasma membrane proteins, unlike cytoplasmic proteins, are glycosylated after their synthesis. It was shown that tunicamycin, an antibiotic that inhibits the N-glycosylation of nascent polypeptides, prevents the secretion of immunoglobulins A and E from plasma cells (Hickman et al., 1977). Furthermore, the regular transport of the G protein of vesicular stomatitis virus from the RER to cell surface requires the N-addition of the carbohydrate moiety (Gibson et al., 1978). However, not all secretory proteins are gylcosylated, such as albumin (Peters, 1971), and Struck et al. (1978) showed that the secretion of four serum glycoproteins by cultured hepatocytes was not interfered with by the presence of tunicamycin. These results suggest that although post-translational glycosylation may be important in the secretion of immunoglobulins, its role in the intra-cellular transport of secretory and membrane proteins cannot be generalized.

It has been demonstrated that certain viral glycoproteins undergo post-translational fatty acid acylation (Schmidt, 1982). The G protein of vesicular stomatitis virus is one of the examples. Zilbestein et al. (1980) reported that the G-protein of a temperature-sensitive mutant of the virus did not reach the cell surface, but instead, accumulated in the Golgi complex. The defective G protein lacks the fatty acid residue, suggesting that post-translational

acylation may play a role in the transport of the G protein to the plasma membrane. However, subsequent studies showed that the defective G protein could be detected on the cell surface of infected cells (Lodish and Kong, 1983). Furthermore, Schlesinger and Malfer (1982) showed that although blockage of acylation by cerulenin would inhibit the formation and release of viral particles from cells infected with vesicular stomatitis virus and Sindbis virus, the non-acylated viral membrane proteins were transported to the cell surface. These results indicate that fatty acid acylation is not essential in the intra-cellular transport of these viral proteins.

Recently, Adams and Rose (1985) found that G protein which has its transmembrane peptide trimmed from 20 amino acids to less than 12 residues was accumulated in the Golgi complex and did not reach the plasma membrane. This result indicates that the primary structure of G protein plays a role in regulating the intra-cellular transport of the protein.

1.2.2 Protein Localization with Post-translational Translocation

Localization of proteins into specific organelles in eukaryotic cells do not always begin with co-translational translocation of the proteins into or across the RER membranes. Many proteins bind to and are translocated across specific organellar membranes after synthesis (Kreil, 1981). These include cytochrome b_5 and its reductase of the RER membranes (Borgese and Gaetani, 1980; Rachubinski et al., 1980), uricase

and catalase of the peroxisomes (Goldman and Blobel, 1978; Miura et al., 1984; Rachubinski et al., 1984), and proteins destined for the semi-autonomous organelles, such as chloroplasts (Highfield and Ellis, 1978; Chua et al., 1980; Schmidt et al., 1981) and mitochondria (Neupert & Schatz, 1981; Schatz & Butow, 1983; Hay et al., 1984). The mechanisms of post-translational translocation are still relatively unknown, and several hypothesis have been proposed.

The Model of Spontaneous Insertion

Integral membrane proteins, such as cytochrome b_5 and NADH-cytochrome b_5 reductase, have a multi-compartment distribution, including the RER, mitochondria and the Golgi apparatus (Oshino, 1978). Both proteins also share the common feature that there are several segments of hydrophobic sequences at the carboxyl terminus of the polypepide chains (Ozoli and Gerard, 1977). These proteins are synthesized on free polysomes without an extension peptide, and incorporated into the membranes post-translationally (Rachubinski et al., 1980). *In vivo* pulse-labeling experiments showed that NADH-cytochrome b_5 reductase is inserted into the various membranes simultaneously, eliminating the possibility that the multi-compartment distribution is due to segregation from a common origin. Furthermore, trypsinization of the membranous fraction did not interfere with the integration of these proteins into the lipid bilayer. Based on these studies, it was postulated that the hydrophobic segment at the C-terminus may act as a post-translational insertion sequence for the

spontaneous incorporation of the two proteins into the various membranes without the mediation of a proteinaceous receptor.

The Membrane Trigger Hypothesis

Studies on the major coat protein of the coliphage M13 led Wickner (1979, 1980) to propose the membrane trigger hypothesis as an alternative mechanism by which proteins with a signal peptide could be inserted into hydrophobic membranes. This protein is an integral membrane protein and is synthesized with a 23-amino acid extension peptide at its N-terminus. However, the precursor protein is integrated into the inner membrane of Escherichia coli post-translationally (Wickner et al., 1978). According to the hypothesis, the function of the N-terminal extension peptide is to trigger conformational changes of the precursor protein when a hydrophobic surface is encountered, resulting in the insertion of the polypeptide into the membrane. The subsequent removal of the extension peptide renders the process irreversible. However, proteins with signal peptides that are post-translationally translocated across the RER membranes have not yet been identified in eukaryotic cells

Import of Proteins into Semi-Autonomous Organelles

There is evidence to suggest that proteins destined for chloroplasts and mitochondria are imported into the organelles post-translationally (Nelson and Schatz, 1979). For instance, the small

subunit of ribulose-1,5-bisphosphate carboxylase in chloroplasts is synthesized on membrane-free polysomes with an amino-terminal extension peptide, and is incoporated into the organelle after the completion of its synthesis (Highfield and Ellis, 1978).

Mitochondria have their own protein synthesizing system. However, more than 90% of mitochondrial proteins are encoded in the nuclear genome (Schatz and Mason, 1974). There is ,so far, no evidence that there is exchange of mRNAs between the nuclear-cytoplasmic and mitochondrial compartments. This indicates that most of the mitochondrial proteins are synthesized on cytoplasmic ribosomes, and then incoporated into the organelle. In vivo pulse-labeled experiments showed that the precursors for the α and β subunits of the ATPase complex in yeasts were imported into mitochondria after a lag (Schatz, 1979). Furthermore, in vitro experiments showed that the uptake of the precursor proteins into mitochondria is not inhibited by cycloheximide. These observations clearly showed that the import process is independent of protein synthesis, suggesting a post-translational mechanism.

1.2.3 Import of Proteins into Mitochondria

The mechanism by which a subset of nuclear-coded proteins are specifically imported into mitochondria has been a major focus in the study of mitochondrial biogenesis during the last few years. Research effort has been concentrated in the following three areas: (1) identifying the physical features of the precursor proteins and the

components on the mitochondrial membranes that are responsible for the specificity of the import process, (2) the details of the translocation process and (3) the mechanisms by which imported proteins are sorted into their respective sub-mitochondrial compartments. Extensive research has been done in Saccharomyces cerevisiae, Neurospora crassa and, recently, in the mammalian system (see reviews by Schatz and Butow, 1981; Neupert and Schatz, 1983; Hay et al., 1984). Studies mainly on the former two organisms will be reviewed in the following, with appropiate papers after the beginning of 1985 being included in the Conclusion.

(A) The Specificity of The Import Process

Physical Features of the Nascent Polypeptides--Most of the mitochondrial precursor proteins, which are synthesized on cytoplasmic ribosomes and destined for the three inner mitochondrial compartments, have an apparent molecular mass larger than their corresponding mature proteins. In cases where the primary structures of the nascent polypeptides are known, such as the proteolipid subunit of the Neurospora ATPase (Viebrock et al.,1982), the additional peptides are located at the amino terminus. It is likely that the extension peptides of the other precursor proteins are located at the same position.

The size of the extension peptide varies widely among the known precursor proteins. It ranges from 0.5 kDa in subunits I and II of the cytochrome bc_1 complex of yeast (Côté et al., 1979) to 10 kDa in δ-aminolevulinate synthase of chicken (Ades and Harpe, 1981).

Furthermore, except for proteins of the outer membrane, no apparent correlation has been observed between the size of the extension peptides and the final locations of the mature proteins (Hay et al., 1984). Even subunits of the same enzyme complex, such as the cytochrome bc_1 complex of Neurospora crassa (Teintze et al., 1982) or cytochrome c oxidase of yeast (Gasser et al., 1982b), have extension peptides of different sizes. Inter-species variation in the size of the extension peptide have also been reported for the matrix enzyme, carbamoyl phosphate synthetase. In rat, the precursor of the enzyme is about 5 kDa larger than the mature form (Mori et al., 1981; Raymond and Shore, 1981; Campbell et al., 1982). However, the precursor of the enzyme in frog has the same size as the mature protein (Mori at al., 1979).

Not all mitochondrial proteins imported from the cytoplasm are synthesized with an extension peptide. Virtually all precursors of outer membrane proteins studied, with one possible exception (Shore et al., 1981), have the same apparent molecular mass as the mature proteins (Freitag et al., 1982; Sagara et al., 1982; Gasser and Schatz, 1983). Although it is uncommon, precursors without an extension peptide have also been observed in proteins located in the other three mitochondrial compartments. These include inner membrane proteins, the adenine nucleotide translocator of Neurospora (Zimmermann et al., 1980) and the uncoupling protein of the rat brown adipose tissue (Freeman et al., 1983; Ricquier et al., 1983), inter-membrane space proteins, cytochrome c of Neurospora and rat (Zimmermann et al., 1979a; Matsuura et al., 1981) and adenylate kinase of yeast (Watanabe and Kubo, 1982), and a

matrix enzyme, 2-isopropyl malate synthase of yeast (Gasser et al., 1982b, Hampsey et al.,1983).

The diversity in the sizes of the extension peptides prompted a search for other common features among the known nascent polypeptides. Comparing the two dimensional electrophoretic patterns of polypeptides from human lymphoid cells in the presence or absence of anti-mitochondrial agents, Anderson (1981) identified a group of mitochondrial nascent polypeptides that have higher isoelectric points than their mature counterparts. He concluded that the increased basicity is due to the presence of 4 to 8 additional lysine or arginine residues in the precursor proteins. This physical feature was later found in the first three cases where the complete amino acid sequences of the nascent polypeptides were determined. These include an inner membrane protein, the proteolipid subunit of the Neurospora ATPase (Viebrock et al., 1982); an inter-membrane space protein, cytochrome c peroxidase of yeast (Kaput et al., 1982), and a matrix protein, ornithine transcarbamylase of humans (Horwich et al., 1984). The amino-terminal extension of all three nascent polypeptides have at least four lysine or arginine residues. The residues have a tendency to appear in groups of two, which are separated by three amino acids at the most. The fact that these three proteins are from three different mitochondrial compartments suggests that this physical feature plays a definite role in the import process. On the other hand, it was reported that the precursor of aspartate aminotransferase has a lower isoelectric point than its mature protein (Kamisaki et al., 1982).

The fact that the extension peptide is removed after the precursor protein is translocated into mitochondria (see below) indicates that its function is limited to the import process. It may serve as recognition signal for the receptor on the mitochondrial membranes, maintain a conformation favourable for the translocation of nascent polypeptide across the mitochondrial membrane, or facilitate the solubility of hydrophobic precursor protein in the cytoplasm. Judging from the diversity in the size as well as the physical properties, it is possible that an extension peptide may have more than one of the above functions, depending on the physical nature, such as hydrophobicity and size, of the nascent polypeptide and the final location of the mature protein. Furthermore, the size of an extension peptide may be proportional to the number of functions it performs. During the preparation of this thesis, several studies showed that extension peptide is essential in targeting several mitochondrial proteins into mitochondria. This will be discussed in more detail in the Conclusion.

As mentioned previously, some imported proteins, including almost all outer membrane proteins, are not made as larger precursor. Whether these proteins have permanent targeting signals that reside in their primary structures remains to be elucidated. Recently, the gene coding for a 70 kDa outer membrane protein of yeast has been cloned (Hase et al., 1983). Its sequence showed there is a stretch of hydrophobic amino acids near the amino terminus, which is flanked on both sides by several basic residues. This segment of peptide may serve

as a membrane anchorage for the protein in the mitochondrial outer membrane (Riezman et al., 1983a). Furthermore, a shortened form of this protein, which has only the amino half of the whole polypeptide, can still be inserted into the outer membrane (Riezman et al., 1983b). This result suggests that the targeting signal of this protein is located in a specific region of its primary structure.

Receptors on the Mitochondrial Membranes: Import of proteins into mitochondria is a very specific process. Only nascent polypeptides destined for mitochondria are translocated into or across the organellar membranes, and these polypeptides do not integrate into other subcellular membranes (Matsurra et al., 1981; Gasser and Schatz, 1983). This stringent specificity requires the presence of specific components on the mitochondrial outer surface for recognizing these mitochondrially targeted polypeptides.

The most convincing evidence indicating the presence of receptors on mitochondrial membranes comes from the studies on apocytochrome c of Neurospora. Apocytochrome c is the precursor of cytochrome c, a protein which is located on the cytoplasmic side of the mitochondrial inner membrane. The precursor has the same apparent size as the mature protein, and the conversion to holocytochrome c is accomplished with the covalent attachment of the heme group (Zimmermann et al., 1979b; Korb and Neupert, 1981). Hennig and Neupert (1981) observed that radiolabeled, in vitro synthesized apocytochrome c bound tightly to isolated mitochondria when the conversion to holoenzyme was

inhibited by deuterohemin, a competitive inhibitor of the heme-attachment step. The binding is reversible and highly specific. It can be competitively decreased by an excess of cold apocytochrome c, but not the mature enzyme. Furthermore, there is a limited number of these binding sites on mitochondria, being approximately 90 pmol/mg of mitochondrial protein (Hening et al., 1983). Mitoplasts, which lack the mitochondrial outer membrane, were also incapable of binding the precursor protein. These results showed definitively the presence of a specific type of receptor on the mitochondrial outer membrane for apocytochrome c.

Similar approaches have also been used to identify the binding sites for apocytochrome c on rat liver mitochondrial membranes. Matsurra et al. (1981) showed that the post-translational uptake of in vitro synthesized rat liver apocytochrome c into isolated mitochondria could be inhibited by an excess of horse heart apocytochrome c, but not the mature heme-containing enzyme. More importantly, they showed that the binding of the newly synthesized precursor can be inhibited by a peptide fragment of the horse heart apocytochrome c, indicating that the uptake of apocytochrome c into mitochondria is mediated through receptors that recognize a specific structural feature on the nascent polypeptide.

In contrast to the studies on apocytochrome c, there is only indirect evidence indicating that import of proteins into the three inner mitochondrial compartments is also mediated through specific

receptors. In Neurospora, mild trypsin treatment of isolated mitochondria abolished the binding and uptake of the adenine nucleotide carrier precursor and the import of the precursors of subunits 2 and 9 of ATPase into the organelles (Zwizinski et al., 1984). Furthermore, there is speculation that different receptors may be present on the outer membrane for importing different proteins because excess apocytochrome c would not inhibit the import of the adenine nucleotide carrier or subunit 9 of ATPase into mitochondria (Zimmermann et al., 1981). However, detailed analysis of these receptors has not been feasible because of the difficulty in obtaining large quantities of cold precursor proteins. Apocytochrome c is a unique case, because the nascent polypeptide can be prepared from the mature enzyme by chemical removal of the heme group.

In yeast, it was shown that the in vitro import of cytochrome b_2 (Gasser et al., 1982a) and the β subunit of the F_1-ATPase (Riezman et al., 1983b) require a protease sensitive component on the mitochondrial outer membrane. However, high affinity binding sites for mitochondrial precursor proteins have yet to be identified in yeast. Subunit 2 of ATPase and cytochrome b_2 have been shown to bind to mitochondrial membranes but the binding is very poor when import is inhibited by an uncoupler (Riezman et al., 1983b). Similar results have also been observed for subunits VI and VII of the Neurospora cytochrome c oxidase complex (Tenitze et al., 1982), and there is a postulate that the binding may be linked to the presence of the membrane potential across the mitochondrial inner membrane (Zwizinski et al., 1984). In

mammals, a proteinaceous component on the mitochondrial outer membrane is also required for the import of rat liver ornithine transcarbamylase (Argan et al., 1983).

At present, it is not clear whether the import of outer membrane proteins is mediated through proteinaceous receptors. In yeast, mild proteolytic treatment did not interfere with the subsequent uptake of these proteins into mitochondria (Hay et al., 1984). However, the import process is very specific. These precursors only integrate into mitochondria or vesicles of mitochondrial outer membranes, but not other subcellular membranes (Gasser and Schatz, 1983). In Neurospora, there is evidence that the import of a major outer membrane protein, porin, is mediated through a receptor because its import is impaired if mitochondria are pretreated with mild trypsin or elastase (Zwizinski et al., 1984).

In secretory proteins, the binding of the growing peptide to the RER membranes is mediated through the signal recognition complex (Walter and Blobel, 1980). The finding that import of some mitochondrial proteins is a receptor-mediated event raised the question whether there is a mediator in the interaction between mitochondrially destined nascent polypeptides and their receptors? Recently, it has been shown that the in vitro import of rat ornithine transcarbamylase into isolated mitochondria requires the presence of a cytosolic factor (Argan et al., 1983, Miura et al., 1983). Similarly, Ohta and Schatz (1984) found that the uptake of the proteolipid subunit of yeast ATPase requires a

cytosolic protein factor. At present, the function of these cytosolic factors is not known. However, it is possible that they may play a role in the binding or the transport of mitochondrial precursors to receptors on the mitochondrial outer membrane.

(B) The Mechanisms of Importing Proteins into Specific Sub-Mitochondrial Compartments

Although there is evidence indicating that nuclear-coded mitochondrial proteins are synthesized on membrane-free polysomes and transported into mitochondria after their synthesis, there is still much to learn about how they are imported into their specific mitochondrial compartments. An in vitro uptake system is used to investigate the import process. In these experiments, radiolabeled in vitro synthesized precursor proteins are incubated with freshly isolated mitochondria under various conditions. Translocation of polypeptides across mitochondrial membranes would be indicated by the inaccessibility of the nascent polypeptides to externally added protease. In cases where the precursor proteins are larger than the mature proteins, proper proteolytic processing of the precursors to the mature size is another criterion. Results obtained from these experiments suggest that differences exist in the import of mitochondrial nascent polypeptides into respective sub-mitochondrial compartments.

Translocation of Proteins into Matrix and Mitochondrial Inner Membranes

Requirement of an electrochemical potential—Import of proteins into the mitochondrial inner membrane and the matrix requires the insertion or the translocation of nascent polypeptides into or across the mitochondrial inner membrane. Earlier studies in yeast spheroplasts showed that the import and processing of the precursors to the α, β, and δ subunits of F_1-ATPase was inhibited in the presence of the uncoupler, CCCP (Nelson and Schatz, 1979). In vivo studies showed that most mitochondrial precursors have very short half-lives and small cytoplasmic pools (Jaussi et al., 1981; Mori et al., 1981). For instance, the half-life of the precursor of the β-subunit of F_1-ATPase is less than one minute. However, Reid and Schatz (1982) showed that, in the presence of CCCP, precursors to cytochrome c, the α and β subunits of F_1-ATPase and superoxide dismutase accumulated in the cytoplasm of a yeast petite mutant. These results indicate that an energized membrane is required for translocating precursor proteins across the mitochondrial inner membrane.

With an in vitro uptake system, Gasser et al. (1982b) showed that it is the electrochemical potential across the mitochondrial inner membrane, but not ATP, that is required for the translocation of the β-subunit of F_1-ATPase into mitochondria. Similar results have also been found in the in vitro import of subunit 9 of Neurospora ATPase (Schleyer et al., 1982). Thus, the post-translational translocation of proteins into the two innermost mitochondrial compartments is dependent on the presence of an electrochemical potential across the inner membrane.

Proteolytic processing of larger precursors--Most, but not all, precursor proteins destined for the mitochondrial inner membrane and the matrix are synthesized with an extension peptide. Translocation or insertion of these nascent polypeptides across or into the inner membrane is usually accompanied by the proteolytic processing to the corresponding mature size. In yeast and Neurospora, the protease responsible for the cleavage is located in the matrix, and requires a metallic divalent cation as a cofactor (Bohni et al., 1980 ; Teintze et al., 1982). The enzyme is specific for mitochondrial precursor proteins. It does not act against other proteins in the cytoplasm, or denatured mitochondrial precursors. Its activity is strongly inhibited by chelators, such as 1,10-o-phenanthroline. Recently, the yeast enzyme has been partially purified, and has a molecular mass of about 59 kDa (McAda and Douglas, 1982; Bohni et al., 1983). A similar endopeptidase has also been identified in the matrix of rat liver mitochondria (Miura et al., 1982).

Translocation, but not proteolytic processing, requires the presence of an electrochemical potential--In an in vitro uptake system, the translocation of larger precursors across the inner membrane is concomitant with the conversion of the precusor to its mature size. It is not clear whether the two steps are necessarily coupled, and which step requires an energized membrane. McAda and Douglas (1982) showed that the precursor to the β-subunit of yeast F_1-ATPase can be cleaved to the mature size by the partially purified enzyme, in the absence of

mitochondria. In Neurospora, Zwizinski and Neupert (1983) showed that the integration of the precursors of the β-subunit of F_1-ATPase and subunit 9 of the F_1F_0-ATPase into isolated mitochondria was not impeded by the presence of a chelator. Furthermore, precursors which have the same size as the mature proteins, such as the adenine nucleotide carrier of Neurospora and 2-isopropyl-malate synthase of yeast, also require a membrane potential for insertion into or translocation across the mitochondrial inner membrane (Schleyer et al., 1982; Hampsey et al., 1983). These results led to the conclusion that translocation can occur independent of the proteolytic process, and the electrochemical potential is only needed for the former process.

Translocation of precursors across two unit membranes--As discussed previously, receptors for import are located on the mitochondrial outer membrane. Thus, precursors destined for the inner membrane and the matrix have to traverse both the inner and outer mitochondrial membranes. It is not clear how this is achieved. Schatz (1979) suggested that these precursors are translocated into their sub-mitochondrial compartments at sites where the two membranes are in close contact. However, at present, no concrete evidence is available to support this hypothesis.

Import of Proteins into the Inter-membrane Space

Different mechanisms exist for the uptake of nascent polypeptides into the inter-membrane space of mitochondria. Precursors

which have the same size as the mature proteins can be imported into the compartment in the absence of an electrochemical potential. These include cytochrome c and adenylate kinase of yeast (Zimmermann et al., 1979a; Matsuura et al., 1981; Watanabe and Kubo, 1982).

On the other hand, an energized membrane is necessary for the import of precursors with extension peptides, such as cytochrome b_2 and cytochrome c_1 of yeast (Daum et al., 1982; Gasser et al., 1982a). These precursors are processed to their mature size by two proteolytic steps. The first cleavage occurs in the matrix by the chelator-sensitive protease. This suggests that at least part of the N-terminal extension of the precursor protein penetrates into the matrix before localizing in the inter-membrane space. The first proteolytic cleavage results in a membrane-bound intermediate form, which is subsequently converted to the mature size by an enzyme located in the inter-membrane space. This inter-membrane space enzyme is not sensitive to chelators and can easily be removed by detergents. It is postulated that the final location of the protein, whether bound to the outer surface of the inner membrane (cytochrome c_1) or within the inter-membrane space (cytochrome b_2), depends on whether a portion of the mature protein is inserted into the inner membrane (Hay et al., 1984). More information was obtained from the nucleotide sequence of cytochrome c peroxidase of yeast. The precursor of this inter-membrane space protein is also converted to its mature size by two proteolytic steps (Reid and Schatz, 1982). The N-terminal extension peptide of this protein consists of 68 amino acid residues, with a stretch of 23 apolar residues from the 19 to 42

positions (Kaput et al., 1982). It is postulated that the translocation of the precursor protein across the mitochondrial inner membrane is halted by the hydrophobic segment of the extension peptide. Thus, only the first 18 amino acids penetrate into the matrix where the first cleavage occurs. The cleavage results in the formation of a membrane-bound intermediate form, consisting of the remainder of the pre and mature sequence. Apart from the hydrophobic portion of the extension peptide, all the remaining sequence is exposed in the intermembrane space, where the second proteolytic cleavage occurs.

Import of Proteins into the Mitochondrial Outer Membrane

Aside from the information that most outer membrane proteins are synthesized without an extension peptide, virtually nothing is known about how these polypeptides are integrated specifically into the mitochondrial outer membrane. In yeast, the process does not require a membrane potential, a trypsin sensitive component on the outer membrane, or proteolytic cleavage of the precursor. At present, the possibility that all outer membrane proteins share a common insertion sequence in their primary structures cannot be overlooked. However, how is the specificity of the integration conferred? In future, it will be essential to investigate whether the phospholipid composition of the mitochondrial outer membrane plays a role in the recognition process. It should be noted that in Neurospora, the uptake of porin is mediated through a protease sensitive component on the outer membrane (Zwizinski et al., 1984).

1.3 OBJECTIVES AND APPROACH

As evident from the previous section, research in Saccharomyces cerevisiae and Neurospora crassa have provided much insight on how proteins are imported into mitochondria. However, progress in the mammalian system has not been as rapid as with the Fungi, and differences could well exist between the two systems as exemplified by the difference between the mitochondrial genome of mammals and that of yeast and Neurospora (see review by Dujon, 1983).

Progress in the mammalian system has been greatly hampered by many unsuccessful attempts to demonstrate import of in vitro synthesized precursor proteins, including malate dehydrogenase, by isolated rat liver mitochondria (Shore et al., 1979; Mihara et al., 1982). At the beginning of this project, in vitro uptake of mammalian mitochondrial precursor proteins into mitochondria had not been demonstrated. During the course of this study (up to the publication of our results), in vitro import of only a few precursors of mammalian matrix proteins were reported. These include precursors of ornithine transcarbamylase (Mori et al., 1980a,b; Conboy & Rosenberg, 1981; Argan et al., 1983; Fenton et al., 1984), carbamoyl phosphate synthase (Campbell et al., 1982), the β-subunit of propionyl coenzyme A carboxylase (Kraus et al., 1983), glutamate dehydrogenase (Miralles et al., 1983), and the uncoupling protein of brown adipose tissue (Freeman et al., 1983). However, only the import of the precursor of ornithine transcarbamylase has been well characterized, and the intermediate steps in the import process are poorly understood. In

addition, in vivo processing of the precursor of carbamoyl phosphate synthase was demonstrated by Raymond and Shore (1979, 1981).

In this project, two cytoplasmically synthesized rat liver mitochondrial proteins, malate dehydrogenase and the adenine nucleotide carrier, were chosen to study the mechanism of importing proteins into mitochondria. Malate dehydrogenase is a matrix enzyme, oxidizing malate to oxaloacetate in the tricarboxylic acid cycle (see review by Banaszak & Bradshaw, 1975). The other protein, the adenine nucleocarrier, is an inner membrane protein. It is responsible for the exchange of one cytoplasmic ADP with one matrix ATP (Klingenberg, 1979a).

In earlier studies, several laboratories used purified enzymes or enzyme subunits to examine the import of mitochondrial proteins. Marra et al. (1978) demonstrated the uptake of mature forms of mitochondrial aspartate aminotransferase and malate dehydrogenase into isolated mitochondria. Our laboratory has examined the binding of mitochondrial malate dehydrogenase to phospholipid vesicles (Webster et al., 1979) and mitoplasts (Strasberg et al., 1979). We have also developed a procedure to purify the enzyme to homogeneity and reported that the enzyme is synthesized as a precursor of larger molecular mass (Aziz et al., 1981). It was, therefore, logical to extend the investigation by probing the molecular aspect of the import of this protein.

The adenine nucleotide carrier is a major mitochondrial protein. In bovine heart mitochondria, it constitutes up to 5% of the total protein

mass. Vignais et al. (1973) reported the use of a gummerifin, carboxyatractyloside (CAT), to inhibit the activity of the protein. Unlike most of the other mitochondrial proteins, the CAT-carrier complex does not bind to hydroxylapatite, and can be purified by a one-step hydroxylapatite chromatography (Riccio et al., 1975a, b). The simplicity of the purification method and the abundance of the protein in mitochondria constitute the major reasons for selecting the adenine nucleotide carrier as a model protein in studying the import of mitochondrial proteins. Furthermore, the carrier is located in the mitochondrial inner membrane. Comparison of the information obtained from malate dehydrogenase and the adenine nucleotide carrier may be very helpful in understanding how proteins imported into mitochondria are sorted into their respective intra-mitochondrial locations.

In this research project, an in vitro approach was used to study the import of the rat liver mitochondrial proteins. The project began with the following objectives: (1) identify the nascent translational products of the mRNAs for the adenine nucleotide carrier and malate dehydrogenase and study the physical characteristics of the nascent polypeptides. (2) Set up an in vitro uptake system, using isolated mitochondria, for investigating the intermediate steps involved in the import of the two proteins. The latter was the prime objective of the project. Specifically, the investigation involved studying the mechanism of transporting the nascent polypeptides of the two proteins from cytoplasm to mitochondria and their binding to mitochondria, the details of their insertion into or translocation across the mitochondrial inner membrane and their subsequent

conversion into a functional protein or an enzyme. Furthermore, the difference in the functional locations of the two proteins would allow the examination of the diversity of the import mechanisms and, possibly, how proteins are sorted into their respective sub-mitochondrial compartments.

METHODS AND MATERIALS

2.1 ISOLATION OF PROTEINS

Isolation of the Rat liver Adenine Nucleotide Carrier

The adenine nucleotide carrier was isolated as a carboxyatractyloside (CAT)-protein complex from Spague-Dawley rat liver mitochondria by hydroxylapatite chromatography (Riccio et al., 1975a,b). Mitochondria were isolated by differential centrifugation and washed three to four times with the Suspension solution. After the final washing, mitochondria were resuspended in 10 ml of the solution and incubated with the inhibitor CAT (25.5 umol/g of mitochondrial protein), at 0°C for 30 min. Mitochondria were then recovered by centrifugation at 5,000 x g for 15 min at 4°C and solubilized with the Solubilization solution containing 0.5 M NaCl and 5% Triton X-100. The Triton/protein ratio was always kept at 3 to 1. Solubilized mitochondria were centrifuged at 100,000 x g for 45 min at 4°C for removal of membrane debris. The supernatant was loaded onto a hydroxylapatite column pre-equilibrated with 2 to 3 bed volumes of the elution buffer. The size of the column required was determined by the amount of protein that would be loaded onto the column. The maximum capacity was 1 mg of extracted mitochondrial protein/ml of bed volume. After loading the sample, the column was washed with the elution buffer and the adenine nucleotide carrier came off at the void volume of the column.

Isolation of the Bovine Heart Mitochondrial Malate Dehydrogenase

Mitochondrial malate dehydrogenase was purified according to the method developed by Aziz et al. (1981). Partially purified bovine heart malate dehydrogenase obtained from Sigma (specific activity approximately 3100 Units/mg protein) in an ammonium sulphate suspension was further purified to homogeneity with a 5'-AMP-Sepharose 4B column. The protein suspension (1 to 2 ml of 0.9 mg/ml) was dialyzed overnight in PEM solution, pH 7.0 and then applied onto a 5'-AMP-Sepharose column (0.9 cm x 30 cm) pre-equilibrated with the same solution. The column was washed with 3 to 4 column volumes of PEM and then bound malate dehydrogenase was eluted with a 500 ml linear gradient of 0 to 100 uM NADH in the PEM. Fractions containing the highest activity of malate dehydrogenase were pooled and dialyzed against saturated ammonium sulphate in PEM. The precipitated protein was centrifuged at 12,000 x g for 45 min and the pellet was resuspended in 1 ml of PEM.

2.2 PREPARATION OF MONOSPECIFIC ANTISERA

Antigen Preparation

The Rat Liver Adenine Nucleotide Carrier--Antibodies were raised against the CAT-protein complex (N-antiserum) as well as the protein denatured by SDS and 2-mercaptoethanol (D-antiserum). For preparation of the inhibitor bound antigen, protein fractions from the hydroxylapatite

column were concentrated to 0.5-1.0 mg protein/ml using a Speed Vac Concentrator (Savant). For the denatured antigen, protein from the column was precipitated with acetone overnight at -20°C, using 4 ml of acetone for each ml of protein solution. The precipitate was collected by centrifugation at 8,000 x g for 30 min at 0°C. The pellet was resuspended to 1 mg/ml in PBS and adjusted to 1% in SDS, 2% in mercaptoethanol (MSH), and boiled for 2 min. The solution was kept at room temperature for 1 h before injection.

Bovine Heart Mitochondrial Malate Dehydrogenase--Antibodies were raised against the denatured form of the enzyme. The purified enzyme (0.5-1.0 mg/ml) from the 5'-AMP-Sepharose column was denatured in a final concentration of 0.5% SDS, 10 mM MSH and 140 mM NaCl and boiled for 2 min. The solution was kept at room temperature for 30 min before injection.

Immunization Procedure

Antibodies against the purified antigens were raised in New Zealand white rabbits (approximately 2.3 kg in weight). Two rabbits were used for each antigen preparation. Before injection, antigen was mixed with complete Freund's adjuvant in equal portions in the syringe. One mg of protein was injected initially, followed by booster injections of 0.5 mg at weekly intervals for two to three weeks. Antisera were collected one week after the second and subsequent booster injections and checked for immunospecificity.

Collection of Antisera

Approximately 20 to 30 ml of blood was collected from the central artery of the rabbit's ear. The blood was kept in an autoclaved 30 ml Corex tube at room temperature for 1 h, and then in the cold room (4°C) for 2 h. After coagulation, the serum was decanted into another sterile 30 ml Corex tube, and clarified by by centrifugation at 2,000 rpm for 20 min at 4°C in a SS 34 rotor using a Sorvall centrifuge. The clarified serum was divided into small aliquots and kept frozen at -20°C. Preimmune sera were collected from non-immunized rabbits using the same procedure before immunization.

2.3 IMMUNOSPECIFICITY OF THE ANTISERA

The specificity of each antiserum against the adenine nucleotide carrier or malate dehydrogenase was tested by its ability to react with the radiolabeled purified protein as well as to recognize the protein from an extract of radiolabeled mitochondrial proteins.

Radiolabeling of the Antigens

Reductive Methylation of Malate Dehydrogenase with [^{14}C]Formaldehyde--The purified bovine heart malate dehydrogenase was radiolabeled by reductive methylation using [^{14}C]formaldehyde according to the procedure of the Rice and Mean (1971). The purified enzyme from

the 5'-AMP column was dialyzed overnight in PEM, pH 7.0, and then adjusted to pH 9.0 with a 1.0 M Tris-base solution in a 1.5 ml microtube. The solution was diluted with 1/4 volume of a 1.0 M borate, pH 9.0, to give a final concentration of 0.2 M borate buffer. Ten ul of 33 mM [^{14}C]formaldehyde (50 mCi/mmol) per 500 ug of protein was added. This was followed within 30 sec by four sequential additions of 2 ul of freshly prepared sodium borohydride (5 mg/ml). The tube was capped and shaken vigorously after each addition. An additional 20 ul was added after 1 min. The solution was then dialyzed overnight against 4 L of PEM, pH 7.0, to remove unreacted [^{14}C]formaldehyde. The radioactivity in 5 or 10 ul of the dialyzed protein solution was determined. The specific activity of the radiolabeled protein was approximately 15,000 cpm/ug protein.

Radiolabeling of Total Mitochondrial Proteins with [^{35}S]Methionine--Chinese hamster ovary (CHO) cells and rat XC cells were grown in suspension culture in β-MEM with 10% fetal calf serum (Stanners et al., 1971). About 2.0 x 10^8 cells were labeled with [^{35}S]methionine. Briefly, CHO cells in log phase were harvested by centrifugation at 500 x g for 5 min at 20°C, and resuspended in 50 ml of methionine-free β-MEM with 10% fetal calf serum. After adding 1 mCi of [^{35}S]methionine (1,000 Ci/mmol), the cells were labeled for two hours at 37°C. The labeling was stopped by the addition of an equal volume of ice-cold PBS. Cells were collected at 500 x g for 5 min and washed twice with 50 ml PBS and once with 10 ml Medium B. After washing, cells were

disrupted in 10 ml of Medium B using an Ultra-Turrax disintegrator, and mitochondria were isolated by differential centrifugation. Mitochondria were further purified on a sucrose step gradient, consisting of 2 ml of 1 M sucrose and 2 ml of 1.5 M sucrose in 2 mM EDTA and 10 mM Tris-HCl, pH 7.5. Centrifugation was at 30,000 rpm for 30 min in a SW 50.1 rotor using a Beckman L2-65B ultracentrifuge. Mitochondria were recovered from the interface between the 1 M and 1.5 M sucrose solutions by puncturing the side of the tube with a 19 or 21 gauge needle fitted with a 1 ml syringe. The collected mitochondrial fraction was diluted with a ten times volume of Medium B and centrifuged at 8,000 rpm in a SS 34 rotor using a Sorvall centrifuge. The radiolabeled mitochondria were used for testing the immunospecificity of antisera as described in the following section.

Immunoreaction Mixture Preparation

Immunoreaction using the Radiolabeled Antigen--The [^{14}C]labeled bovine heart malate dehydrogenase was denatured before immunoprecipitation. About 5-10 ug of the labeled protein in 0.3 ml of PEM was denatured by adjusting the solution to 4% (w/v) SDS and 0.1 M MSH in distilled water. The solution was boiled in a water bath for 5 to 10 min. After cooling to room temperature, the solution was adjusted to 1 ml with the Denaturing Adjusting solution, containing 0.15 M NaCl, 1% Triton X-100, 5 mM EDTA and 50 mM Tris-HCl, pH 7.2. The mixture was left at room temperature for at least 30 min before antiserum or control serum was added.

Immunoreactions using Radiolabeled Mitochondrial Proteins--The [^{35}S]methionine labeled mitochondrial pellet was solubilized with non-ionic detergent or denatured with SDS and MSH for immunoreaction. In the former case, the mitochondrial pellet was solubilized with the Native Adjusting solution at a Triton/protein ratio of 3 to 1 at 0°C for 30 min. Solubilized proteins were collected in the supernatant after a clarifying spin in an Eppendorf centrifuge for 30 min. The supernatant was divided into aliquots. Each aliquot was adjusted to 1 ml with the same solution. Five ul of soybean trypsin inhibitor (10 mg/ml) was added and the mixture was kept on ice before the addition of antiserum or control serum.

In experiments with denatured mitochondrial proteins, the mitochondrial pellet was solubilized with 300 ul of a solution containing 4% SDS and 10 mM MSH in distilled water and boiled for 5 min. After cooling to room temperature, the solution was divided into aliquots and each aliquot was adjusted to 1 ml with the Denaturing Adjusting solution. Five ul of soybean trypsin inhibitor (10 mg/ml) was added to each reaction mixture, and the mixtures were left at room temperature for at least 30 min before the addition of antiserum or control serum.

Immunoprecipitation

Immunoreactions were initiated by the addition of appropriate

antiserum or control serum (usually 20 ul was used) to an immunoreaction mixture. The immunoreaction was carried out in a 1.5 ml microtube for 12 h at 4°C with continuous rotation. This was followed by the addition of 20 ul of protein A-Sepharose CL-4B, which was pre-swollen in either Native or Denaturing Adjusting solution. After rotating for 3 more hours, the protein A-Sepharose was recovered using an Eppendorf centrifuge, and washed repeatedly with 1 ml of the appropriate adjusting solution. After washing, the ternary complexes of protein A-Sepharose-antibody-antigen were dissociated in 100 ul of a solution containing 8% (w/v) SDS, 6.5% (v/v) MSH and 20 mM Tris-HCl, pH 6.8.

2.4 IN VITRO SYNTHESIS OF THE PRECURSOR PROTEINS OF THE RAT LIVER ADENINE NUCLEOTIDE CARRIER AND MALATE DEHYDROGENASE

Peparation of Rabbit Reticulocyte Lysate

Rabbit reticulocyte lysate was prepared according to Villa-Komaroff et al. (1974). Acetyl-phenylhydrazine (1.2 g) was dissolved in 100 ml sterile distilled water and the pH was adjusted to 7.0 with 1.0 M Hepes, pH 7.5. Two 2.3 kg New Zealand white rabbits were injected subcutaneously with the 1.2% acetyl-phenylhydrazine solution according to the following schedule :

Days	Amount injected (ml)
1	2.0
2	1.8
3	1.5
4	1.1
5	1.5
6	2.0.
7	hematocrit checked

About 1 to 2 ml of blood was collected from the ear vein of each rabbit on day 7 for checking the hematocrit value. The test was done in a clinical laboratory. If the hematocrit value was below 30%, terminal bleeding was carried out on the following day. Otherwise, injection would be continued until the hematocrit value reached the desired level. Blood was collected from the common carotid artery into a 50 ml sterile syringe containing 10 to 20 ml of chilled saline with 0.001% heparin. Blood from each rabbit was pooled and filtered through a piece of steriled cheese-cloth. The filtrate was centrifuged at 3,500 rpm in a SS 34 rotor for 5 min at 4°C. The pelleted red blood cells were washed three times with saline solution, containing 0.001% heparin. After the last wash, cells were collected at 7,000 rpm for 5 min at 4°C in the same rotor. Packed cells were lysed with an equal volume (cell volume) of cold sterile distilled water, and then centrifuged at 12,000 x g for 20 min. The supernatant (lysate) was divided into small aliquots (1 ml), frozen and stored immediately in liquid nitrogen.

Isolation of Rat Liver Total RNA

The method was that of Haffner et al. (1978). One Sprague-Dawley

rat (100-150 g) was fasted for 12 h and killed by a blow to the head. Excised liver (approximately 6.0 g) was frozen immediately in liquid nitrogen, crushed into small pieces on dry ice within 30 sec and then blended by five 4 sec bursts in an emulsion prepared by mixing 70 ml 90% (v/v) phenol with 70 ml 0.1 M Tris-HCl, pH 9.0, at room temperature. Redistilled phenol was used and the 90% phenol solution was made by adding sterile water just before use. The Tris-HCl solution was precooled in the cold room.

The emulsion was centrifuged at 1,800 rpm in a SS-34 rotor using a Sorvall centrifuge for 5 min at 0°C. The aqueous phase (40 ml) was collected, and the phenolic phase re-extracted with 70 ml of 0.1 M Tris-HCl, pH 9.0. The aqueous phase from the two extractions were pooled (total 100 ml), and re-extracted twice with an equal volume of 90% phenol. Potassium acetate (2 M, 6.25 ml) was added to each 50 ml of the re-extracted aqueous phase, and then mixed with two volumes of 95% ethanol. The resulting suspension was left one hour at -20°C. The precipitate was collected by centrifugation at 1,800 rpm in a SS-34 rotor for 15 min at 0°C. The pellet was dissolved in 20 ml sterile distilled water and made 2.5 M in NaCl by adding solid salt. After 18 h at 0°C, a pellet of insoluble RNA (without tRNA) was recovered by centrifugation at 15,000 rpm in a SS-34 rotor for 15 min at 0°C. The pellet was washed twice with 67% ethanol, twice with ethanol and twice with anhydrous ether. After the final wash, the pellet was air-dried to a white powder. The powder was dissolved in 1 ml sterile distilled water and divided into small aliquots which were frozen immediately in liquid

nitrogen and stored at -70°C. The concentration of the RNA solution was determined by its absorbance at 260 nm.

Isolation of Rat Liver Free and Bound Polysomes

Free and bound polysomes were isolated from Sprague-Dawley rats by the method of Blobel (1967). In this method, polysomes were prepared in the presence of a high-speed supernatant fraction of rat liver, which contained a RNase inhibitor.

The high-speed supernatant (S3) fraction containing the RNase inhibitor was prepared as follows: Sprague-Dawley rats were fasted 12 h before the experiment. The livers were removed and chilled in ice-cold 0.25 M sucrose-TKM solution at pH 7.5, minced with scissors, and homogenized in 2 volumes of the same solution with 10 strokes of a Potter-Elvehjem homogenizer with Telfon pestle. The homogenate was centrifuged at 12,000 rpm in a SS 34 rotor using a Sorvall centrifuge for 10 min 4°C. The post-mitochondrial supernatant was centrifuged at 105,000 x g for 4 h at 0°C. The resulting supernatant is the S3 fraction containing the RNase inhibitor. It was used either fresh or stored frozen at -20°C.

For the preparation of free and bound polysomes, a post-mitochondrial supernatant fraction was obtained as described above except that the liver was homogenized in a S3 fraction-containing sucrose solution (9 parts of 0.25 M sucrose-TKM with 1 part of S3

fraction). Six ml of the post-mitochondrial supernatant fraction was layered over a two-layer discontinuous gradient with 3 ml of 2.0 M sucrose-TKM-S3 solution and 3 ml of 1.38 M sucrose-TKM-S3 solution. The latter sucrose containing solutions were prepared from a stock solution of 2.3 M sucrose-TKM, with appropriate dilution using the S3-high speed supernatant fraction. The gradient was centrifuged for 24 h at 40,000 rpm in a Ti 65 rotor in a Beckman L2-65B ultracentrifuge. Free polysomes were pelleted under this condition and resuspended in approximately 500 ml of TKM. The 1.38 M sucrose-S3 fraction layer of the discontinuous gradient contained the bound polysomes, which was collected in a 10 ml syringe by side puncture, using a 21 gauge needle. A detergent mixture of 20% (w/v) Triton-X 100 and 5% (w/v) sodium deoxycholate was added to give a final concentration of 4% Triton X-100 and 1% sodium deoxycholate. The mixture was homogenized with 10 strokes of a Potter-Elvejhem homogenizer. The resultant homogenate was layered onto 3 ml of 2.0 M sucrose-S3 fraction in a polyallomer tube and centrifuged at 105,000 x g for 24 h. The ribosomes that were originally bound to membranes were pelleted and resuspended in a minimum volume of TKM. Concentrations of both free and bound polysomes were determined by measuring their absorbance at 260 nm using a Cary spectrometer.

In vitro Synthesis of Rat Liver Proteins

Rat liver proteins were synthesized in vitro in a rabbit reticulocyte lysate primed with rat liver total RNA, or with free or bound polysomes, according to Pelham and Jackson (1976). The lysate used

was treated with micrococcal nuclease so that protein synthesis was dependent on the amount of exogenous mRNA added.

Nuclease Treatment—Lysate (960 ul) was thawed and immediately made up to 150 ug creatine kinase/ml and 150 uM in hemin with stock solutions of creatine kinase (5 mg/ml) and hemin (5 mM). About 5% of the resulting mixture was saved for estimation of the endogenous activity of protein synthesis in the lysate. The rest of the lysate was supplemented with 10 ul of 0.1 M $CaCl_2$ and 10 ul of a solution of 1 mg micrococcal nuclease/ml. The mixture was incubated for 15 min at room temperature, and the nuclease was then inactivated with a final concentration of 0.1 M EGTA.

In vitro Protein Synthesis—Total RNA, or free or bound polysomes was translated in the nuclease-treated rabbit reticulocyte lysate. A typical translation mixture contained approximately 0.05–0.1 A_{260} units of free or bound polysomes/ml or 0.02–0.04 A_{260} units of total RNA/ul. The translation was carried out in the presence of [^{35}S]methionine (1 uCi/ul) for 60 min at 25°C. The relative percentage of each component in the translation mixture is shown in the following table :

Translation Mixture	Relative Percentage
Nuclease-treated lysate	70%
Master mix (cf next table)	5%
Potassium chloride (1.9M)	5%
[^{35}S]methionine	10%
Calf liver tRNA	1%
Total RNA or free or bound polysomes	0-9%
Sterile distilled water	9-0%

Master mix	Final Concentration in the Translation Mixture
ATP (pH 7.0)	900 uM
GTP pH 7.0)	175 uM
Creatine Phosphate	11 mM
2-Mercaptoethanol	5 mM
Hepes (pH 7.6)	12 mM
Amino Acid mixture without methionine	120 uM
Magnesium acetate	2 mM

Translation was started by the addition of [^{35}S]methionine. Incoporation was checked at 30 min intervals by hot trichloroacetic acid-precipitatable counts as described below.

Immunoprecipitation of the two Mitochondrial Precursor Proteins

Translation was stopped by transfering the translation mixture from 25°C to 0°C. The mixture was transfered into a 0.8 ml ultracentrifuge tube, which was centrifuged at 37,500 rpm for 30 min at 2°C in a SW 50.1 rotor—using a 3 ml adaptor tube. The post-ribosomal supernatant obtained was divided into portions, usually of 100 to 200 ul depending on the extent of incorporation of [^{35}S]methionine. For

immunoprecipitation of precursor proteins without prior protein denaturation, each portion was adjusted to 1.0 ml with the Native Adjusting Solution. To prevent proteolysis, 10 ul each of phenylmethylsulfonyl fluoride (5 mg/ml) and soybean trypsin inhibitor (10 mg/ml) was added. This was followed by the addition of 20 ul of appropriate antiserum or preimmune serum. For experiments in which *in vitro* synthesized proteins were denatured before immunoprecipitation, each aliquot of the post-ribosomal supernatant was adjusted to 4% SDS and 0.1 M MSH in sterile distilled water. The solution was boiled for 5 min and cooled to room temperature before adjusting to 1 ml with the Denature Adjusting solution. The two protease inhibitors mentioned above were also added and the solution was left at room temperature for 30 min before the addition of 20 ul antiserum against the adenine nucleotide carrier or malate dehydrogenase, or 20 ul of preimmune serum. Immunoprecipitation was carried out as described in the previous section. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli, 1970) with subsequent fluorography of the dried gel (Bonner and Laskey, 1974).

2.5 ESTABLISHMENT OF PRECURSOR AND MATURE PROTEIN RELATIONSHIP

Competition Experiment

Competition for immunoprecipitation of the precursor for the rat liver adenine nucleotide carrier was carried out in the presence of an excess amount of unlabeled rat liver adenine nucleotide carrier (10 ug)

which had been isolated by hydroxylapatite chromatography. The immunoreaction was in Native Adjusting solution with N-antiserum raised against the rat liver CAT-adenine nucleotide carrier complex. The unlabeled adenine nucleotide carrier was mixed with the lysate containing *in vitro* synthesized [^{35}S]methionine-labeled proteins before the addition of the antiserum. A precursor-protein relationship would be indicated by the reduction in the amount of the radiolabeled precursor protein immunoprecipitated in the presence of excess cold protein.

Peptide Mapping

Precursor-protein relationship can also be established by comparing the primary structure of the two proteins using peptide mapping. However, the major obstacle in performing this type of experiment is the minute amount of the precursor precipitated by the antiserum as well as its low level of radioactivity. Thus, both the precursor protein and the mature protein were iodinated before digestion with *Staphylococcus aureus* V8 protease.

Iodination of the Precursor Protein and the Mature Protein--The [^{35}S]methionine-labeled precursor of the rat liver adenine nucleotide carrier or malate dehydrogenase was immunoprecipitated by the specific antiserum and visualized after electrophoesis on a SDS-polyacrylamide gel by impregnating the gel with diphenyloxazole, and followed by fluorography. A sample of the corresponding rat liver mature protein, having the same level of radioactivity as in the immunoprecipitate was

applied on the same gel. After determining the positions of the protein and its precursor on the gel, the appropriate sections of the gel were excised. They were rehydrated in water, washed in fresh dimethylsulfoxide to remove the diphenyloxazole, and then in 25% (v/v) ethanol and 10% (v/v) methanol for 30 min each. The gel slices were then dried under a heat lamp and transfered to separate siliconized screw-cap vials. The proteins were iodinated separately with sodium [^{125}I]iodide in the gel slices by the chloramine T method (Elder et al., 1977). Briefly, 20 ul of 0.5 M sodium phosphate buffer (pH 7.5) was used to rehydrate the gel pieces in the vial. This was followed by the sequential addition of 10 ul of sodium [^{125}I]iodide (100 mCi/ml) and 5 ul of chloramine T (1 mg/ml). The reaction was carried out in a fume hood at room temperature for 30 min. Sodium bisulfite (1 ml of 1 mg/ml) was used to stop the reaction. After iodination, the gel slices were dialyzed in 10% (v/v) methanol for 12 h to remove excess free iodide. Fresh dialysis solution was used every 2 to 3 h. The gel slices were dried under a heat lamp and then transfered to siliconized screw-cap vials.

Two-Dimensional Peptide Mapping--The procedure used was adapted from the published method of Enequist et al. (1981). The gel slices containing the iodinated precursor and the mature protein were rehydrated in separate siliconized screw-cap vials with 0.5 ml of 50 mM sodium bicarbonate, pH 7.8 and the proteins were digested with 10 ug of S. aureus V8 protease for 16 h at 37°C with constant shaking. The solutions were then lyophilized overnight. Fragments of the digested

proteins were resolved on 20 cm x 20 cm silica-gel coated thin-layer chromatography plates. The first dimension was in methanol/chloroform/concentrated ammonia (2:2:1 ; v/v/v) and the second dimension was in pyridine/glacial acetic acid/n-butanol/water (40:14:68:25 ; v/v/v/v). The plates were air-dried and the peptides visualized by autoradiography at -80°C for 1 to 2 weeks.

2.6 ESTIMATION OF THE SIZE OF THE TWO PRECURSOR PROTEINS IN THE POST-RIBOSOMAL SUPERNATANT

Sephadex G-100 Chromatography—Translation products from rat liver free polysomes were fractionated on a Sephadex G-100 (coarse) column (43 cm x 2 cm), equilibrated with 0.3 M KCl and 50 mM Tris-HCl, pH 7.2. The column was precalibrated with 0.5 ml of a mixture of [^{14}C]labeled marker proteins, consisting of aldolase (150 kDa), bovine serum albumin (68 kDa), carbonic anhydrase (30 kDa) and cytochrome c (12 kDa), and non-radioactive horse hemoglobin (58 kDa). After synthesis, the translation mixture was centrifuged at 100,000 x g for 30 min at 2°C and the post-ribosomal supernatant containing [^{35}S]labeled proteins was adjusted to 0.3 M KCl, and 0.5 ml of the solution was applied to the column. The column was washed subsequently with a solution containing 0.3 M KCl and 50 mM Tris-HCl, pH 7.2, at a flow rate of 20 ml/h. Fractions of 2.8 ml were collected and one ml of each fraction was used for immunoprecipitation with appropriate antiserum after adjusting to 1% (w/v) Triton X-100.

Sucrose Density Gradient Centrifugation--A linear 5 to 25% (w/v) sucrose density gradient was formed in a solution containing 0.3 M KCl, 5 mM EDTA and 10 mM Tris-HCl, pH 7.2. The solution also contained 1 mM of each of the following protease inhibitors: N-p-tosyl-L-lysine chloromethyl ketone-HCl, L-1-tosylamide-2-phenylethylchloromethyl ketone, phenylmethylsufonyl fluoride and soybean trypsin inhibitor. The post-ribosomal supernatant of a reticulocyte lysate primed with rat liver RNA was adjusted to 0.3 M KCl, layered on top of the gradient, and centrifuged in a SW 40.1 rotor at 36,000 rpm for 16 h at 4°C in a Beckman L2-65B ultracentrifuge. The sucrose density gradient was calibrated by centrifuging a solution of unlabeled cross-linked bovine serum albumin on a parallel gradient at the same time. Fractions of 1 ml were collected using a sucrose-density gradient fractionator. Fractions from the calibrated gradient were analyzed by SDS-polyacrylamide gel electrophoresis. Fractions from the gradient with radiolabeled proteins were subjected to immunoprecipitation. Half of each fraction was adjusted to 1 ml with a solution containing 0.3 M KCl, 5 mM EDTA, 2% Triton X-100 and 100 mM Tris-HCl, pH 7.2 before the addition of appropriate antiserum. The immunoprecipitates were also analyzed by SDS-polyacrylamide gel electrophoresis with subsequent fluorography.

2.7 IN VITRO IMPORT SYSTEM

In Vitro Import of Mitochondrial Precursor Proteins--Freshly isolated mitochondria from CHO cells were used for importing in vitro synthesized [^{35}S]methionine-labeled rat liver mitochondrial precursor

proteins. CHO cells were grown in suspension culture and approximately 1 to 2 x 10^8 cells were used for each experiment. Cells were disrupted with an Ultra-Turrax disintegrator and mitochondria were isolated by differential centrifugation. After the last centrifugation, the mitochondrial pellet was resuspended in 1 to 1.5 ml ice-cold Medium B and the suspension was applied onto two sucrose-step gradients as mentioned in Section 2.3. After centrifugation at 30,000 rpm in a SW 50.1 rotor at 2°C for 30 min, mitochondria were recovered by side-puncture from the interface between the 1.0 M and 1.5 M sucrose layer. The mitochondrial fraction collected (about 2.0 ml) was diluted with at least 10 ml sterile ice-cold Medium B solution and centrifuged in a 50 ml sterile Sorvall tube at 8,000 rpm for 20 min at 2°C using a SS 34 rotor. The mitochondrial pellet was resuspended in about 0.5-1.0 ml ice-cold sterile Medium B solution and duplicate samples of 5 ul were used for protein determination by the method of Lowry et al. (1951). The rest of the sample was kept in a 1.5 ml microtube on ice in the cold room.

Rat liver free polysomes were used for the in vitro synthesis of mitochondrial precursor proteins as well as other cellular proteins. A typical translation mixture was shown in Section 2.4. Translation was initiated only after the protein determination of the purified mitochondrial fraction had been started. The translation was carried out for 60 min at 25°C and stopped by transfering the reaction mixture onto ice. The post-ribosomal supernatant was obtained by centrifugation at 120,000 x g for 30 min at 2°C as described. Incorporation of

[^{35}S]methionine into newly synthesized proteins was determined during the 30 min of centrifugation by hot trichloroacetic acid-precipitatable radioactivity.

The mitochondrial suspension was adjusted to 450 to 600 ug of protein/100 ul with Medium B. Usually, the concentration was within this range and adjustment was not necessary. For the in vitro import system, 100 ul of the mitochondrial suspension was added to about 1-3 x 10^7 cpm of [^{35}S]methionine-labeled polysomal translation products in a 1.5 ml microtube. The mixture was adjusted to a final volume of 0.3 ml with Medium B if necessary, so that the ratio of Medium B to lysate was about one to one. All additions were made on ice. The reaction was started by incubating the reaction mixture at 30°C and carried on for 30 to 60 min with constant shaking in a water-bath. At appropriate time intervals, mitochondria were removed by centrifugation in an Eppendorf centrifuge for 15 min at 4°C. The supernatant containing [^{35}S]methionine-labeled proteins was transfered to another 1.5 ml microtube. The mitochondrial pellet was washed once with ice-cold Medium B and repelleted by the same procedure. The supernatant fraction was adjusted to 4% (w/v) SDS and 10 mM MSH with stock solutions of 30% SDS and 0.1 M MSH. The washed mitochondrial pellet was solubilized in the microtube with 150 ul of a solution containing 4% SDS and 10 mM MSH. The microtubes were capped and holes were punched in the cap with a 19 gauge needle. The solutions were then boiled for 5 to 10 min. After cooling to room temperature, one ml of Denaturing Adjusting solution was added to each solution, followed by the addition of 5 ul soybean trypsin inhibitor (10 mg/ml). The solutions

were left at room temperature for 30 min before the addition of antiserum or preimmune serum. Usually, 50 ul of serum was added. Immunoprecipitation was carried out as described in Section 2.3. Fifty ul of protein-A Sepharose was used and washing was carried out with the Denaturing Adjusting solution supplemented with 10 mM methionine.

Protease Protection of the Newly Imported Mitochondrial Precursor Proteins--Mitochondria (400 to 600 ug) in 100 ul of Medium B were incubated with the post-ribosomal supernatant containing [^{35}S]methionine-labeled proteins (1-3 x 10^7 cpm) for 30 min at 30°C. The reaction was stopped by chilling in an ice-water bath. Freshly prepared trypsin solution (1 mg/ml) was added to the mixture in a ratio of 6 ug trypsin to 100 ug mitochondrial protein. Proteolysis was carried out in an ice-water bath for 30 min. The reaction was stopped by the addition of 50 ul of soybean trypsin inhibitor (10 mg/ml) and, in some cases, 10 ul of phenylmethylsufonyl fluoride (1 mg/ml). The mixture was left in the ice-water bath for another 5 min before centrifugation in an Eppendorf centrifuge for 5 min at 4°C. The mitochondrial pellet as well as the supernatant were adjusted to 4% SDS and 10 mM MSH, and boiled for 5 min. Immunoprecipitation was carried out as described before.

In Vitro Import in the presence of Carbonyl cyanide m-chlorophenylhydrazone (CCCP) and o-phenanthroline--Stock solutions of 1 mM CCCP and 0.1 M o-phenanthroline were prepared in 100% ethanol. The energy dependence of the import process and the chelator sensitivity of the protease involved in the process were examined by adding 1% of the

final volume of the appropriate inhibitor to the reaction mixture. Reaction and immunoprecipitation were carried out as described previously.

2.8 STUDY ON THE BINDING OF THE PRECURSOR OF RAT LIVER MALATE DEHYDROGENASE TO ISOLATED MITOCHONDRIA

Binding Study—The ribosomal-free supernatant containing the radiolabeled precursor of mitochondrial malate dehydrogenase was incubated with freshly-isolated mitochondria (300 to 600 ug protein) in the presence of 10 uM CCCP at 30°C for 60 min in a 1.5 ml microtube. The uncoupler was added from a 1 mM solution in analytical grade absolute ethanol. In many experiments, approximately equal volumes of mitochondria and ribosomal-free supernatant were used and the final volume, if necessary, was adjusted to 300 ul with Medium B. The mitochondrial pellet recovered after centrifugation as well as the supernatant were denatured in a final concentration of 4% SDS and 10 mM MSH. Immunoprecipitation was carried out as described previously and monospecific antibodies against the denatured form of malate dehydrogenase were used, with 15 ul for the supernatant and 30 ul for the mitochondrial fraction.

Protease Sensitivity of the Bound Precursor of Malate Dehydrogenase—The binding assay was carried out as described in the above paragraph. Proteolytic treatment of the reaction mixture with trypsin followed that described in Section 2.7.

Saturation of the Binding Sites--Mitochondria (150 ug protein/assay) were incubated with varying amounts of post-ribosomal supernatant containing radiolabeled proteins in the presence of 10 uM CCCP. The final volume of each reaction mixture was adjusted to 300 ul, using non-translated, nuclease-treated reticulocyte lysate. Incubation was carried out for 30 min at 30°C. Mitochondria were recovered by centrifugation, and washed once with Medium B. Both supernatant and mitochondrial fractions were subjected to immunoprecipitation as described in Section 2.7. Five ul from each immunoprecipitation was counted in 5 ml of an aqueous counting scintillant.

The Direct Processing of the Bound Precursor of Malate Dehydrogenase--Experiments were carried out to show that the processing of the bound precursor of malate dehydrogenase was independent of the volume of the reaction mixture. The precursor of malate dehydrogenase was bound to mitochondria by incubating gradient purified mitochondria from CHO cells with rat liver free polysomal translation products for 5 min at 0°C. Mitochondria were recovered by centrifugation for 15 min in an Eppendorf centrifuge, and the sides of the microtubes were rinsed with small volumes of Medium B before the mitochondrial pellet was resuspended in Medium B.

In one experiment, the mitochondrial fraction was divided into five aliquots. One was processed for immunoprecipitation directly. The rest were divided into two sets. One set was adjusted to a final volume

of 0.4 ml and the other set to 2.0 ml, with 50% of nuclease-treated, non-translated lysate. One reaction mixture from each set was incubated at 0°C and the other at 30°C. Incubation was carried out for 30 min and mitochondria were recovered by centrifugation and subjected to immunoprecipitation.

In another experiment, the amount of processing of the bound precursor was compared to that of unbound precursor. Mitochondria with bound precursor were prepared as described above, and incubated in a final volume of 0.2 ml or 2.0 ml, with 50% by volume of Medium B and 50% of nuclease-treated, non-translated lysate. The same amount of mitochondria without any pretreatment were incubated in a final volume of 0.2 ml or 2.0 ml, with 50% by volume of Medium B and 50% of lysate, containing radiolabeled post-ribosomal translation products. The reaction mixtures were incubated at 30°C for only 5 min and mitochondria were recovered by centrifugation and subjected to immunoprecipitation.

2.9 CONFORMATIONAL STUDY ON THE 'PRECURSOR OF RAT LIVER MITOCHONDRIAL' MALATE DEHYDROGEANSE AND THE NEWLY IMPORTED PROTEIN

Conformational Specificity of the Antibody Preparation--The antibody preparation used was obtained by immunizing rabbits with SDS-denatured bovine malate dehydrogenase. It was used to immunoprecipitate both the mature enzyme from [^{35}S]methionine-labeled mitochondrial proteins and the [^{35}S]methionine-labeled *in vitro* synthesized rat liver free polysomal translation products, with or

without prior denaturation. Immunoprecipitation of the mature enzyme followed the procedure described in Section 2.3. Similar procedures were used for immunoprecipitation of the precursor protein as described in Section 2.4. Immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis.

Affinity Chromatography of the Newly Imported Precursor of Rat Liver Malate dehydrogenase and the Precursor Protein--In vitro synthesized malate dehydrogenase precursor was imported into freshly isolated mitochondria as described in Section 2.7. Mitochondria (1.76 mg) were incubated with 3×10^7 cpm of in vitro synthesized rat liver proteins for 60 min at 30°C. Mitochondria were recovered and washed once with Medium B. They were then suspended in 0.6 ml of the binding solution which contained 4 mM EDTA and 20 mM sodium phosphate, pH 7.0 and disrupted by sonication in a bath sonicator at 0°C six times for 1 min each, with a one min interval. One half of the supernatant fraction after centrifugation for 30 min in an Eppendorf centrifuge at 4°C was loaded onto a 0.5 ml affinity column of 5'-AMP-Sepharose 4B equilibrated with the binding solution. The column was then washed with 4 bed volumes and bound material eluted with the same solution but containing 40 uM NADH. Fractions of 0.5 ml were collected and assayed for malate dehydrogenase activity. Proteins in the fractions were analyzed by SDS-polyacrylamide gel electrophoresis as well as by immunoprecipitation with subsequent gel electrophoresis and fluorography.

The affinity of the newly synthesized precursor of malate

dehydrogenase to the 5'-AMP-Sepharose column was also examined. About 7.6×10^7 cpm of [^{35}S]methionine-labeled translation products in the post-ribosomal supernatant were applied to a 0.5 ml of the affinity column. The chromatographic procedure followed that described in the above paragraph and fractions were analyzed by immunoprecipitation and gel electrophoresis.

2.10 MISCELLANEOUS

N-[^{14}C]Ethyl-Maleimide Labeling of Mitochondrial Membrane Proteins--Mitochondria were isolated from rat liver and resuspensed in Suspension solution (10 mg protein/ml). Four one ml samples of mitochondrial suspension were placed in 1.5 ml microtubes and kept on ice. Fifty ul of CAT in Suspension solution (4 mg/ml) was added to two of the samples. All four samples were incubated at 0°C for 30 min. At the end of this time, 50 ul of mersalylic acid (2 mg/ml in water) was added to one of the samples without CAT, and all four samples were incubated at 20°C for 5 min. Then, 100 ul of 3 mM ADP in water was added to the sample with mersalylic acid, and 100 ul of water was added to the rest. This was followed by the addition of 100 ul N-[^{14}C]ethyl-maleimide (10 uCi of 23.7 mCi/mmol) to all of the samples. The labeling was carried out at 20°C for 5 min and stopped by the addition of 100 ul of 15 mM N-ethyl-maleimide and 100 ul of 75 mM cysteine. Mitochondria were recovered by centrifugation and the mitochondrial pellet was washed once with Suspension solution. The mitochondria were then solubilized with Solubilization solution at a Triton to protein ratio of 3 to 1. The

extract from each sample was loaded onto a hydroxylapatite column (1 cm x 15 cm) and washed with the elution buffer. The flow through fractions were analyzed by gel electrophoresis according to the method of Swank and Munkres (1971). Protein bands were visualized by Coomassie blue R250 staining as well as fluorography.

[^{14}C]Reductive Methylation of Molecular Mass Markers for Gel Electrophoresis--The set of molecular mass markers used included bovine serum albumin (68 kDa), ovalbumin (43 kDa), c-subunit (34 kDa) and r-subunit (17 kDa) of aspartate transcarbamoylase, carbonic anhydrase (30 kDa), chymotrypsinogen A (26 kDa) and cytochrome c (12.5 kDa). Each protein was dissolved or adjusted to a concentration of approxiamtely 5 mg/ml in 0.2 M borate buffer, pH 8.0. Ten ul of 33 mM [^{14}C]formaldehyde (50 mCi/mmol) was added per 500 ug of protein in a 1.5 ml microtube. This was followed by the sequential additions of 4 x 2 ul freshly prepared sodium borohydride (5 mg/ml) within 30 sec with capping of the tube and mixing between each addition. Twenty ul of the same solution was added 1 min later. The reaction mixture was then dialyzed against PBS overnight to remove excess formaldehyde, and a 5 ul aliquot was used for determination of the extent of labeling. The above procedure was adapted from Rice and Means (1971).

Trichloroacetic Acid-Precipitable Counts--The radioactive samples (2 to 5 ul) were spotted on Whatman 3 MM filter paper discs and air dried. The dried discs were washed once in boiling 5% (w/v) trichloroacetic acid for 5-10 min and then once at 0°C with the same

solution. The discs were then washed twice with ethanol:di-diethyl ether (1:1), and once with di-diethyl ether. The washes were done with constant shaking for approximately 5 min per wash. After the last wash, the discs were air-dried and counted with 5 ml toluene-omniflour scintillant (4 g omniflour per liter of toluene). This procedure followed that of Mans and Novelli(1961).

Enzymzatic Assay for Malate Dehydrogenase Activity—The enzyme was assayed in 0.1 M sodium phosphate, pH 7.5 containing 160 uM NADH and 120 uM oxaloacetic acid. A 10 ul enzyme sample was added to this assay mixture and the change in absorbance at 340 nm was monitored using a Cary spectrometer. One unit of activity is defined as the amount of enzyme that will catalyze the oxidation of 1 umol of NADH in 1 min at 25°C.

SDS-Polyacrylamide Gel Electrophoresis and Fluorography—Unless otherwise stated, gel electrophoresis was performed according to the method of Laemmli (1970) using 10% or 12.5% polyacrylamide gels. Fluorography was done according to Bonner and Laskey (1974).

Protein Determination—Protein was determined according to the method of Lowry et al. (1951).

2.11 COMPOSITION OF SOLUTIONS

Solutions were adjusted to the correct pH with sodium hydroxide (for phosphate buffers) or hydrochloric acid (for Tris buffers).

Rat Liver Adenine Nucleotide Carrier Isolation

Suspension soluton	0.25 M sucrose
pH 6.8	0.5 mM EDTA
	2.5 mM $MgCl_2$
	10 mM NaH_2PO_4
Solubilization solution	0.5 M NaCl
pH 7.2	0.5 mM EDTA
	5%(w/v) Triton X-100
	10 mM NaH_2PO_4
Elution buffer	0.1 M NaCl
pH 7.2	0.5 mM EDTA
	0.5%(w/v) Triton X-100
	10 mM NaH_2PO_4

Bovine Heart Malate Dehydrogenase Isolation

PEM	1 mM EDTA
pH 7.0	2 mM MSH
	100 mM NaH_2PO_4
TKM	25 mM KCl
pH 7.2	5 mM Mg acetate
	50 mM Tris

Immunoprecipitation

Native Adjusting solution	0.3 M KCl
pH 7.2	5 mM EDTA
	1%(w/v) Triton X-100
	10 mM Methionine
	10 mM Tris
Denaturing Adjusting Solution	0.15 mM NaCl
pH 7.2	5 mM EDTA
	I%(w/v) Triton X-100
	10 mM Methionine
	50 mM Tris

Others

Medium B	2 mM Na_2EDTA
pH 7.4	0.3 M Sucrose
	2 mM Tris
Phosphate buffered saline	0.14 M NaCl
pH 7.4	27 mM KCl
	1.5 mM KH_2PO_4
	8 mM Na_2HPO_4

2.12 MATERIALS

The sources of materials used were :

Aqueous counting scintillant	Amersham Coporation
5'-AMP-Sepharose 4B	Pharmacia Fine Chemicals
Bovine heart malate dehydrogenase	Sigma Chemical Co.
Calf liver tRNA	Boehringer Mannheim
Carboxylatractyloside	Boehringer Mannheim
Creatine kinase	Sigma Chemical Co.
Creatine phosphate	Sigma Chemical Co.
Hydroxylapatite	Biorad Laboratories
Micrococcal nuclease	Boehringer Mannheim
Protein A-Sepharose CL-4B	Pharmacia Fine Chemicals
Radioisotopes	New England Nuclear
Sephadex G-100 (coarse)	Pharmacia Fine Chemicals
Staphylococcus aureus V8 protease	Boehringer Mannheim

Most other chemicals are obtained from either Sigma Chemical Co. or Fischer Scientific.

RESULTS AND DISCUSSION

3.1 IN VITRO IMPORT OF THE PRECURSOR OF RAT LIVER MALATE DEHYDROGENASE

(I) THE IN VITRO IMPORT SYSTEM

Malate dehydrogenase is the enzyme responsible for the oxidation of L-malate to oxaloacetate in eukaryotic cells. There are two distinct forms of the enzyme. One is located in the matrix of mitochondria and the other in the cytoplasm. Both forms take part in the shuttling of malate across the mitochondrial inner membrane, which is essential in the transfer of electrons from cytoplasmic NADH into the matrix of mitochondria. However, the key role of the mitochondrial form is in the tricarboxylic acid cycle.

Both malate dehydrogenases are dimeric enzymes of identical subunits. The monomeric molecular mass of the mitochondrial form is about 34 kDa, and that of the cytoplasmic form is 36 kDa (Banaszak et al., 1971; Noyes et al., 1974). They are encoded in the nuclear genome by two separate genes, and synthesized on cytoplasmic ribosomes (see review by Edwards & Hopkinson, 1977).

Our laboratory is interested in the mechanism by which the newly synthesized mitochondrial form of the enzyme is located in the organelle. Aziz et al. (1981) showed that the putative precursor of rat

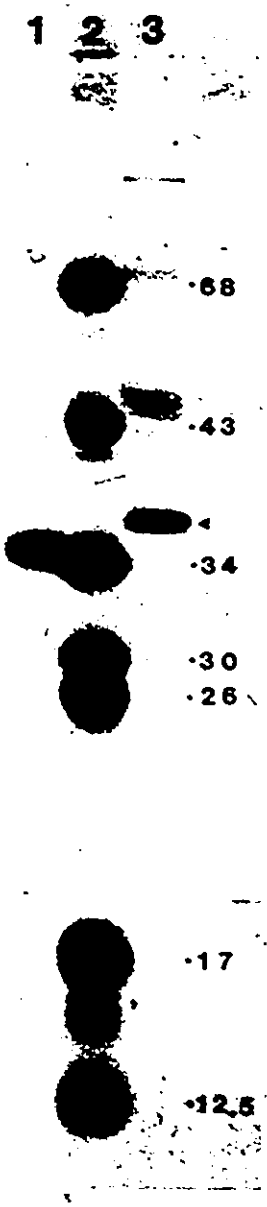
liver mitochondrial malate dehydrogenase is about 1.5 to 2.0 kDa larger than the mature enzyme. The aim of the research presented in this part of the thesis was to (1) establish the relationship between the putative precursor and the mature enzyme, (2) determine the type of ribosomes on which the synthesis of the precursor occurs, (3) estimate the size of the precursor in the post-ribosomal supernatant, (4) establish an *in vitro* system, using isolated mitochondria, for the import and processing of the precursor to its mature size.

RESULTS

Precursor Form of Mitochondrial Malate Dehydrogenase—The precursor form of malate dehydrogenase was synthesized *in vitro* in a nuclease-treated reticulocyte lysate with [^{35}S]methionine primed with rat liver total RNA. The putative form of the enzyme was identified by immunoprecipitating the precursor with a monospecific antiserum against the SDS-denatured mature protein and analysed on a SDS-polyacrylamide slab gel. As shown in Fig. 1, the precursor of malate dehydrogenase (lane 3) immunoprecipitated by the antiserum is about 1.5 to 2.0 kDa larger than the mature form of the enzyme (lane 1).

The relationship between the putative precursor and mature malate dehydrogenase was further confirmed by two-dimensional peptide mapping. Both malate dehydrogenase and its precursor were labeled with [^{125}I]iodide in the gel slices and digested with *S. aureus* V8 protease. The resulting peptides were eluted from the gel slices, lyophilized, and

Fig. 1. Immunoprecipitation of the precursor form of rat liver mitochondrial malate dehydrogenase synthesized in vitro. Rat liver RNA (2 A_{260} units/100 ul) was translated in a nuclease-treated rabbit reticulocyte lysate with [^{35}S]methionine (10 Ci/ul) for 60 min at 25°C. Postribosomal supernatant was obtained after centrifugation at 120,000 x g for 30 min. Translation products were immunoprecipitated from the post-ribosomal supernatant with monospecific antibodies against malate dehydrogenase. The immunoprecipitates were analyzed by SDS-10% polyacrylamide gel electrophoresis with subsequent fluorography of the dried gel. Lane 1, [^{14}C]formaldehyde-labeled rat liver malate dehydrogenase; lane 2, [^{14}C]formaldehyde-labeled molecular mass markers; lane 3, anti-malate dehydrogenase serum with the arrow indicating the position of pMDH. Numbers to the right of lane 3 indicate the molecular mass marker proteins (kDa): bovine serum albumin, ovalbumin, c-subunit of aspartate transcarbamoylase, carbonic anhydrase, chymotrypsinogen A, r-subunit of aspartate transcarbamoylase, and cytochrome c.


1 2 3
·68
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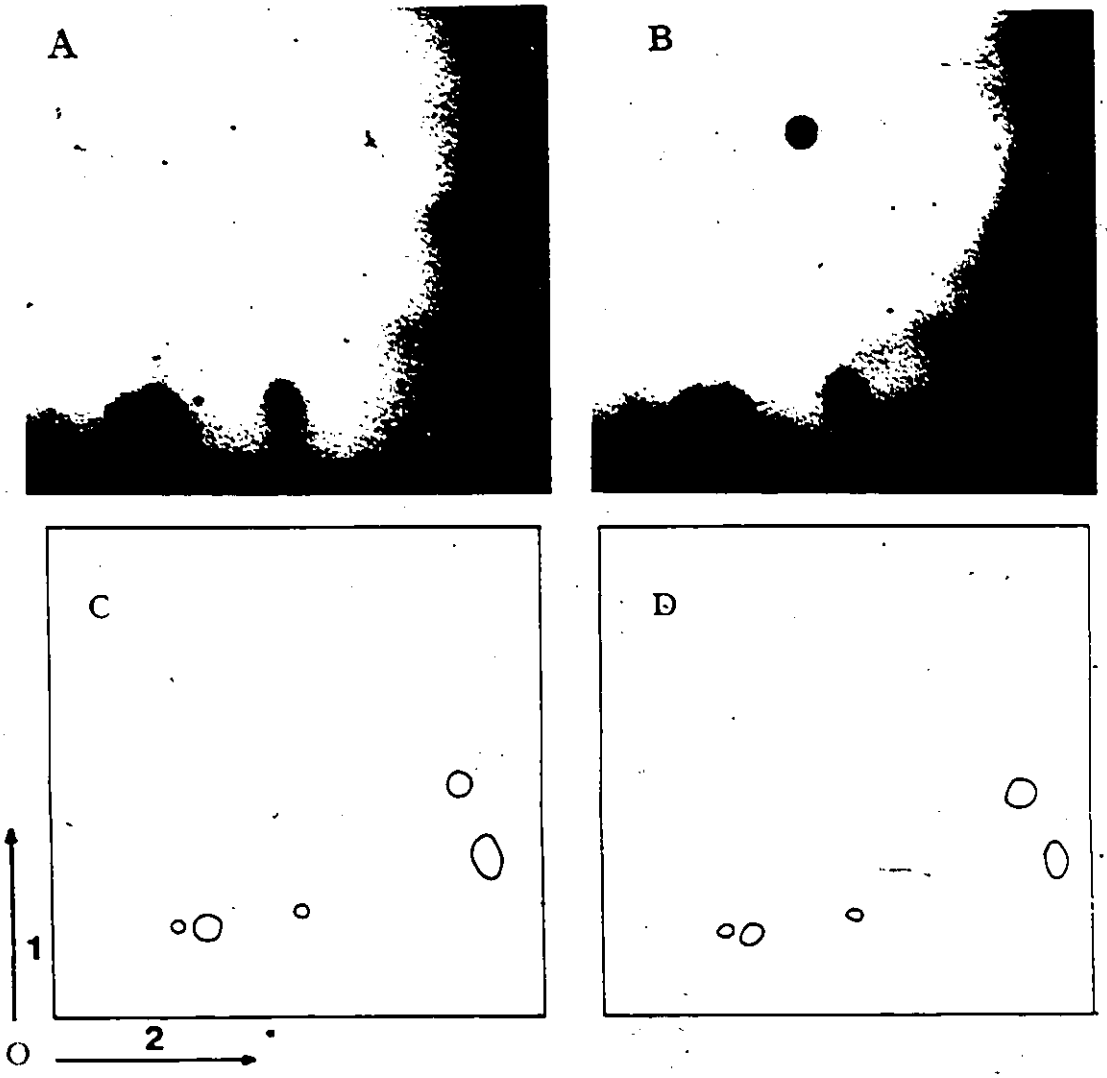
resolved by two-dimensional thin layer chromatography. As seen in Fig. 2, the peptide patterns of the two proteins were very similar, with most major spots being identical. This strongly suggests that most of the primary structure of the putative precursor is identical to malate dehydrogenase.

Subcellular Site of Synthesis of Malate Dehydrogenase—Rat liver free or bound polysomes were used for the synthesis of proteins in a rabbit reticulocyte lysate with [^{35}S]methionine. As shown in Fig. 3, the malate dehydrogenase precursor could only be detected when the translation was primed with free polysomes (lane 4), but not with membrane-bound polysomes (lane 9). The purity of the free and bound polysomal preparations was indicated by the relative amount of albumin precursor synthesized in the respective translation mixture. Albumin, a protein synthesized on the RER and secreted from liver cells, was predominantly recovered from the translation mixture of bound polysomes (lane 2 versus lane 7).

The results shown in Fig. 3 were also quantitated by trichloroacetic acid precipitable counts. Approximately 0.02% of the total acid-insoluble translation products of free polysomes was immunoprecipitated by the anti-malate dehydrogenase serum, whereas, no precipitatable counts were obtained when translation was primed with bound polysomes. In contrast, 0.04% and 1.7% of the products of free and bound polysomes were immunoprecipitated by anti-albumin serum respectively. These results showed definitively that malate

Fig. 2. Comparison of two-dimensional peptide maps of rat liver malate dehydrogenase (A and C) with its precursor (B and D). Malate dehydrogenase and its precursor were radio-iodinated and then digested with S. aureus V8 protease within gel slices. The peptides were then analyzed by two-dimensional thin layer chromatography and visualized by autoradiography. Detailed procedures are given under 'Methods and Materials'. A, Autoradiogram of the peptide fragments of malate dehydrogenase; B, autoradiogram of the peptide fragments of the precursor; C and D are tracings of the autoradiograms of malate dehydrogenase and its precursor, indicating the probable identical spots.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B
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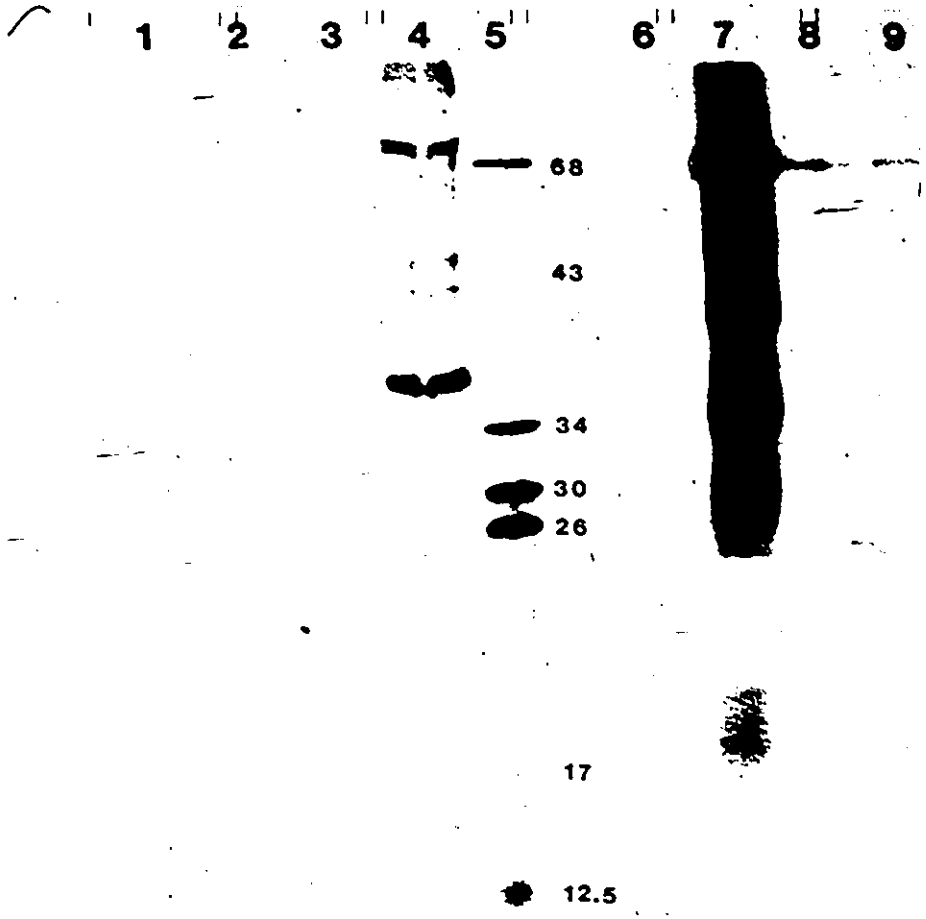
Fig. 3. Subcellular site of synthesis of the precursor form of malate dehydrogenase. Cell-free translations were primed either with rat liver free polysomes (3.8 A_{260} units/100 ul lysate) or with bound polysomes (6.5 A_{260} units/ 100 ul lysate). Translation was for 60 min at 25°C. The mixture was centrifuged at 120,000 x g for 30 min. Immunoreaction mixtures contained 100 ul of the supernatant and 20 ul of one of preimmune serum, anti-rat albumin serum or anti-malate dehydrogenase serum. The immunoprecipitates were analyzed by SDS-10% polyacrylamide gel electrophoresis with subsequent fluorography of the dried gel. Lanes 1-4, free polysomes; lanes 6-9, bound polysomes; Lanes 1, 3, 6 and 8, preimmune serum; lanes 2 and 7, anti-rat albumin serum; lanes 4 and 9, anti-malate dehydrogenase serum; lane 5, [^{14}C]formaldehyde-labeled molecular mass markers.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12.5

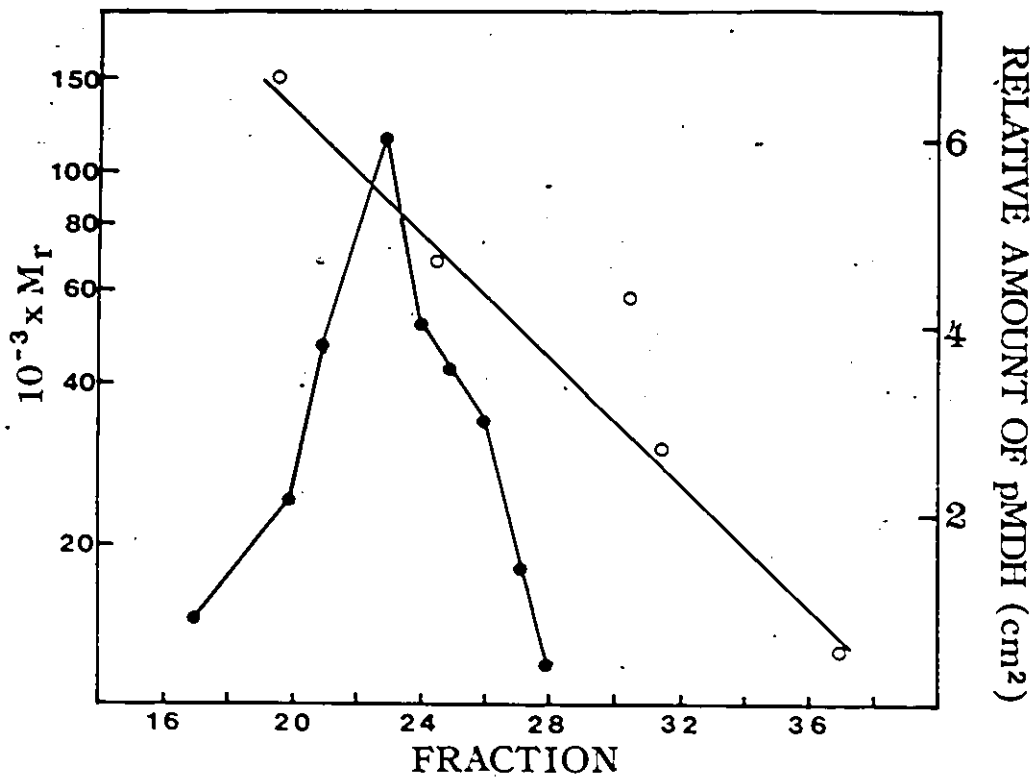
dehydrogenase is synthesized on membrane-free polysomes.

Size of the Precursor in the Post-ribosomal Supernatant--The post-ribosomal supernatant of a free polysomal translation mixture was fractionated by molecular sieving chromatography on a Sephadex G-100 column. The column was precalibrated with a mixture of radioactive molecular mass marker proteins (for details see the legend of Fig. 4). The eluted fractions were subjected to immunoprecipitation by the anti-malate dehydrogenase serum. Fig. 4 shows the distribution of pMDH upon gel filtration. The precursor was fractionated as a complex with an apparent molecular mass centered around 90 kDa, which is slightly larger than the size of the dimeric form of the mature enzyme (about 70 kDa). A similar result was obtained when the size of the precursor was estimated by equilibrium centrifugation in a sucrose density gradient.

Processing of The Precursor of Malate Dehydrogenase by Isolated Mitochondria—As mentioned in the Introduction, there has been only limited success in the *in vitro* import of mitochondrial precurosor proteins into rat liver mitochondria. It is possible that mitochondria isolated from actively growing cells would be more efficient in the uptake of proteins than mitochondria from rat liver. Mitochondria isolated from Chinese hamster ovary cells were, therefore, used for the examination of the *in vitro* import and processing of the precursor of malate dehydrogenase.

The post-ribosomal supernatant of a reticulocyte lysate

Fig. 4. Estimation of the size of the precursor of malate dehydrogenase by Sephadex G-100 chromatography. The G-100 column was precalibrated with 0.5 ml of a mixture of [^{14}C]-labeled proteins, aldolase (150 kDa), bovine serum albumin (68 kDa), carbonic anhydrase (30 kDa), and cytochrome c (12.5 kDa), and non-radioactive hemoglobin (58 kDa). Although the position of hemoglobulin was anomalous, it was used as an internal marker to compare the calibration and sample elutions. No significant difference was observed in the eluting volume of hemoglobin between the two cases. Cell-free translations were primed with rat liver free polysomes (3.8 A_{260} units/100 ul of lysate). After synthesis the postribosomal supernatant containing [^{35}S]methionine-labeled proteins was adjusted to 0.3 M KCl, and 0.5 ml of the solution was applied to the column. Fractions were subjected to immunoprecipitation and the immunoprecipitates were separated by SDS-10% polyacrylamide gel electrophoresis and visualized by fluorography. The amount of pMDH in each fraction was estimated by densitometric scanning of the pMDH band and then calculating the area under the peaks.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80
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$10^{-3} \times M_r$
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FRACTION
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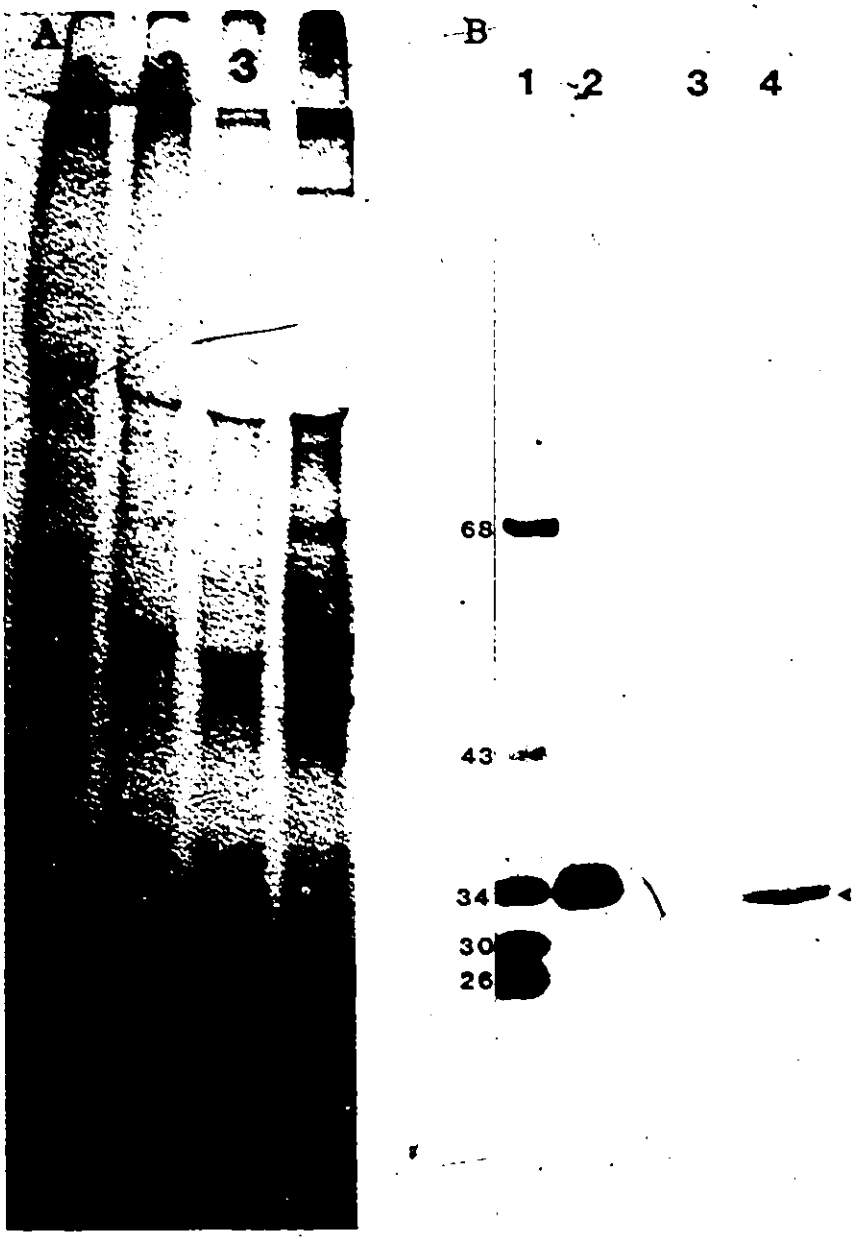
4
2
RELATIVE AMOUNT OF pMDH (cm²)

containing newly synthesized, radioactive precursor was incubated with freshly isolated and gradient purified mitochondria. After incubation, mitochondria were separated from the post-ribosomal supernatant by centrifugation, and both fractions were subjected to immunoprecipitation. As shown in Fig. 5A, newly processed malate dehydrogenase (closed arrow) and a very small amount of precursor (open arrow) could be immunoprecipitated from the mitochondrial fraction by the anti-malate dehydrogenase serum, but not by pre-immune serum (lane 3 versus lane 4). The processed protein had the same size as the [^{14}C]labeled mature form (Fig.5B, lane 4 versus lane 2). Both pMDH and a small amount of newly processed malate dehydrogenase could be immunoprecipitated from the supernatant (Fig. 5A, lane 2). The presence of the newly processed malate dehydrogenase in the supernatant was possibly due to leakage from the mitochondria. Processing was also observed with mitochondria that were not purified by sucrose density gradient centrifugation or with mitochondria isolated from rat XC cells grown in suspension culture; with mitochondria isolated from rat liver, much less processing was observed (results not shown). It should be noted that a protein of about 28 kDa was non-specifically immunoprecipitated, in some cases, by both preimmune serum (Fig. 5A, lane 4) and the anti-serum (Fig. 5A, lane 3, and also in Fig. 6).

The processing of the precursor of malate dehydrogenase was shown to be due to import into mitochondria by the following criteria. First, unlike the precursor form in the supernatant fraction (Fig. 5B, lane 3), newly processed malate dehydrogenase was inaccessible to the

Fig. 5. Processing in vitro of the precursor of malate dehydrogenase by mitochondria isolated from Chinese hamster ovary cells. [^{35}S]Methionine-labeled proteins from cell-free translation primed with rat liver free polysomes were incubated with CHO cell mitochondria. Unless mentioned specifically, incubations were at 30°C for 60 min. A typical reaction mixture contained 1-3 x 10^7 cpm and 300-600 ug of mitochondrial protein in a volume of less than 500 ul. Mitochondria were recovered by centrifugation after the incubation. Both the supernatant and mitochondrial fractions were subjected to immunoprecipitation as described under 'Methods and Materials'. Twenty ul of anti-malate dehydrogenase serum was used for immunoprecipitation from the supernatant, and twice this amount was used for the mitochondrial fraction because of endogenase malate dehydrogenase. Panel A compares immunoprecipitates from preimmune serum (lanes 1 and 4) and anti-malate dehydrogenase serum (lanes 2 and 3); lanes 1 and 2, supernatant (s); lanes 3 and 4, mitochondrial (m) fraction. Panel B shows the protease resistance of the processed malate dehydrogenase. In this case, mitochondria were incubated with the radiolabeled proteins for 30 min and then treated with trypsin (6 ug/100 ug of mitochondrial protein) for 30 min at 0°C. Proteolytic action was stopped with protease inhibitors. Lane 1, molecular mass markers; lane 2, [^{14}C]-labeled malate dehydrogenase; lane 3, supernatant; lane 4, mitochondrial fraction. Panel C demonstrates the inhibitory effect of 10 uM CCCP on processing in vitro of pMDH. The stock CCCP was 1 mM in ethanol; the incubation contained 1% ethanol which had no effect in a control experiment. Lanes


A
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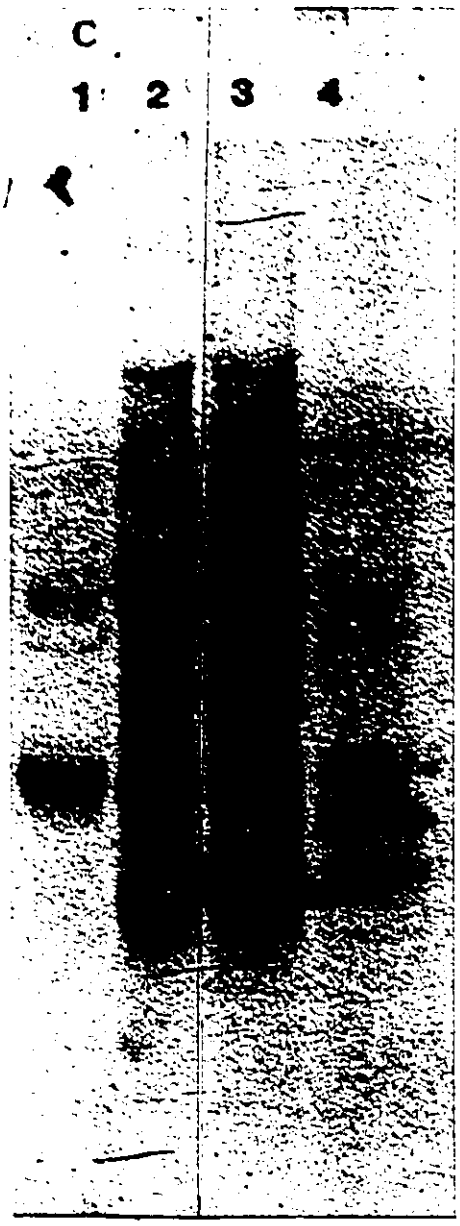
1 and 2, immunoprecipitates from the reaction containing 10 uM CCCP; lanes 3 and 4, immunoprecipitates from the reaction without the uncoupler; lanes 1 and 3, supernatant; lanes 2 and 4, mitochondrial fraction. Panel D shows the effect of o-phenanthroline and EDTA on processing in vitro of pMDH. The incubations contained either 1 mM o-phenanthroline or EDTA. Lanes 1 and 2, immunoprecipitation from the reaction containing o-phenanthroline; lanes 3 and 4, immunoprecipitation from the reaction containing EDTA. Lanes 1 and 3, supernatant; lanes 2 and 4, mitochondrial fraction. (▷), indicates positions of pMDH; (▶), indicates positions of newly processed malate dehydrogenase.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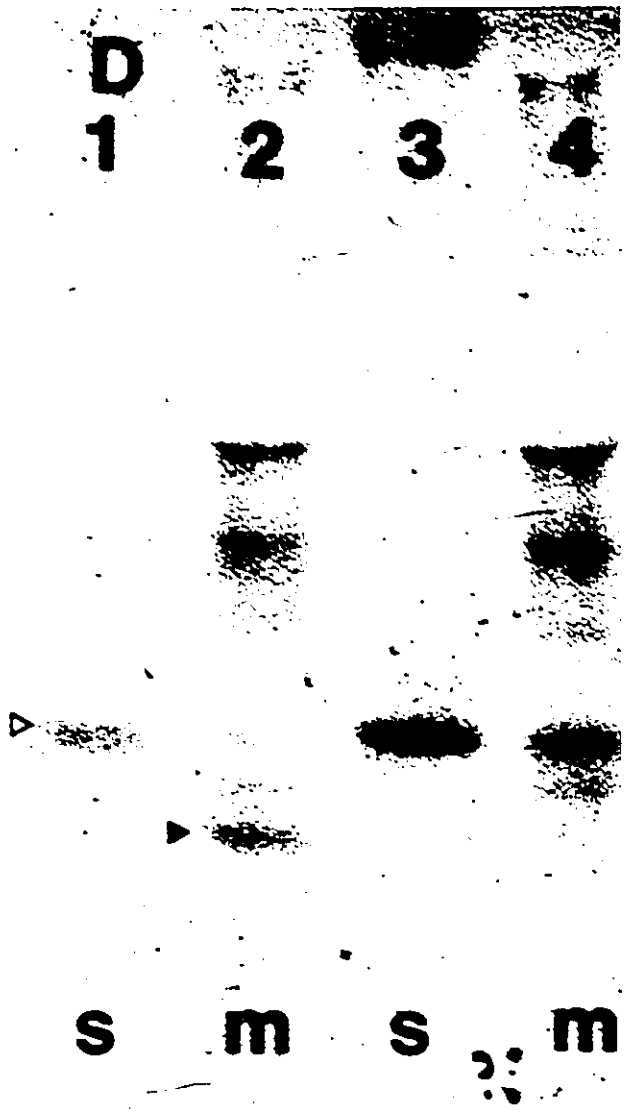
D
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s m s m

proteolytic action of added trypsin (Fig. 5B, lane 4), indicating that it was translocated into mitochondria and, thus, protected from the protease by mitochondrial membranes. Second, processing was inhibited by the uncoupler CCCP (Fig. 5C, lane 2 versus lane 4). As mentioned previously, the translocation of mitochondrial precursor proteins into mitochondria requires the presence of an electrochemical potential across the mitochondrial inner membrane. The inhibition of the processing of the precursor by the uncoupler indicates that the cleavage is a specific event related to the import of the precursor protein. Third, the cleavage of the precursor was inhibited by the membrane permeable chelator, o-phenanthroline, but not by EDTA (Fig. 5D). There is evidence that the protease responsible for the cleavage of larger precursors destined for the inner membrane and the matrix of mitochondria is located in the matrix and requires a metallic cation as cofactor (Bohni et al., 1980; Conboy et al., 1982; McAda et al., 1982; Miura et al., 1982). The inhibition of the processing of pMDH by o-phenanthroline, but not EDTA, indicates the cleavage occurred intra-mitochondrially.

As shown in Fig. 5, in addition to the newly processed form, pMDH was also recovered from the mitochondrial fraction. The nature of this possibly mitochondrially bound pMDH in the import process will be the subject of the next part of Results and Discussion.

The Conversion of the Precursor of Malate Dehydrogenase to the Mature Size is a Direct One Step Process—The cleavage of pMDH to the mature

form by isolated mitochondria at 30°C was followed in a time-course study. As shown in Fig. 6, the processing was essentially completed within 10 min in this experiment, and 30 min in a repeat experiment. Furthermore, no intermediate form was identified in the experiment, suggesting that the conversion of pMDH to the mature form is a one step process.

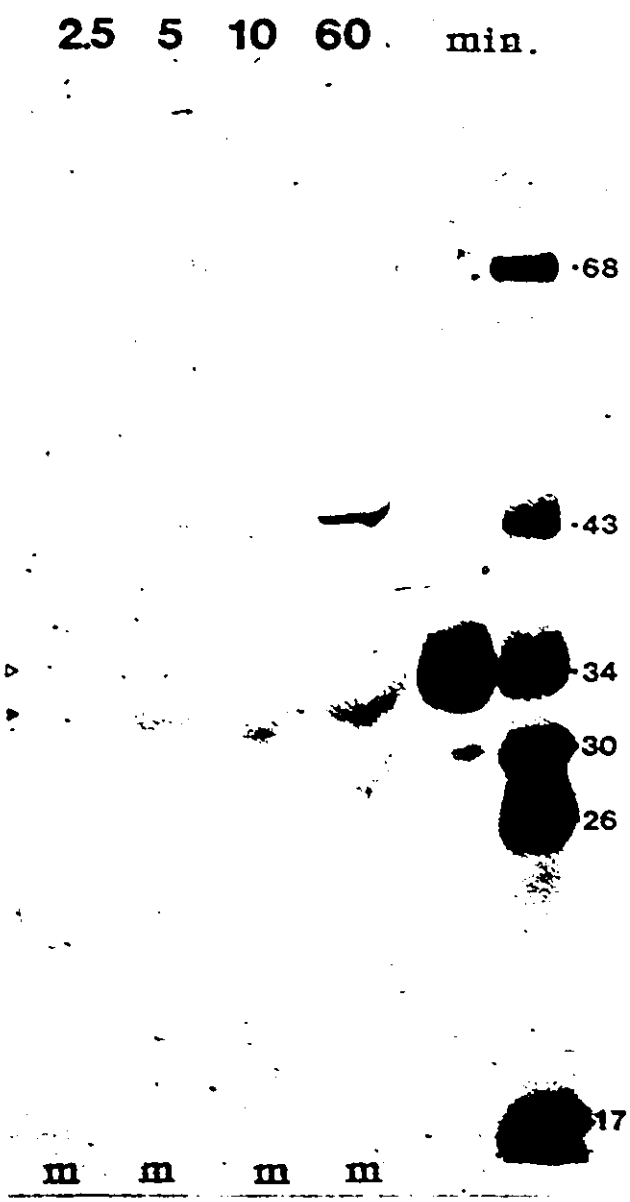
DISCUSSION

The comparison of the two-dimensional peptide maps of the [^{125}I]iodinated malate dehydrodenase and its putative precursor revealed a striking similarity in their primary structures. This result, coupled with the previous finding that immunoprecipitation of the precursor could be competitively inhibited by excess cold mature enzyme (Aziz et al., 1981), strongly established the precursor-protein relationship between the two proteins.

Based on the differential electrophoretic mobility on SDS-polyacrylamide gels, the precursor is about 1.5 to 2 kDa larger than the mature protein. This observation was subsequently confirmed by two other groups (Mihara et al., 1982; Grant et al., 1983). Recently, Gietl et al. (1985) reported that mitochondrial malate dehydrogenase of watermelon is also synthesized as a precursor of larger size.

As discussed previously, many mitochondrial precursor proteins of Fungi and mammals, which are synthesized on cytoplasmic ribosomes,

Fig. 6. Kinetics of processing of the precursor of malate dehydrogenase by isolated mitochondria at 30°C. Mitochondria were incubated at 30°C with an equal volume of *in vitro* synthesized radioactive rat liver protein for various periods of time as indicated on the top of the figure. Each incubation mixture contained 480 ug of mitochondrial protein and 1.4×10^7 cpm of radioactive protein in a final volume of 220 ul. Mitochondria were recovered at the times indicated, and pMDH and newly processed malate dehydrogenase were immunoprecipitated from the mitochondrial (m) fractions by monospecific anti-malate dehydrogenase serum. The immunoprecipitates were analyzed by SDS-10% polyacrylamide gel electrophoresis and fluorography. (▷), pMDH; (▶), newly processed malate dehydrogenase.


2.5 5 10 60 min.
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are larger than their mature forms. Although the size of additional peptides on precursors of matrix enzymes vary widely, most of them fall within the range of 1.5 to 3 kDa (see review by Hay et al., 1984). Results presented here showed that the size of the additional peptide of the precursor of malate dehydrogenase is within this range. It should be noted that not all precursors of matrix enzymes are larger than their mature counterparts. For instance, matrix enzymes, such as 2-isopropylmalate synthase of yeast (Gasser et al., 1982b; Hampsey et al., 1983), and carbamoyl-phosphate synthetase of frog (Mori et al., 1979), are of the same size as the mature proteins.

At present, the location of the additional peptide of pMDH has not yet been determined. In cases where the complete or partial primary structure of precursor proteins is known, the additional peptide is located at the amino terminus (Arends et al., 1984; Hase et al., 1984; Maarse et al., 1984; Sadler et al., 1984; Suissa et al., 1984). It is likely that the additional peptide of pMDH is located at the same position.

The results presented here also indicated that rat liver pMDH, like the other mammalian nuclear-coded mitochondrial proteins, is synthesized on membrane-free polysomes (Mihara et al., 1982). In Neurospora, mitochondrial proteins are also synthesized on the same type of ribosomes. However, the sub-cellular sites of synthesis of yeast mitochondrial proteins is not as clear cut as in Neurospora or in mammals. Butow and his co-workers showed the association of cytoplasmic

ribosomes with the outer surface of mitochondria (Kellems et al., 1972,1974,1975). These ribosomes are particularly enriched in mRNAs that code for mitochondrial proteins and 80% of their translation products are sequestered into mitochondria (Ades and Butow, 1980a,b). However, a study on the distribution of mRNAs of twelve yeast mitochondrial proteins between these mitochondrially bound polysomes and cytoplasmic free polysomes showed that none of the mRNAs studied had an exclusive or predominant association with the mitochondrially bound polysomes (Suissa et al., 1982). It was concluded that these proteins are predominantly synthesized on membrane-free polysomes and that under certain conditions some bind to mitochondria before the polypeptide chain is completed; however this binding is not necessary for import.

The mechanism by which mitochondrial precursor proteins reach the outer surface of mitochondria is still poorly understood. It was shown in the Results that pMDH in the post-ribosomal supernatant has an apparent molecular mass of about 90 kDa, suggesting the precursor may be transported to the outer surface of mitochondria by either interacting with itself or with other cellular components. Rosenberg and his co-workers found that the precursors of rat ornithine transcarbamylase and methylmalonyl-CoA mutase also exist as complexes several times larger than their mature monomeric forms (Fenton et al., 1984; Kalousek et al., 1984). In Neurospora, the precursor of the adenine nucleotide carrier in the post-ribosomal supernatant was found to have a size about five times its mature monomeric form. However, in all cases, the nature of the cellular components interacting with the precursor proteins is

not known.

Recently, the import of rat liver ornithine transcarbamylase into mitochondria was found to require a cytosolic factor (Argan et al., 1983; Miura et al., 1983). In yeast, Ohta and Schatz (1984) showed that the translocation of the purified precursor of the β-subunit of F_1-ATPase into mitochondria also requires the presence of a protein factor with an apparent molecular mass of about 40 kDa. Furthermore, it was reported that the in vitro import of the precursor of rat ornithine transcarbamylase was inhibited by high concentration of RNase (100 ug/ml reaction mixture), indicating that the process may require a RNA-sensitive component (Firgaira et al., 1985). Thus, it seems that both proteinaceous and perphaps RNA-sensitive factors are required for the import of at least some mitochondrial precursor proteins. It is not known whether these factors interact directly with the precursor proteins or facilitate the import indirectly. In the case of secretory proteins, the growing polypeptide chain on a free ribosome is directed to the membrane of rough endoplasmic reticulum by a signal recognition particle which consists of six polypeptides and a 7S RNA particle (Walter et al., 1982).

The most significant result presented here is the import of the in vitro synthesized precursor of malate dehydrogenase into isolated mitochondria. The import occurs in the absence of protein synthesis and results in the processing of the precursor to its mature size. As shown in Fig 5B, only the processed precursor was protease protected,

indicating that it was translocated into mitochondria. Translocation into mitochondria was inhibited by an uncoupler. This result correlates with the general finding in yeast and Neurospora that the translocation of precursor proteins across the mitochondrial inner membrane requires the presence of an electrochemical potential across the membrane.

No intermediate form was identified in the time course study on the processing of pMDH by isolated mitochondria (Fig. 6). This indicates the conversion of the precursor to the mature size is a direct one step process. Most mitochondrial precursor proteins are processed to their mature size in a single step. However, exceptions have been found in some inter-membrane space proteins, cytochrome c_1 and cytochrome b_2 (Daum et al., 1979; Gasser et al., 1982a) and the precursor of subunit 9 of Neurospora ATPase. These proteins are converted to their mature size by two proteolytic cleavages, the second of which occurs in the inter-membrane space and leads to the final localization of the proteins. In the case of ornithine transcarbamylase, an intermediate form was observed during import and processing of the precursor protein by rat liver mitochondria in vitro (Kolansky et al., 1982; Morita et al., 1982). However, Argan et al. (1983) showed that the intermediate form is sensitive to exogenous protease and does not convert to the mature form when added to fresh mitochondria after recovery from an in vitro import assay. At present, it is not clear whether the processing of the precursor is necessarily a two-step event.

In summary, the results presented here demonstrated that rat

liver malate dehydrogenase is synthesized on free polysomes as a precursor of about 1.5-2.0 kDa larger than the mature monomeric form. The precursor exists as a 90 kDa complex in the post-ribosomal supernatant, and can be imported into isolated mitochondria in the absence of protein synthesis. The in vitro translocation of the precursor into mitochondria requires the presence of an electrochemical potential across the mitochondrial inner membrane, and the precursor is converted to the mature size with a single proteolytic cleavage, which is performed by a chelator sensitive protease in the matrix. At present, it is not known how the polypeptide is translocated across the two mitochondrial membranes. Nor is it clear whether the proteolytic processing occurs during or after the translocation. It is possible that the translocation of the precursor across the mitochondrial membranes requires transmembrane proteins that are located near possible sites of apposition of the two membranes and that the processing of the extension peptide occurs as soon as it penetrates into the matrix.

In addition, the demonstration of uptake and processing of the precursor of malate dehydrogenase by isolated mitochondria from CHO cells showed that the in vitro import system can be used to probe the details of the import mechanism of mitochondrial proteins.

(II) BINDING OF THE PRECURSOR TO MITOCHONRIA—AN INTERMEDIATE STEP IN IMPORT

Experiments with the in vitro import of the precursor of malate dehydrogenase showed that both the precursor and the newly processed form could be recovered in the mitochondrial fraction (c.f. Fig. 5). The association of pMDH with mitochondria could be due to non-specific interaction between the precursor and the mitochondrial outer membrane, or a specific interaction, which would subsequently results in the transfer of the precursor into the matrix. The latter hypothesis imply the presence of specific receptors on mitochondria for pMDH.

A genuine ligand-receptor interaction would have the following characteristics: (i) the binding process is temperature insensitive, energy independent and is completed rapidly; (ii) there is a specific recognition between the ligand and the receptor; (iii) there is a finite number of binding sites, demonstrating saturation in the presence of excess ligand; (iv) the interaction would result in a subsequent biological effect (Cuatrecasas, 1974; Kahn and Roth, 1975).

In this part of Results and Discussion, the binding of pMDH to mitochondria is characterized according to the above criteria. In vitro synthesized, [^{35}S]methionine-labeled rat liver proteins were incubated with isolated mitochondria under a variety conditions to examine the different properties of the interaction. The aim was to investigate whether the binding of pMDH to mitochondria is an obligatory step in the

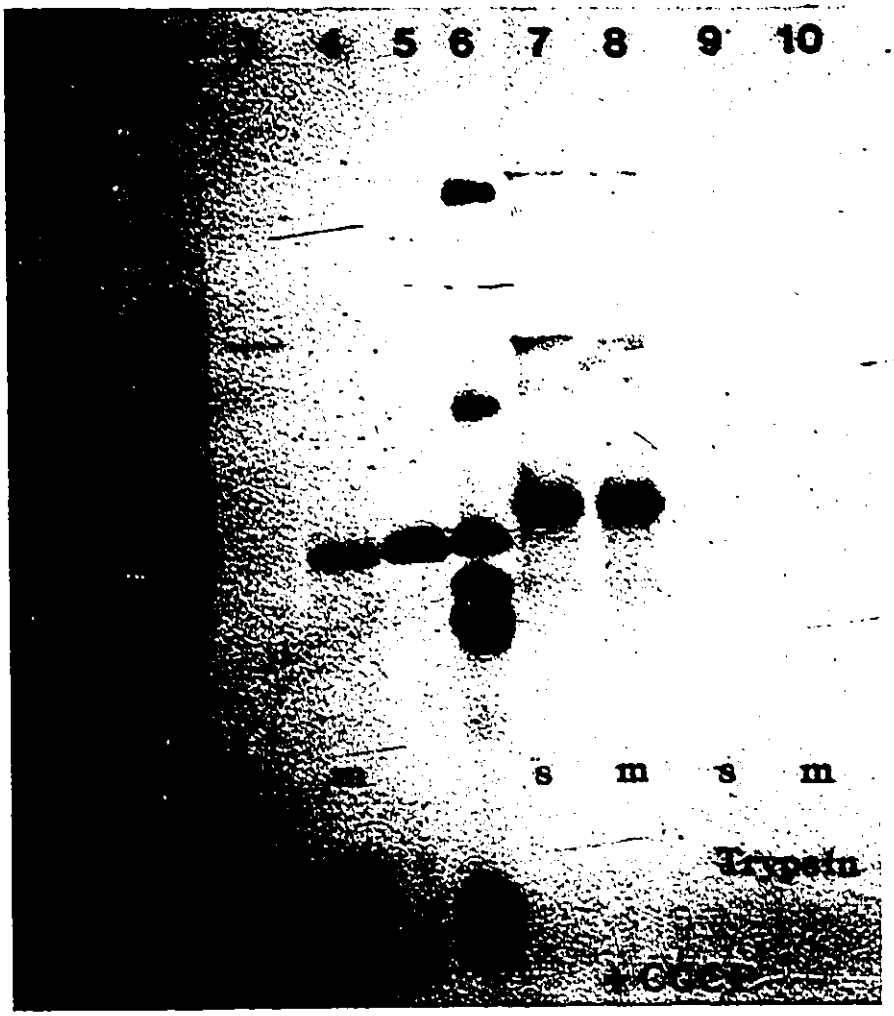
import of the precursor protein into mitochondria.

RESULTS

The Precursor of Malate Dehydrogenase in the Mitochondrial Fraction is Protease Sensitive—The newly processed form of malate dehydrogenase was insensitive to trypsin added to the in vitro import system, indicating that it was inside mitochondria (Fig. 7 lane 4). However, pMDH recovered in the mitochondrial fraction (lanes 2, 8 versus lanes 4, 10), like those in the supernatant (lanes 1, 7 versus lanes 3, 9), was degraded by the externally added protease. This indicates that the precursor in the mitochondrial fraction is located on the mitochondrial outer membrane. Furthermore, only the precursor of malate dehydrogenase (open pointer) was detected in the mitochondrial fraction in the presence of the uncoupler CCCP (lane 8). However, in the absence of the uncoupler (lane 2), a significant amount of newly processed form (closed pointer) was detected in the mitochondrial fraction, with a concomitant decrease in the amount of the precursor. This phenomenon was observed repeatedly in different experiments (see Fig. 5) and suggests that binding of the precursor to the mitochondrial outer membrane may be an early obligatory step in the import process, prior to the translocation of the precursor across the membranes. The amount of the newly processed form obtained from different experiments varied from 50% to 100% of the two forms in the mitochondrial fraction.

The Binding of The Precursor of Malate Dehydrogenase is Temperature

Fig. 7. Malate dehydrogenase precursor, but not newly processed malate dehydrogenase in the mitochondrial fraction, is protease sensitive. Mitochondria (480 ug of protein in 150 ul) were incubated with an equal volume of rat liver free polysomal translation products (2.7×10^7 cpm) at 30°C, in the absence (lanes 1-4) or in the presence of 10 uM CCCP (lanes 7-10). The incubation was stopped after 45 min by rapid cooling in an ice-water bath. One-half of each reaction mixture was treated with trypsin (15 ul of 1 mg/ml) at 0°C for 30 min. Proteolysis was stopped by adding soybean trypsin inhibitor (30 ul of 10 mg/ml) and phenylmethylsulfonyl fluoride (10 ul of 1 mg/ml) to the appropriate reaction mixtures, and after 5 min, mitochondria were recovered by centrifugation and washed as described under 'Methods and Materials'. The initial supernatant (s) and the mitochondrial (m) fractions of all four reaction mixtures were subjected to immunoprecipitation with 15 ul and 30 ul respectively of monospecific anti-malate dehydrogenase serum. The immunoprecipitates were analyzed by SDS-10% polyacrylamide gel electrophoresis with subsequent fluorography. Lanes 1-4 were the reaction mixtures in the absence CCCP, without (lanes 1 and 2) or with (lanes 3 and 4) subsequent trypsin treatment. Lanes 7-10 were the reaction mixtures in the presence of CCCP, without (lanes 7 and 8) or with (lanes 9 and 10) subsequent trypsin treatment. Lane 5, [^{14}C]formaldehyde-labeled molecular mass markers: bovine serum albumin (68 kDa), ovalbumin (43 kDa), c-subunit of aspartate transcarbamoylase (34 kDa), carbonic anhydrase (30 kDa), chymotrypsinogen A (26 kDa), and r-subunit of aspartate transcarbamoylase (17 kDa). ( ), malate dehydrogenase precursor; ( ), newly processed malate dehydrogenase.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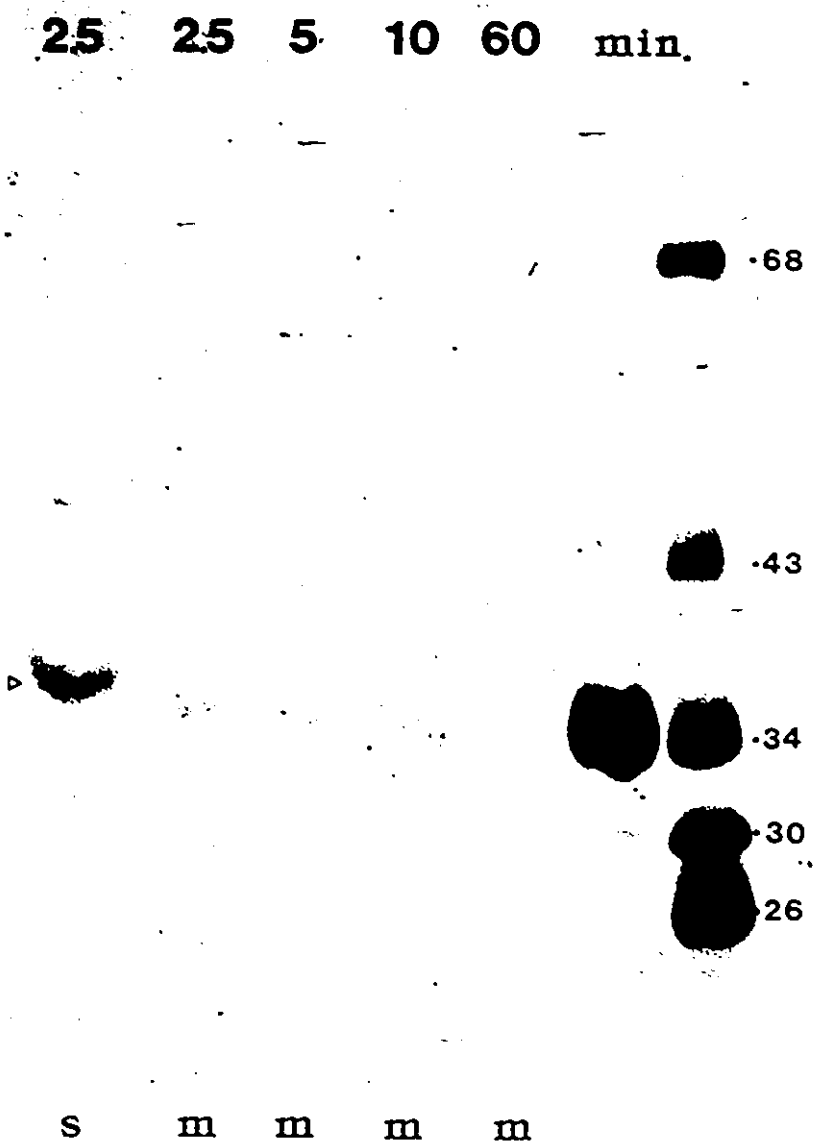
Trypsin
+Odcp

Independent—When isolated mitochondria were incubated at 0°C with in vitro synthesized rat liver proteins, the major form of malate dehydrogenase in the mitochondrial fraction was the larger precursor (Fig. 8, lane 1). The sensitivity of this form to protease indicates that the precursor was on the cytoplasmic side of the mitochondrial outer membrane (Fig. 9, lane 1 versus lane 2). The binding occurred very rapidly at 0°C and was essentially completed within 2.5 min. Although pMDH could still be detected in the supernatant (Fig. 8, lane 2.5 min, S), no further increase in the amount of bound precursor was observed after 2.5 min. Furthermore, no noticeable difference in the amount of pMDH bound at 0°C (Fig. 8) or at 30°C as described previously (Fig. 6), was observed. The two experiments were performed at the same time with the same preparation of mitochondria. These results suggest that the binding of the precursor of malate dehydrogenase to mitochondria is not an energy dependent process.

On the other hand, the rate of processing of the precursor of malate dehydrogenase to the mature size was greatly dependent on temperature. Only a minute amount of newly processed form could be detected after a 10 or 60 min incubation at 0°C (Fig. 8). This is in sharp contrast to the observation of the large amount of newly processed form in the mitochondrial fraction when the experiment was performed at 30°C (Fig 6).

Saturation of The Binding Sites—In order to determine whether there is a saturable number of binding sites for the precursor of malate

Fig. 8. Binding of the precursor of malate dehydrogenase to mitochondria at 0°C. Mitochondria were incubated at 0°C for various times with an equal volume of *in vitro* synthesized radioactive rat liver protein for various times as indicated on the top of the figure. This experiment was done in parallel to that in Fig. 6. The experimental details are the same except for the temperature of incubation and the additional immunoprecipitation of the 2.5 min supernatant (s) fraction. Molecular mass markers are shown.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68
43
34
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s m m m m

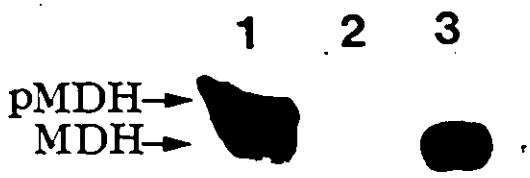


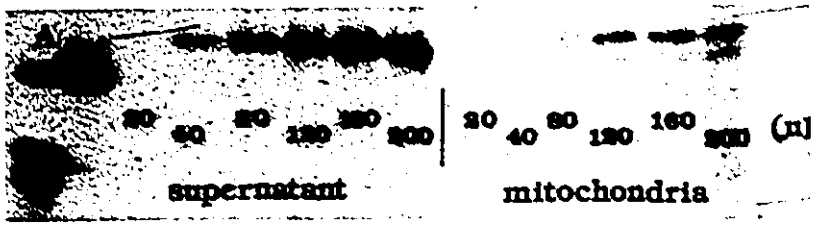
Fig. 9. Precursor of malate dehydrogenase bound to mitochondria at 0°C is protease sensitive. Mitochondria (1500 ug of protein in 0.3 ml) were incubated with an equal volume of rat liver free polysomal translation products (1.0×10^7 cpm) at 0°C for 5 min. The incubation was divided into two fractions, and one was treated with trypsin (6 ug/100 ug of mitochondrial protein) (lane 2) as described in Fig. 7. Mitochondria were recovered and subjected to immunoprecipitation and the immunoprecipitates were analyzed by SDS-10% polyacrylamide gel electrophoresis with subsequent fluorography. Lanes 1 and 2; reaction mixtures without and with trypsin treatment respectively; lanes 3, [^{14}C]formaldehyde-labeled bovine malate dehydrogenase. pMDH, precursor of malate dehydrogenase; MDH, mature form of malate dehydrogenase.

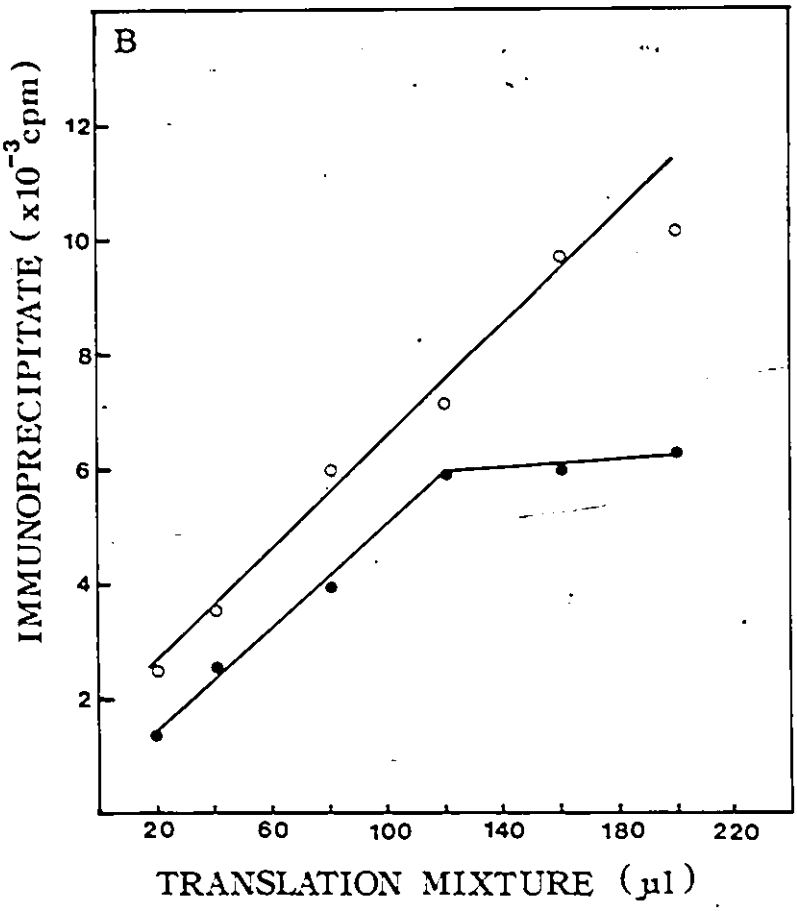
dehydrogenase on the mitochondrial outer membrane, a defined system that examines only the binding process is required. The facts that binding, but not processsing, occurs in the absence of an electrochemical potential (Fig. 7) and that pMDH can easily be distinguished from its newly processed form by SDS-polyacrylamide gel electrophoresis allow the constitution of an in vitro system for studying binding without the influence of the processing step.

As shown in Fig. 10, when a small fixed amount of mitochondria was incubated with increasing amounts of in vitro synthesized, [^{35}S]methionine rat liver protein in the presence of CCCP, the amount of bound precursor increased linearly, but then leveled off at a large amount of radiolabeled protein (Fig. 10 B). This saturation phenomenon was observed in three separate experiments and indicates that there is a limited number of binding sites on the mitochondrial outer membrane. In addition, the bound pMDH was found to be tightly associated with mitochondria, since after the initial washing, there was a complete recovery on the second wash and 92% recovery on the third wash. It should be noted that at low amounts of radiolabeled protein, there was a significant amount of precursor recovered from the supernatant after incubation with mitochondria (Fig 10A), though more binding sites for the precursor should still be available. The reason for this is not known.

Specificity of the Binding Sites--Since only approximately 0.02% of the incorporated cpm in an in vitro translation mixture of rat liver free

Fig. 10. Saturation of binding sites for malate dehydrogenase precursor on mitochondria by increasing amounts of translation mixture. Mitochondria (150 ug of protein from a suspension at 3.7 mg/ml) were incubated in the presence of 10 uM CCCP with a range of volumes of translation mixture containing radioactive *in vitro* synthesized rat liver proteins (4.3×10^6 cpm/ul) as indicated on the abscissa. The final volume of each reaction mixture was adjusted to 300 ul using non-translated nuclease-treated reticulocyte lysate. Incubations were for 30 min at 30°C. Mitochondria were recovered by centrifugation and washed once with a solution containing 0.3 M sucrose, 2 mM EDTA, and 2 mM Tris-HCl, pH 7.2. Immunoprecipitation was carried out on both the supernatant and the mitochondrial fractions. Five per cent of each immunoprecipitate was counted (B) and the rest was analyzed by SDS-10% polyacrylamide gel electrophoresis with subsequent fluorography (A). The band below the precursor in the mitochondrial fractions is the result of non-specific binding; its size is greater than that of mature malate dehydrogenase. (O), Total counts of immunoprecipitates from the supernatant; (●), total counts of immunoprecipitates from the mitochondrial fractions. In A, the molecular mass markers of 26 kDa, 30 kDa, and 34 kDa, are shown as well as mature malate dehydrogenase (▶) and malate dehydrogenase precursor (▷).


supernatant
mitochondria
(µl)


B
IMMUNOPRECIPITATE (x10^{-3}cpm)
12
10
8
6
4
2
20
60
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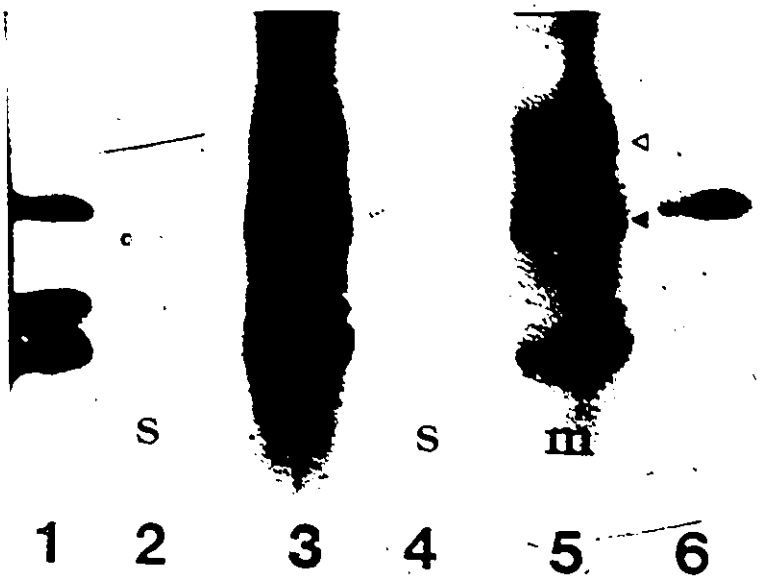
140
180
220
TRANSLATION MIXTURE (µl)

polysomes is in the precursor of malate dehydrogenase, it is very difficult, at present, to obtain a large quantity of pure pMDH. This precludes the possibility of demonstrating the specificity of the binding sites by using a large amount of cold ligand to compete with radioactive ligand for the binding sites. However, the specificity of the binding sites was tested indirectly by examining the effect of excess cold mature malate dehydrogenase on the binding as well as the import process.

As shown in Fig. 11, preincubation of mitochondria with 60 ug of cold bovine mature enzyme at 0°C for 15 min before addition of in vitro synthesized radiolabeled protein (lane 3), or mixing 12.5 ug of the cold enzyme with mitochondria just prior to addition of radiolabeled protein (lane 5), did not affect the binding of the precursor (open pointer) or its processing (closed pointer) by isolated mitochondria (cf. Fig. 7). The amount of malate dehydrogenase used in both situations was probably in great excess of the total amount of pMDH present in the reaction mixture.

In contrast to the mitochondrial fraction, pMDH was not detected in the supernatant under either condition (Fig. 11, lanes 2 and 4). The apparent absence of the precursor in the supernatant was the result of competition of the precursor with the externally added cold enzyme for immunoprecipitation. The lack of competition in the mitochondrial fraction indicates that most of the added cold enzyme remained in the

Fig. 11. Mature malate dehydrogenase does not inhibit binding or processing of the precursor of malate dehydrogenase by isolated mitochondria. Mitochondria were either co-incubated or pre-incubated with mature malate dehydrogenase to examine its effect on the import of pMDH. The co-incubation contained 12.5 ug bovine heart malate dehydrogenase, 600 ug mitochondrial protein, and 3.9×10^7 cpm of *in vitro* synthesized rat liver proteins in a final volume of 300 ul. The incubation was for 60 min at 30°C. For the pre-incubation, 60 ug of bovine heart malate dehydrogenase was incubated at 0°C for 15 min with 600 ug mitochondrial protein in 300 ul of a solution containing 0.3 M sucrose, 2 mM EDTA and 10 mM Tris-HCl, pH 7.2. Mitochondria were recovered by centrifugation, washed once in the above medium, and resuspended in100 ul of this medium. The mitochondria were incubated with 200 ul of *in vitro* synthesized rat liver protein (4.3×10^7 cpm) for 60 min at 30°C. Supernatant (s) and mitochondria (m) were separated, subjected to immunoprecipitation and analyzed by SDS-10% polyacrylamide gel electrophoresis. Lane 1, [^{14}C]formaldehyde-labeled molecular mass markers, c-subunit of aspartate transcarbamoylase (34 kDa), carbonic anhydrase (30 kDa) and chymotrypsinogen A (26 kDa); lanes 2 and 3, supernatant and mitochondrial fractions respectively from the co-incubation assay; lanes 4 and 5, supernatant and mitochondrial fractions respectively from the pre-incubation assay; lane 6, [^{14}C]formaldehyde-labeled malate dehydrogenase.


S
S
m
1
2
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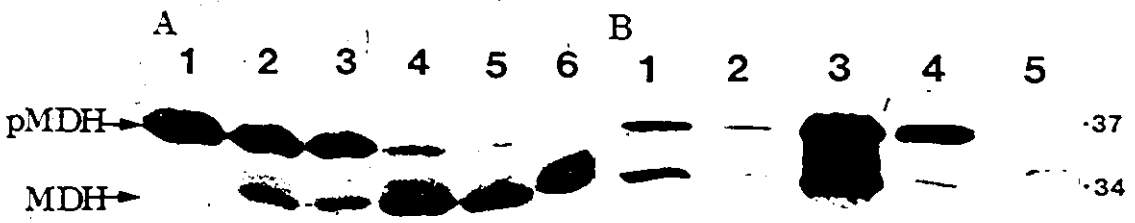
6

supernatant. If there was any of it in the mitochondrial fraction, the amount was probably negligible. These results showed that mature enzyme does not interact with mitochondria, and reflect indirectly the specificity of the binding of pMDH to the mitochondrial outer membrane. This conclusion is based on the assumption that there is no species or tissue specificity involved in the binding or processing of pMDH. This is probably valid since the rat liver pMDH can be imported into mitochondria of CHO cells. A similar lack of inhibition by mature protein on import of mitochondrial protein precursor has also been observed in yeast (Butow and Schatz, 1983).

Processing of Mitochondrially Bound Malate dehydrogenase Precursor—If binding is an intermediate step in import, the bound form of the precursor should be directly processed and the rate of processing should be independent of the volume of the reaction mixture. This was examined by allowing binding, but not processing, at 0°C, re-isolating the mitochondria, and incubating at 30°C to permit processing. As shown in Fig. 12A, when the initial reaction mixture was incubated at 0°C for 5 min, the precursor was the predominant form in the reisolated mitochondria. After reincubation of the mitochondria in 0.4 ml (lane 4) and 2.0 ml (lane 5) at 30°C for 30 min, 80% of the malate dehydrogenase recovered was in the processed form in both cases. In contrast, after reincubation of the mitochondria at 0°C for 30 min, only 25% was in the processed form (lanes 2 and 3). At both temperatures, there was lower recovery (83%) of both forms of malate dehydrogenase when the incubation was done in 2 ml (lanes 3 and 5). Thus, the results in Fig. 12A indicate

Fig. 12. Processing of prebound precursor but not unbound precursor is independent of reaction volume. Panel A, mitochondria with bound precursor were prepared by incubating 0.8 ml of gradient purified mitochondria (4.2 mg/ml) with an equal volume of [^{35}S]methionine-labeled rat liver free polysomal translation products (2 x 10^7 cpm) for 5 min at 0°C. Mitochondria were recovered by centrifugation in an Eppendorf centrifuge for 15 min. The pellet was rinsed two times with 1 ml of a solution containing 0.3 M sucrose, 2 mM EDTA, and 2 mM Tris-HCl, pH 7.2. The mitochondrial pellet was then resuspended in 0.8 ml of the same solution and divided into five aliquots. Two aliquots were adjusted to 0.4 ml with nuclease-treated, non-translated lysate and two were adjusted to 2 ml with 0.84 ml of the sucrose-containing solution and 1.0 ml of nuclease-treated, nontranslated lysate. The remaining aliquot was processed immediately for immunoprecipitation. One reaction mixture of 0.4 ml and one of 2.0 ml was incubated at 0°C for 30 min and the other set of 0.4 and 2.0 ml reaction mixtures was incubated at 30°C for the same time. Subsequently, mitochondria were recovered by centrifugation, processed for immunoprecipitation and analyzed on SDS-10% polyacrylamide gels. Lane 1, immediate immunoprecipitation; lanes 2 and 3, incubation at 0°C for 30 min in 0.4 and 2.0 ml, respectively; lanes 4 and 5, incubation at 30°C for 30 min in 0.4 and 2.0 ml, respectively; lane 6, [^{14}C]formaldehyde-labeled bovine malate dehydrogenase. Panel B, in a similar experiment mitochondria containing bound precursor were prepared and reincubated as described above except that the incubations were at 30°C for 5 min in 0.2 ml (lane 1) and 2.0 ml (lane 2). Mitochondria

were also incubated with an equal volume of rat liver free polysomal translation products at 30°C for 5 min in 0.2 ml (lane 3) and 2.0 ml (lane 4). In all cases, mitochondria were recovered and processed for immunoprecipitation. Lane 5, [^{14}C]formaldehyde-labeled bovine malate dehydrogenase. The position of molecular mass markers (kDa) are shown on the right side of panel B.



that processing of pre-bound precursor was independent of reaction volume.

This result was extended by comparing the extent of processing of the bound pMDH to that of unbound precursor at 30°C for 5 min. As shown in Fig. 12B, the proportion of prebound precursor processed in incubations of 0.2 ml (lane 1) and 2.0 ml (lane 2) was about the same, being 55% and 50% respectively. In contrast, the proportion of the processed form was significantly decreased when previously unbound precursor was bound and processed in a larger volume (lane 4 versus lane 3). In these cases, the proportion of the processed form in incubations of 0.2 ml (lane 4) and 2 ml (lane 5) was 43% and 19% respectively.

DISCUSSION

The results presented above strongly suggest that the binding of the precursor of malate dehydrogenase to the mitochondrial outer membrane is a specific event. Import of *in vitro* synthesized rat liver pMDH into mitochondria can be arrested at the binding stage either by abolishing the electrochemical potential, in the presence of an uncoupler, or by lowering the temperature to 0°C. The binding process fulfills most of the criteria for a specific ligand-receptor interaction. Binding, unlike processing, was temperature insensitive, and completed very rapidly (within 2.5 min) at 0°C. There is an apparent saturable number of binding sites. The specificity of these binding sites is reflected, though indirectly, by the lack of

interference by mature malate dehydrogenase with the binding or the processing of the precursor of malate dehydrogenase.

The most significant result presented here is the finding that prebound precursor was processed to the mature size and that the rate of its processing, unlike that of unbound precursor, is independent of the volume of reaction mixture. This strongly suggests that the binding of pMDH to mitochondria is an intermediate step in the import process. Furthermore, the protease sensitivity of the bound precursor indicates that the presumptive receptors for pMDH are located on the mitochondrial outer membrane.

Although the results presented indicate that there is a limitied number of binding sites for pMDH on the mitochondrial outer membrane, the specificity of these binding sites is not known. The binding sites could be specific for the precursor alone or, for example, could be specific for one or all groups of mitochondrial precursor proteins (e.g. matrix proteins and/or inner membrane proteins). For instances, binding sites for the precursor of ornithine transcarbamylase and cytochrome c have also been identified on the outer membrane of rat liver mitochondria (Matsuura et al., 1981; Argan et al., 1983). In the case of ornithine transcarbamylase, these binding sites are protease sensitive (Argan et al., 1983). However, it is not known whether these precursors share the same binding sites for import. Thus, the saturation phenomenon observed for the binding of pMDH could reflect either saturation of binding sites by the precursor or by all or some of the nuclear-coded

mitochondrial precursor proteins.

In Neurospora, Neupert and his co-workers showed that there are different receptors on the mitochondrial outer membranes for importing precursor proteins. Zimmermann et al. (1981) showed that excess amount of unlabeled chemically prepared apocytochrome c, which would inhibit the import of in vitro synthesized apocytochrome c, did not interfere with the translocation of the precursor of the adenine nucleotide carrier or subunit 9 of ATPase into isolated mitochondria. Furthermore, Zwisinski et al. (1984) showed that the receptors for the latter two precursors have different sensitivity towards the protease, elastase.

Receptors for mitochondrial precursor proteins have also been found on the mitochondrial outer membrane of yeast (Riezman et al., 1983a,b). The precursor of cytochrome b_2 was shown to bind specifically to the outer membrane, but not to the inner membrane or microsomes. Furthermore, mature cytochrome b_2 did not compete with its precursor for the binding sites.

In this part of Results and Discussion, binding sites for pMDH on the mitochondrial outer membrane were demonstrated and characterized by arresting the import process prior to translocation. However, it seems that not all precursor proteins bind to mitochondria in the absence of import. For instances, the precursors of yeast ATPase subunit 2 and cytochrome b_2, as well as the precursors of subunits I and VII of the Neurospora cytochrome bc_1 complex bind poorly to mitochondria in the

presence of an uncoupler (Daum et al., 1982; Gasser et al., 1982b; Teintze et al., 1983). These observations have led to the suggestion that binding of some mitochondrial precursor proteinsmay require an electrochemical potential across the mitochondrial inner membrane.

In summary, the results presented here strongly suggest the binding of pMDH to specific receptors on the mitochondrial outer membrane is an obligatory step in the import of the precursor protein. The specific interaction between pMDH and its receptor would trigger the electrochemical-dependent transfer of the precursor across the mitochondria membrane to the matrix side, where removal of the extension peptide occurs.

(III) ACQUSITION OF A NAD^+-BINDING SITE BY THE NEWLY PROCESSED PROTEIN

The results presented above indicate that isolated mitochondria import and process the precursor of malate dehydrogenase to its mature size. However, it is essential to show that the import reconstituted in the in vitro system adequately reflects the process that occurs in vivo. This is demonstrated in this part of the Results and Discussion by examining the affinity of the precursor of malate dehydrogenase and its in vitro processed form to the 5'-AMP-Sepharose affinity column. Rat liver malate dehydrogenase binds to 5'-AMP-Sepharose because it requires NAD^+ (NADH) as a co-enzyme and each of its monomeric subunit contains a binding site for the co-enzyme (see review by Banaszak and Bradshaw, 1975). The bound enzyme can be eluted from the affinity column with 40 uM NADH (Aziz et al., 1981). Demonstration of a NAD^+-binding site on the newly processed form, but not the larger precursor form, would indicate that the in vitro imported protein has probably been transformed into its functional form and reflects the genuineness of the in vitro import system.

In addition, possible conformational changes of the precursor protein during its import were followed by examining the reaction of the larger precursor and its newly processed form with the antiserum raised against the denatured enzyme. As reported previously, the antiserum recognizes the denatured form, but not the native form, of the mature enzyme (Aziz et al., 1981).

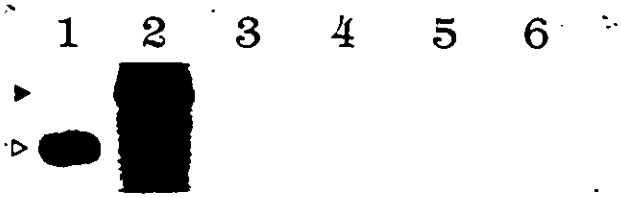
RESULTS

The Precursor of Malate Dehydrogenase Does Not Bind to an Affinity Column of 5'-AMP-Sepharose--The mitochondrial form of malate dehydrogenase has been purified to homogeneity by the 5'-AMP-Sepharose affinity column in a solution containing 4 mM MSH, 2mM EDTA and 20 mM sodium phosphate, pH 7.0 (Aziz et al., 1981). The enzyme can be eluted by washing the column with 40 uM NADH. However, as shown in Fig. 13, in vitro synthesized pMDH, in the translation mixture, did not bind to the same affinity column. Instead, the precursor was recovered by immunoprecipitation in the flow through fractions collected when the column was washed with 40 uM NADH (Fig. 13, lanes 3-6). The lack of binding did not seem to be because of the increased ionic strength in the translation mixture (0.18 M KCl), since a control experiment showed that the mature enzyme added to a translation mixture did bind to the affinity column and could be eluted by 40 uM NADH (see legend to Fig. 13). These results strongly suggest that the binding sites for NAD^+, which are present on the functional protein, are absent or not available in the precursor form of the enzyme.

The Newly Processed Form of The Precursor of Malate Dehydrogenase Shows Affinity for 5'-AMP-Sepharose--The radioactive precursor form of malate dehydrogenase was synthesized in vitro and imported into isolated mitochondria as described previously. The newly processed form, together with the mature endogenous enzyme, was released from mitochondria by

Fig. 13. The precursor of malate dehydrogenase does not bind to an affinity column of 5'-AMP-Sepharose. Rat liver free polysomes were translated in a rabbit reticulocyte lysate as described under 'Methods and Materials'. About 7.6 x 10^7 cpm of [^{35}S]methionine-labeled translation products in the post-ribosomal supernatant were applied to a 0.5 ml column of 5'-AMP-Sepharose equlibrated with binding solution containing 4 mM mercaptoethanol, 2 mM EDTA and 20 mM sodium phosphate, pH 7.0. The column was washed with 4 bed volumes of the binding solution, and then eluted with the same solution but containing 40 uM NADH after the washing solution was collected. In total, fourteen 0.5 ml fractions were collected. For immunoprecipitation, 0.2 ml of each fraction was adjusted to 4% SDS and 10 mM mercaptoethanol, boiled for 5 min and cooled to room temperature. Then 1 ml of adjusting buffer containing 0.15 M NaCl, 5 mM EDTA, 1% Triton X-100 and 50 mM Tris-HCl, pH 7.2 was added, followed by 20 ul of antiserum after 30 min. Immunoprecipitates, recovered by protein A-Sepharose, were pooled in groups of three fractions and analyzed by SDS-10% polyacrylamide gel electrophoresis. In a control experiment, a rat liver mitochondrial extract containing 19 units of malate dehydrogenase was added to a transaltion mixture and applied to an identical column and the column was washed with the 20 mM sodium phosphate binding solution. No enzyme activity was recovered in fractions 1 to 4. However, application of the binding solution containing 40 uM NADH eluted the following percentage of the applied enzyme activity in fractions 5 to 13: 0, 0, 15, 15, 15, 11, 9, 6 and 6. Lane 1, [^{14}C]formaldehyde-labeled bovine malate

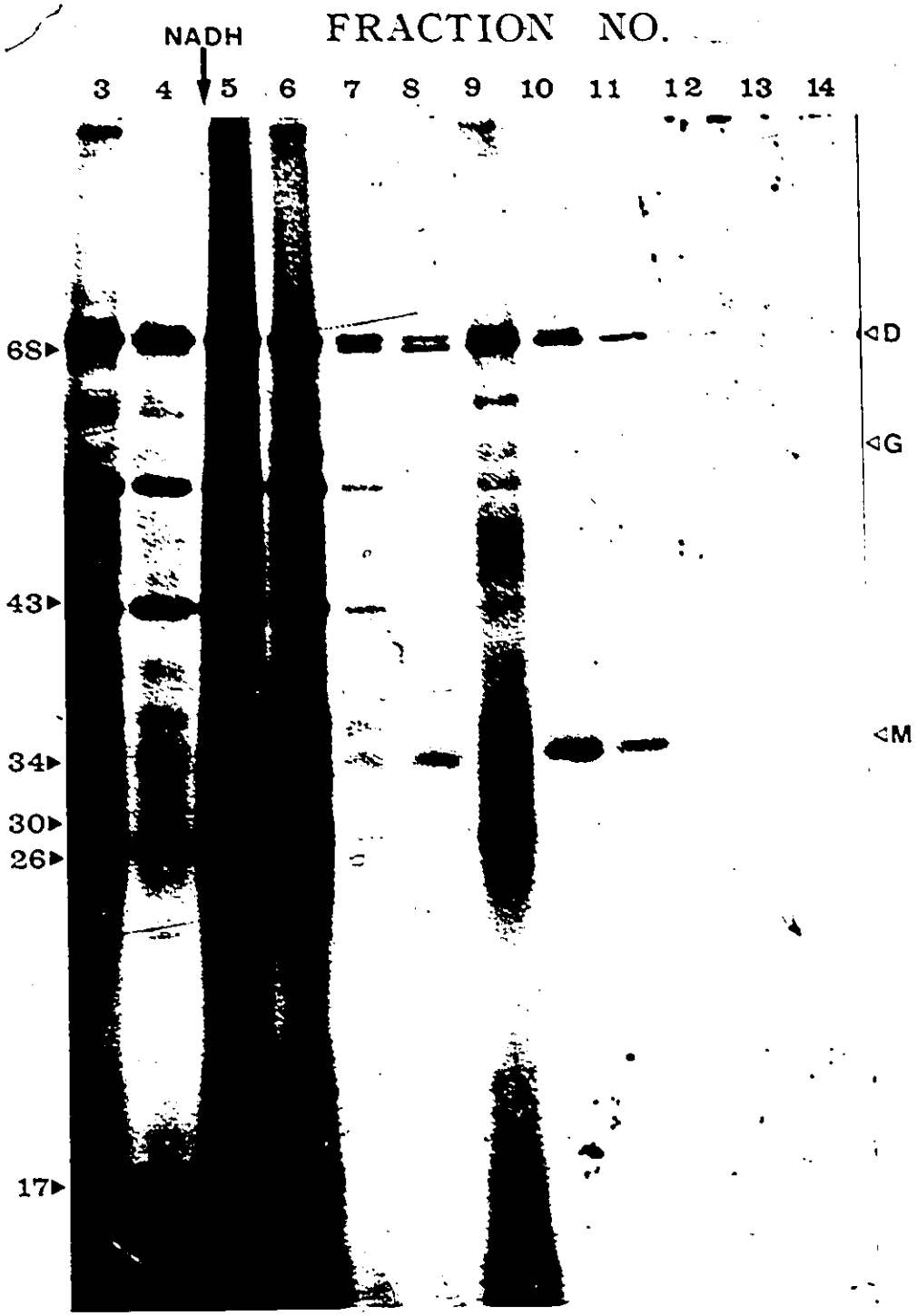
dehydrogenase; lanes 2-6, immunoprecipitates from pooled samples of fractions 1-3, 4-6, 7-9, 10-12, and 13-14, respectively.



sonication. After centrifugation, more than 90% of the enzyme activity and of the newly processed form were recovered in the supernatant fraction. This fraction was applied to an affinity column and the same chromatographic procedure as described in Fig. 13 was followed. A sample from each collected fraction was analyzed by electrophoresis on a SDS-polyacrylamide gel with subsequent fluorography. The resultant fluorogram is shown in Fig. 14. Elution with 40 uM NADH yielded a distinct radioactive band, with an apparent molecular mass of about 34 kDa, in fractions 7 to 13. The amount of the radioactive protein in each fraction was quantitated by densitometric scanning, and compared with the activity of malate dehydrogenase measured in the fraction. As shown in Fig. 15, there was a striking similarity between the amount of the enzyme activity measured and the amount of the 34 kDa protein band in each fraction. The correlation extended even to the drastic decrease of both parameters in fraction 9, in which proteolytic degradation of the processed precursor and of the mature enzyme was suspected. These observations suggest that the 34 kDa protein band is likely the newly processed pMDH.

Antiserum specific for malate dehydrogenase was used to investigate the nature of the 34 kDa protein. Fractions collected from the above experiment were pooled in groups of three and subjected to immunoprecipitation by the anti-malate dehydrogenase serum under denaturing condition. As shown in Fig. 16, not only the radioactive protein was recognized by the antiserum, but also the amount of protein recovered from immunoprecipitation correlated with the relative

Fig. 14. Newly processed precursor of malate dehydrogenase shows affinity for 5'-AMP-Sepharose. Mitochondria (1.76 mg) were incubated with 3 x 10^8 cpm of *in vitro* synthesized rat liver proteins. After 60 min, mitochondria were isolated as described in the 'Methods and Materials'. One-half of the sonicated mitochondria was applied to a 0.5 ml column of 5'-AMP-Sepharose. Chromatographic procedures were exactly the same as described in Fig. 13. Fifty ul of each 0.5 ml fraction was separated on a SDS-10% polyacrylamide gel. Fractions 1 and 2 were omitted from the figure because these were flow-through fractions which contained large amounts of radioactivity resulting in over-exposure because of the time needed for identifying components in the other fractions. The nature of D and G are discussed in the text.


FRACTION NO.
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D
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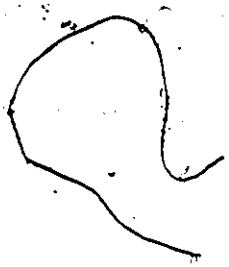
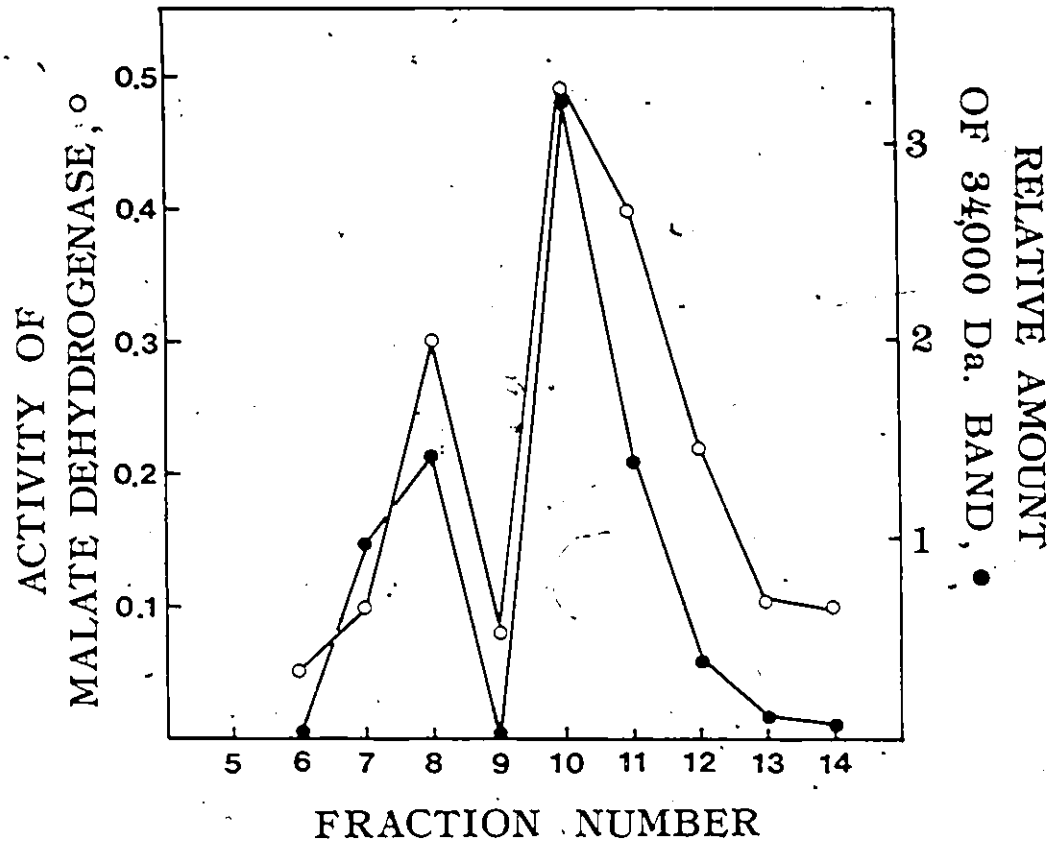


Fig. 15. Correlation of malate dehydrogenase activity with the elution profile of the 34 kDa radioactive protein band. Malate dehydrogenase activity was measured with 30 ul of each fraction collected from the 5'-AMP-Sepharose column as described in the 'Methods and Materials'. The activity of the enzyme is expressed in terms of units defined as the amount of enzyme that oxidizes 1 umol of NADH in 1 min. The amount of the 34 kDa radioactive protein band in each fraction was estimated by densitometric scanning of the band and is represented in terms of the area of the scanned peak. No 34 kDa protein band was detected in fraction 9 as shown in Fig. 14 and very little malate dehydrogenase activity was measured in the same fraction. The reason for this anomaly is not known. However, a prominent protein band at about 28 kDa was present in fraction 9. It is possible that this band arose from the 34 kDa band by proteolysis.


ACTIVITY OF
MALATE DEHYDROGENASE, ○
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0.2
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RELATIVE AMOUNT
OF 34000 Da. BAND, ●
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FRACTION NUMBER

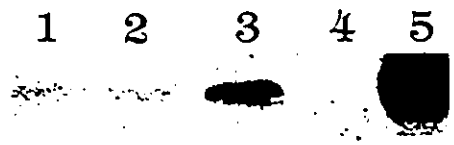

Fig. 16. Immunoprecipitation from fractions collected from the 5'-AMP-Sepharose affinity column. A 200 ul sample from each fraction was denatured and immunoprecipitated with 50 ul of anti-malate dehydrogenase serum as described in Fig. 13. Immunprecipitates were recovered, pooled in groups of three as in Fig. 13, and analyzed on a SDS-10% polyacrylamide gel. Lanes 1-4 are immunoprecipitates from fractions 4-6, 7-9, 10-12, 13-14, respectively. No newly processed malate dehydrogenase was detected in fractions 1-3. However, these fractions were omitted because of high background radioactivity as described in Fig. 14.

intensity of the 34 kDa radioactive band in the fractions (cf Fig. 14). These results strongly suggest that the radioactive 34 kDa protein is malate dehydrogenase. Quantitation of the immunoprecipitation results showed that 40% of the applied newly processed precursor was eluted by 40 uM NADH in fractions 7 to 14.

It should be noted that some proteins were also bound to the affinity column and were eluted, in part at least, past the flow-through fractions (Fig. 14). The elution of some of these as well as a general increase in elution of non-specifically bound proteins were enhanced when 40 uM NADH was added to the elution buffer (fraction 5). In addition to malate dehydrogenase, the elution of at least two other proteins, labeled D and G in the figure, was initiated by NADH. Neither of these proteins were observed when the affinity column was loaded with the translation mixture (results not shown). From the size of G (approximately 54 kDa), it could be newly processed glutamate dehydrogenase. The mature form of the enzyme also has a NAD^+-binding site. The enzyme is synthesized as a larger precursor (Mihara et al., 1982; Miralles et al., 1983), and the precursor can be processed to the mature size by isolated rat liver mitochondria (Miralles et al., 1983). The nature of D is not known, but it is of interest that its size (approximately 68 kDa) is twice the monomeric mass of malate dehydrogenase. It should be emphasized that the nature of D and G are not known, and the above discussion is purely speculative.

Different Antigenic Sites are Exposed on Mature Malate Dehydrogenase

and Its Precursor--It had been shown previously that antiserum raised against denatured malate dehydrogenase recognized the mature enzyme only if it had been denatured before immunoprecipitation (Aziz et al., 1981). The antiserum would not immunoprecipitate the native form of the enzyme. This was observed with both purified rat and bovine malate dehydrogenase using antiserum prepared against the respective denatured enzyme. These results showed that the antiserum is specific for a group of antigenic determinants that are present on the denatured mature enzyme, but not present or exposed when the enzyme is in the native form.

The anti-bovine malate dehydrogenase serum was used to immunoprecipitate the in vitro synthesized pMDH with and without prior denaturation. The results are presented in Fig. 17. As shown in the figure, pMDH could be immunoprecipitated with or without prior denaturation of the protein (lane 7 and lane 3). This is in contrast to the result obtained with the mature enzyme, which requires prior denaturation for immunoprecipitation (lane 9 versus lane 2). This observation was obtained repeatedly, indicating that there are certain antigenic sites that are present or exposed only on pMDH, but not on the mature endogenous enzyme. This would imply that the precursor has a different tertiary structure from the mature protein.

A similar experiment was carried out to examine the reaction of the newly processed form of the precursor of malate dehydrogenase towards the anti-bovine malate dehydrogenase serum. As shown in Fig. 18, immunoprecipitation of the newly processed form released from

Fig. 17. Immunoprecipitation of malate dehydrogenase and its precursor with or without prior denaturation. Mature malate dehydrogenase was immunoprecipitated from mitochondria isolated from [^{35}S]methionine-labeled Chinese hamster ovary cells as described in the 'Methods and Materials'. Malate dehydrogenase precursor was recovered from 1.75 x 10^6 cpm of the [^{35}S]methionine-labeled post-ribosomal supernatant of a cell-free reticulocyte system primed with rat liver free polysomes. The mitochondria and post-ribosomal supernatants were either denatured (lanes 6, 7, 9, 10) by boiling for 5 min in 4% SDS and 10 mM mercaptoethanol before immunoprecipitation or immunoprecipitated directly (lanes 1-4). Immunoprecipitates were analyzed by SDS-10% polyacrylamide gel electrophoresis with subsequent fluorography. Lanes 1, 2, 9 and 10 are from the mitochondrial samples. Lanes 3, 4, 6 and 7 are from the post-ribosomal supernatant after cell-free translation. Pre-immune serum was used in lanes 1, 4, 6 and 10; and anti-malate dehydrogenase serum in lanes 2, 3, 7 and 9. Lane 5 is [^{14}C]formaldehyde-labeled bovine malate dehydrogenase and lane 8 is [^{14}C]formaldehyde-labeled molecular mass markers at 34, 30 and 26 kDa. ( ), pMDH, ( ), malate dehydrogenase.

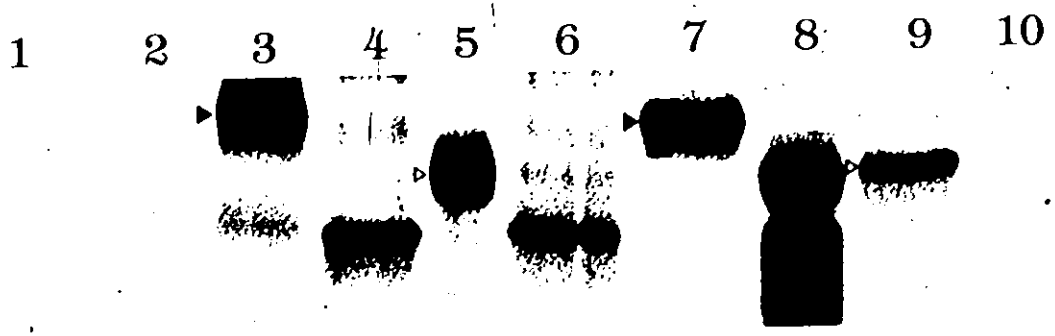
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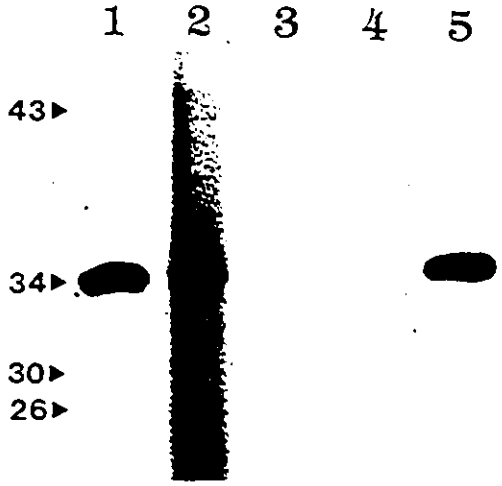
Fig. 18. Immunoprecipitation of the newly processed precursor of malate dehydrogenase with or without prior denaturation. Gradient purified mitochondria isolated from CHO cells (1.2 mg of protein in 300 ul) were incubated with an equal volume of translational products using mRNA (8.2×10^6 cpm) at 30°C for 30 min. After the incubation, mitochondria were recovered by centrifugation and resuspended in 600 ul of 5 mM EDTA, 10mM mercaptoethanol, 0.1% Triton X-100 and 20 mM sodium phosphate, pH 7.6. The mitochondrial suspension was sonicated in a bath sonicator at 0°C six times for 1 min each, with a one min interval, and then centrifuged for 30 min in an Eppendorf centrifuge at 4°C. The resultant supernatant was divided into two aliquots. One aliquot was adjusted to 4% SDS and 10 mM mercaptoethanol (lane 2). This sample and the pellet, which was solubilized in 300 ul of 4% SDS and 10 mM mercaptoethanol (lane 4) were boiled for 5 min. One ml of the solution containing 150 mM NaCl, 5 mM EDTA, 10 mM mercaptoethanol, 1% Triton X-100 and 50 mM Tris-HCl, pH 7.6, was added to the untreated portion of the supernatant (lane 3) and the boiled samples, after they were cooled to room temperature and adjusted to 1% in Triton X-100. Immunoprecipitation was carried out with anti-malate dehydrogenase serum as described in the 'Method and Materials'. The immunopreciptates were analyzed on a SDS-10% polyacrylamide gel. Lanes 1 and 5, [^{14}C]formaldehyde-labeled bovine heart malate dehydrogenase; lanes 2 and 3, immunoprecipitates from the supernatant with or without denaturation of sample respectively; lane 4, immunoprecipitate of the solubilized and denatured pellet.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mitochondria by sonication required prior denaturation of the protein before immunoreaction (lane 2 versus lane 3). The higher background in lane 2 did not account for the result because the newly processed form was present only in lane 2, whereas several minor proteins were non-specifically immunoprecipitated and present in lanes 2-4. This observation is identical to that of the mature endogenous enzyme, but not the precursor protein, suggesting that the newly processed form had undergone conformational changes and lost certain antigenic determinants that are present on the precursor protein, but not the mature enzyme.

DISCUSSION

The fact that newly processed pMDH acquires a NAD^+-binding site, which is not present on pMDH, indicates that in vitro processing of pMDH by isolated mitochondria is accompanied by a conformational change, which results in the formation of the binding site for the co-enzyme. This conclusion is supported by the experiment showing that certain antigenic sites are present or exposed on pMDH, but not the mature enzyme or the newly processed form, indicating that there is a conformational difference between the precursor protein and the latter two forms of the enzyme. These results show that the proteolytic processing of the precursor of malate dehydrogenase is accompanied with conformational changes, resulting in the formation of the NAD^+-binding site.

Furthermore, the acquisition of the binding site for NAD^+ by the newly processed pMDH in isolated mitochondria indicates that the in vitro import system used reflects the genuine processes occuring in an intact cell.

It should be noted that binding sites for substrates or specific ligands have also been identified on the in vitro imported adenine nucleotide carrier of Neurospora (Schleyer and Neupert, 1984) and ornithine transcarbamylase of rat (Kalousek et al., 1984).

3.2 IN VITRO IMPORT OF THE PRECURSOR OF THE RAT LIVER ADENINE NUCLEOTIDE CARRIER

(I) ISOLATION AND CHARACTERIZATION OF THE RAT LIVER ADENINE NUCLEOTIDE CARRIER

The transport of ADP and ATP across the mitochondrial inner membrane is mediated by an integral membrane protein, the adenine nucleotide carrier (Klingenberg, 1976). The carrier is the most abundant protein found in bovine heart mitochondria (Klingenberg et al., 1979a). Its activity can be non-competitively inhibited by carboxyatractyloside (CAT), a plant toxin from Atractyles gummifera which binds to the protein on the cytoplasmic side of the mitochondrial inner membrane (Vignais et al., 1973; Klingenberg et al., 1979b). Riccio et al. (1975a,b) showed that the CAT-carrier complex could be isolated by passing a Triton-X 100 mitochondrial extract through a hydroxylapatite column. The CAT-carrier complex, unlike most of the mitochondrial proteins, does not bind to the column and can be recovered in the void volume.

The adenine nucleotide carrier of bovine heart mitochondria has been well characterized (Riccio et al., 1975b; Kramer & Klingenberg, 1977a,b; Aquila et al., 1978). The complete amino acid sequence of the bovine carrier has also been published (Aquila et al., 1982). It is a dimeric protein with monomeric molecular mass of approximately 30 kDa.

Another aspect of this research was to study the biogenesis of the rat liver adenine nucleotide carrier. Antibody was raised against the rat liver protein isolated according to the method of Riccio et al. (1975a,b). The newly synthesized protein was identified by the antibody and its import into mitochondria was studied with the in vitro import system developed in the study of malate dehydrogenase.

RESULTS

Isolation of The Rat Liver Adenine Nucleotide Carrier—Mitochondria isolated from rat liver were incubated with the inhibitor, carboxyatractyloside, for 30 min at 0°C, and then solubilized with the Solubilization solution with a Triton/protein ratio of 3 to 1. After a clarifying centrifugation at 100,000 x g, the solubilized extract was passed through a hydroxylapatite column, and the column was washed with the Elution buffer. Lanes 3 and 4 of Fig. 19 show the proteins in the extract applied onto the column. Protein determination of the collected fractions indicated a protein peak in the flow-through fractions. Proteins in these fractions were separated on a SDS-12.5% polyacrylamide gel, and then stained with Coomassie blue. As shown in Fig. 19, two protein bands with similar molecular mass were identified (lanes 1 and 2). The major protein is approximately 30 kDa, and the other is about 3.5 kDa larger in size. The relative ratio of the two proteins in the gel shown is 93:7. The ratio was obtained by quantitating the Coomassie blue stained protein bands with a densitometer. The peaks on the recording paper were excised and weighed. It should be noted that the

Fig. 19. Isolation of the rat liver adenine nucleotide carrier.

Mitochondria were isolated from livers of Sprague Dawley rats. After incubation with carboxyatractyloside (16 umol/g mitochondrial protein) for 30 min at 0°C in the Suspension solution, mitochondria were solubilized in the Solubilization solution for 30 min, with a Triton/protein ratio of 3 to 1. The supernatant obtained after a clarifying centrifugation at 100,000 x g was loaded onto a hydroxylapatite column pre-equilibrated with the Elution buffer. The column was washed with the same buffer and fractions, with volume proportional to the size of the column, were collected. Proteins in fractions containing the flow through volume were analyzed by SDS-12.5% polyacrylamide gel electrophoresis. Lanes 1-2, flow through fractions from the hydroxylapatite column; lane 3, mitochondrial extract applied onto the hydroxylapatite column and 100 ul of the flow through fraction in lane 1; lane 4, the mitochondrial extract; lane 5, molecular mass markers, 68, 43, 34, 26, 17 and 12.5 kDa.


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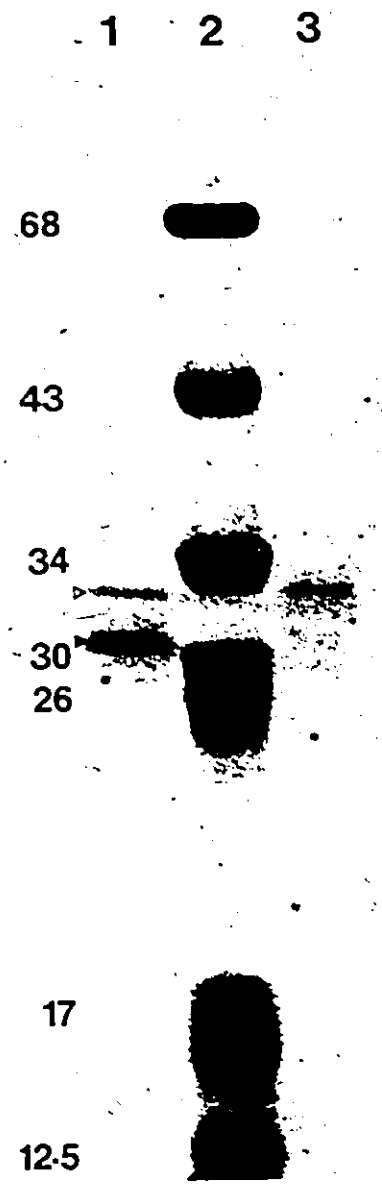
ratio was not constant for different experiments. The 30 kDa protein constituted from 50% to 95% of the sum of the two proteins. The nature of the two proteins were further investigated by the following experiments.

Isolation of the Adenine Nucleotide Carrier in the Absence of Carboxyatractyloside--Riccio et al. (1975b) showed that the binding of CAT to the adenine nucleotide carrier is essential in preventing the denaturation of the carrier during the solubilization process and allowing the protein to pass through a hydroxylapatite column without retardation. In the absence of carboxyatractyloside the carrier would lose its native conformation and bind to hydroxylapatite. The nature of the two rat liver proteins which flowed through the hydroxylapatite column was, therefore, investigated by solubilizing rat liver mitochondria which were not pretreated with carboxyatractyloside. The resultant mitochondrial extract was also incubated at room temperature instead of at 0°C for 20 min to facilitate the denaturation of the adenine nucleotide carrier. Lane 3 of Fig. 20 showed the proteins in the flow-through fractions of a hydroxylapatite column loaded with the extract. The major protein band detectable by Coomassie blue staining was the 33.5 kDa protein. No protein band with a molecular mass of 30 kDa was observed. However, when mitochondria were preincubated with carboxyatractyloside, the 30 kDa protein was the predominant protein in the flow through fractions (lane 1). These results showed that the stability of the 30 kDa protein is dependent on carboxyatractyloside, suggesting that it is the rat liver mitochondrial adenine nucleotide

Fig. 20. Isolation of the rat liver adenine nucleotide carrier in the absence of carboxyatractyloside. Rat liver mitochondria were incubated with or without CAT (26 umol/g mitochondrial protein) for 30 min at 0°C. Mitochondria were recovered and solubilized as described in Fig. 19. The resultant extract was applied to a hydroxylapatite column which was pre-equilibrated and washed subsequently in the Elution buffer. Lane 1, eluate from a hydroxylapatite column loaded with carboxyatractyloside-treated mitochondrial extract; lane 3, eluate from a hydroxylapatite column loaded with non-CAT treated mitochondrial extract; lane 2, molecular mass markers, 68, 43, 34, 26, 17 and 12.5 kDa.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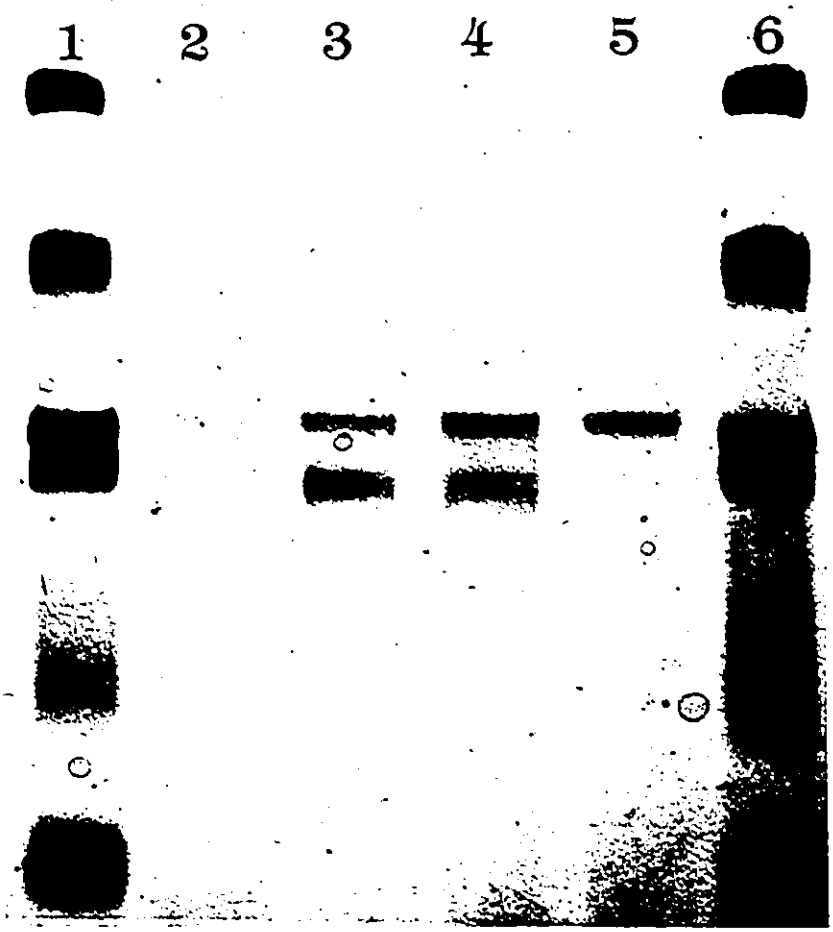
carrier. Furthermore, the results showed that the 33.5 kDa protein is a distinct protein different from the adenine nucleotide carrier, since its binding to hydroxylapatite is independent of carboxyatractyloside.

Labeling of Rat Liver Mitochondria with N-[^{14}C]Ethyl-Maleimide--It was shown that the adenine nucleotide carrier has free sulphydryl groups which are sensitive to reducing reagents, such as N-ethyl-maleimide (Leblanc and Clauser, 1972; Klingenberg and Appel, 1980). However, the two sulphydryl groups are protected in the presence of carboxyatractyloside. The nature of the 30 kDa and 33.5 kDa proteins were further investigated by examining their sensitivity towards N-ethyl-maleimide.

Rat liver mitochondria were treated with carboxyatractyloside before being labeled with N-[^{14}C]ethyl-maleimide for 5 min at 20°C. The reaction was stopped by adding excess cold N-ethyl-maleimide to the reaction mixture. Mitochondria were solubilized and the extract chromatographed on a hydroxylapatite column as described in the legend of Fig. 19. Proteins in the fractions containing the void volume were analyzed on a Swank and Munkres gel. Both the 30 kDa and 33.5 kDa proteins were detected by Coomassie blue staining (Fig. 21A lanes 3 and 4). However, only the 33.5 kDa protein, but not the putative adenine nucleotide carrier, was radiolabeled by the N-[^{14}C]ethyl-maleimide (Fig. 21B lanes 3 and 4). The radiolabeling of the 33.5 kDa protein could be prevented by incubating mitochondria with mersalylic acid before the addition of N-[^{14}C]ethyl-maleimide (lane 2). If mitochondria were

Fig. 21. N-[^{14}C]ethyl-maleimide labeling of rat liver mitochondria. Rat liver mitochondria (10 mg/ml) were labeled with N-[^{14}C]ethyl-maleimide with or without preincubation with carboxyatractyloside and mersalylic acid. Each reaction mixture contained 10 mg of mitochondrial protein and 100 ul of N-[^{14}C]ethyl-maleimide (0.1 mCi/ml). Labeling was for 5 min at 20°C and was stopped by adding 100 ul of 15 mM cold N-ethyl-maleimide and 100 ul of 75 mM cysteine. Mitochondria were recovered after 5 min at 20°C and solubilized with the Solubilization solution as described in Fig. 19. The resultant extracts were passed through 10 ml hydroxylapatite columns with the Elution buffer. Fractions containing the flow-through volume were analysed by SDS-10% polyacrylamide gel electrophoresis (Swank and Munkrès, 1972). Proteins were identified by Coomaisse-blue staining (A) and subsequently with fluorography (B). Lanes 1 and 6, molecular mass markers (68, 43, 34, 26, 17 and 12.5 kDa); lane 2, mitochondria without preincubation with carboxyatractyloside, were treated with 50 ul of mersalylic acid (2 mg/ml) for 5 min at 20°C before labeling; lanes 3 and 4, mitochondria were incubated with 50 ul of carboxyatractyloside (4 mg/ml) before labeling; lane 5, mitochondria were labeled without preincubation with carboxyatractyloside or mersalylic acid.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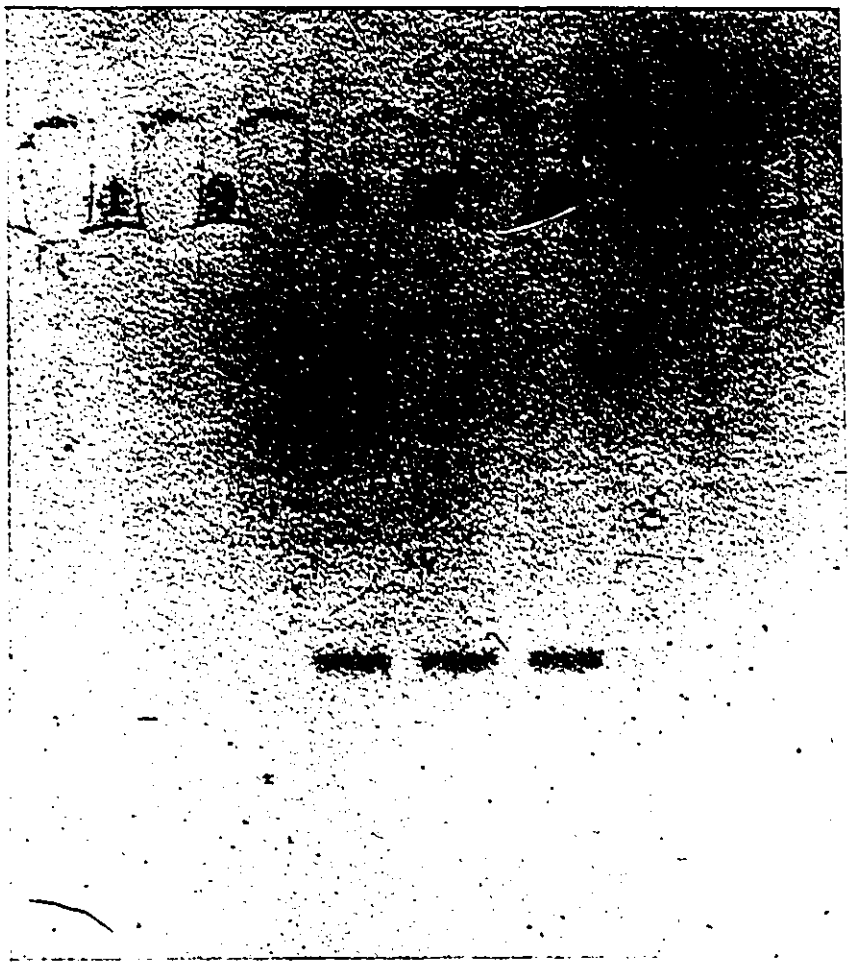

labeled without preincubation with carboxyatractyloside, only the labeled 33.5 kDa protein was found in the void volume (lane 5). The difference in the reactivity of the two proteins towards N-[^{14}C]ethyl-maleimide strongly supports the conclusion drawn from the previous experiment that the two proteins are distinct.

DISCUSSION

The above results showed that isolation of the 30 kDa protein by hydroxylapatite chromatography requires pretreating mitochondria with carboxyatractyloside, indicating that this protein is the rat liver mitochondrial adenine nucleotide carrier. Based on its electrophoretic mobility on SDS-polyacrylamide gels, the protein is about the same size as its counterpart in bovine heart mitochondria (Riccio et al. 1975b). However, it is smaller than the carrier in mitochondria of Fungi. The monomeric molecular mass of the carrier in Neurospora is 32 kDa (Zimmermann et al., 1979a) and is 37 kDa in yeast (Lauquin et al., 1979).

In contrast, isolation of the 33.5 kDa protein is independent of carboxyatractyloside and the protein is sensitive to N-ethyl-maleimide. With hydroxylapatite chromatography, Wohlrab (1980) isolated a protein of about 34 kDa from bovine heart mitochondria which catalyzes phosphate-phosphate exchange when reconstituted into liposomes. The activity of this phosphate exchange protein is sensitive to sulphydryl reagents, but not carboxyatractyloside (Hadvary & Kadenbach, 1976;

Wohlrab and Flowers, 1982). Thus, it is likely that the 33.5 kDa protein identified in the above experiments is the phosphate transport protein of rat liver mitochondria, which copurified with the adenine nucleotide carrier.

(II) SYNTHESIS IN VITRO OF THE RAT LIVER ADENINE NUCLEOTIDE CARRIER

At the beginning of this research, experiments showed that synthesis of the adenine nucleotide carrier in CHO cells is inhibited by cycloheximide which acts on cytoplasmic ribosomes, but not by tevenel, a chloramphenicol analogue, which is a specific inhibitor for mitochondrial protein synthesis (Chien et al., 1981). Thus, the protein is coded by the nuclear genome and imported into mitochondria. In Neurospora, the adenine nucleotide carrier is synthesized on membrane-free cytoplasmic ribosomes with a size identical to the mature monomeric form, and is imported into mitochondria post-translationally (Zimmermann et al., 1979, 1980). As part of the study on the biogenesis of the rat liver adenine nucleotide carrier, antibodies raised against the carrier isolated according to the method of Riccio et al. (1975b), were used to identify the precursor form of the protein.

RESULTS

Specificity of The Antisera against the Rat Liver Adenine Nucleotide Carrier—The rat liver adenine nucleotide carrier was stabilized with CAT and isolated by passage through a hydroxylapatite column (Riccio et al., 1975a,b). Antibodies were raised against the CAT-stabilized form (N-antiserum), as well as the denatured protein (D-antiserum). Examination of the specificity of the antisera required radiolabeled carrier. However, experiments showed that radiolabeled CAT-stabilized carrier with high specific radioactivity could not be obtained by

reductive methylation using [^{14}C]formaldehyde (see legend of Fig. 22). Thus CHO cell adenine nucleotide carrier labeled in vivo was used for examining the specificity of the antisera. As shown in Fig. 22, the carrier (solid pointer) isolated from CHO (lane 1) and rat XC (lane 5) tissue culture cells has the same size as the carrier isolated from rat liver (lane 3).

The specificities of the two antisera were examined by immunoprecipitation from a Triton X-100 solubilized mitochondrial extract of [^{35}S]methionine-labeled CHO cells (Fig. 23, lane 5). CHO cells were chosen, instead of rat XC cells, because they contain more mitochondria and can be easily labeled in suspension culture. Both the N-antiserum (lane 2) and D-antiserum (lane 4), but not preimmune serum (lane 1), precipitated a protein (solid pointer) with the same molecular mass as the carrier purified from a Triton X-100 extract of rat liver mitochondria treated with CAT before solubilization (Fig. 22, lane 3). Thus the antisera were monospecific. It is estimated that 10 ul of N-antiserum would immunoprecipitate about 0.1 ug of adenine nucleotide carrier. Both antisera could also immunoprecipitate the carrier from SDS-treated mitochondria. Thus, neither of the antisera appear to be specific for a particular conformation of the protein.

Synthesis in vitro of the Rat Liver Adenine Nucleotide Carrier--Both N- and D-antiserum were used to immunoprecipitate the newly synthesized form of the carrier from a reticulocyte lysate protein synthesizing system primed with rat liver free polysomes (Fig. 24). A major protein

Fig. 22. Isolation of [^{35}S]methionine-labeled adenine nucleotide carrier from mitochondria of Chinese hamster ovary cells and rat XC cells. About 4.0 x 10^8 cells of each cell type was labeled with 1 mCi of [^{35}S]methionine for 2 h at 37°C. Mitochondria were incubated with CAT and solubilized with 0.3 M KCl, 1% Triton X-100, 5 mM EDTA and 10 mM Tris-HCl, pH 7.2 at a Triton:protein ratio of 3:1. The adenine nucleotide carrier was then isolated from the extract by hydroxylapatite chromatography. The solid pointer indicates the adenine nucleotide carrier and the open pointer the presumptive phosphate carrier. Lane 1, adenine nucleotide carrier (6,900 cpm, 7 ug) from CHO cells. Lanes 2 and 4, [^{14}C]-labeled molecular mass markers whose size in kDa are indicated to the left of the figure. Lane 3, [^{14}C]-reductively methylated rat liver adenine nucleotide carrier (1,500 cpm, 10 ug). The rat liver mitochondrial adenine nucleotide carrier was isolated by hydroxylapatite chromatography and was purified by polyacrylamide gel electrophoresis. To ensure homogeneity, the carrier was excised from the gel and extracted in 10 mM NH_4HCO_3, 1 mM EDTA, 1 mM phenylmethylsufonyl fluoride and 1% (w/v) sodium dodecyl sulfate. The protein was then labeled by reductive methylation using [^{14}C]formaldehyde. The specific radioactivity was approximately 150 cpm/ug protein, which is about 100 fold less than that obtained with soluble proteins. Lane 5, adenine nucleotide carrier (5,000 cpm, 7 ug) of rat XC cells. Lane 6, the mitochondrial extract (15,000 cpm, 9 ug) of rat XC cells. Lanes 1-3, and lanes 4-6 were from different parts of the gel and the two sections were brought together for the figure.

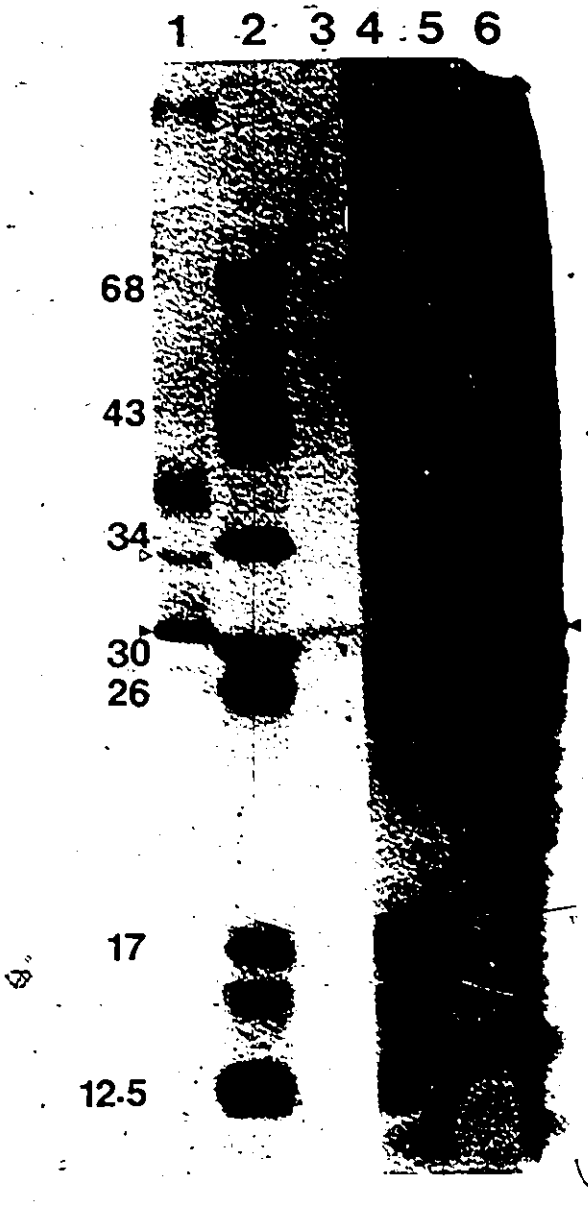
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Fig. 23. Specificity of the antisera against the rat liver adenine nucleotide carrier. The antisera were used to immunoprecipitate the adenine nucleotide carrier from mitochondria of [^{35}S]methionine-labeled Chinese hamster ovary cells. CHO cells (4.5 x 10^8) were labeled with .1 mCi of [^{35}S]methionine for 2 h at 37°C. Mitochondria were isolated as described in 'Methods and Materials'. Mitochondria (2.7 mg protein) were treated with carboxyatractyloside and solubilized as described in the legend of Fig. 19. The solubilized extract was recovered after centrifugation. Each immunoprecipitation mixture contained 100 ul of the extract (5 x 10^6 cpm) with 20 ul antiserum or preimmune serum. Immunocomplexes were recovered with 30 ul protein A-Sepharose. The immunoprecipitates were analyzed by SDS-12.5% polyacrylamide gel electrophoresis and subsequent fluorography of the dried gel. The solid pointer indicates the carrier. Lane 1, preimmune serum; lane 2, antiserum against the native form of the carrier; lane 3, molecular mass markers, 68, 43, 34, 30, 26, 17, and 12.5 kDa; lane 4, antiserum against the denatured form of the carrier; lane 5, protein profile of the mitochondrial extract used for immunoprecipitation. This lane is from a separate gel.

band (solid pointer) with an apparent molecular mass of about 1.5 kDa larger than the mature monomeric form (open pointer) was observed with both antisera (Fig. 24A, lanes 2 and 3 versus lane 5, and Fig. 24B, lane 2 versus lane 1). This protein was not immunoprecipitated by preimmune serum (Fig. 24A, lane 1). These results suggested that the protein is likely the precursor of the rat liver adenine nucleotide carrier. This was supported by a competition experiment in which immunoprecipitation was carried out with a limited amount of radioactive, in vitro synthesized protein and excess amount of non-radioactive, hydroxylapatite-isolated rat liver carrier. As shown in Fig. 25, the putative precursor (lane 3) was not immunoprecipitated by preimmune serum (lane 5) or by N-antiserum in the presence of excess unlabeled carrier (lane 4).

Finger-Print Analysis of The Putative Precursor of The Rat Liver Adenine Nucleotide Carrier--The relationship between the immunoprecipitated putative precursor and the mature carrier was further confirmed by finger-print analysis of the two proteins. Both proteins were radio-iodinated in gel slices and, then digested with S. aureus V8 protease. As shown in Fig. 26, the peptide patterns of the radio-iodinated rat liver adenine nucleotide carrier (A) and its putative precursor (B) were very similar. All the major spots were identical as sketched in Fig. 26 C and D. Similarity was also observed among the minor spots. Differences could reflect different extents of digestion or difference in the primary structures of the two proteins. In particular, in both this experiment and a repeat experiment, there

Fig. 24. Synthesis of the putative precursor of the adenine nucleotide carrier in a reticulocyte lysate primed with rat liver free polysomes. Rat liver free polysomes (3.8 A_{260} units/100 ul lysate) were translated in 0.5 ml of a nuclease-treated reticulocyte lysate for 60 min at 25°C. Post-ribosomal supernatant was obtained after centrifugation at 120,000 x g for 30 min, and 100 ul samples of the supernatant were boiled for 5 min after adjusting to 4% SDS and 2 mM in mercaptoethanol. The sample was cooled to room temperature and then 0.9 ml of a solution containing 0.15 M NaCl, 5 mM EDTA, 1% (v/v) Triton X-100, and 50 mM Tris-HCl, pH 7.2 was added. Immunoreaction was started 30 min later by the addition of 20 ul of preimmune serum, N-antiserum or D-antiserum. Immunoprecipitates were analyzed as in Fig. 23. Panels A and B were obtained from different experiments. The solid and open pointers indicate the precursor and mature forms of the carrier, respectively. Panel A, lane 1, preimmune serum; lane 2, N-antiserum; lane 3, D-antiserum; lane 4, molecular mass markers; lane 5, CHO cell mature adenine nucleotide carrier. Panel B, lane 1, mature adenine nucleotide carrier; lane 2, N-antiserum.

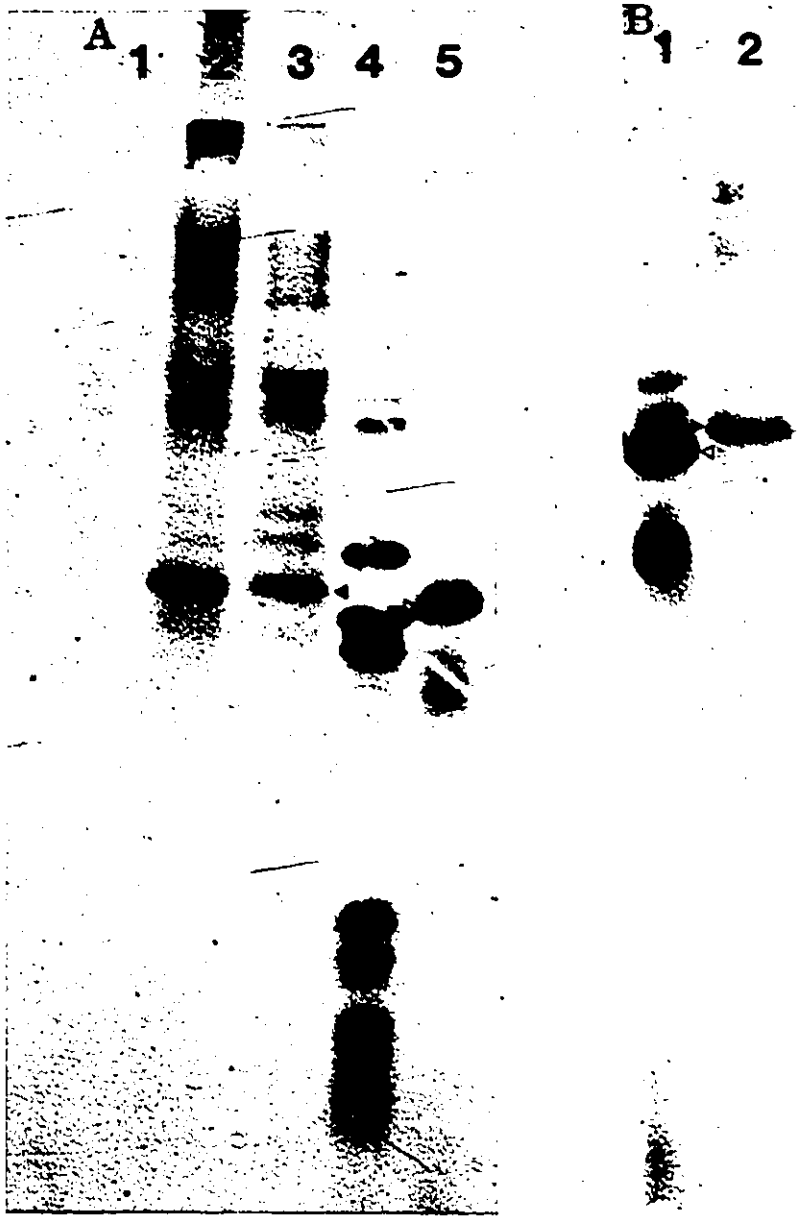
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Fig. 25. Demonstration of the relationship of the newly synthesized adenine nucleotide carrier and the mature protein by competition for immunoprecipitation. Rat liver total RNA (10 A_{260} units) was translated in the presence of [^{35}S]methionine (10 uCi/ul) in 0.5 ml of a nuclease-treated reticulocyte lysate for 60 min at 25°C. Post-ribosomal supernatant was obtained after centrifugation at 120,000 x g for 30 min. Each immunoreaction mixture contained 100 ul of the supernatant (7 x 10^6 cpm) and 20 ul of N-antiserum or preimmune serum. Immunoprecipitates were separated by SDS-12.5% polyacrylamide gel electrophoresis and analyzed by fluorography. Lane 1, the mature form of the carrier from CHO cells; lane 2, molecular mass markers; lane 3, the N-antiserum; lane 4, same as lane 3, but the immunoreaction mixture contained 10 ug of unlabeled rat liver adenine nucleotide carrier; lane 5, immunoprecipitation with preimmune serum.

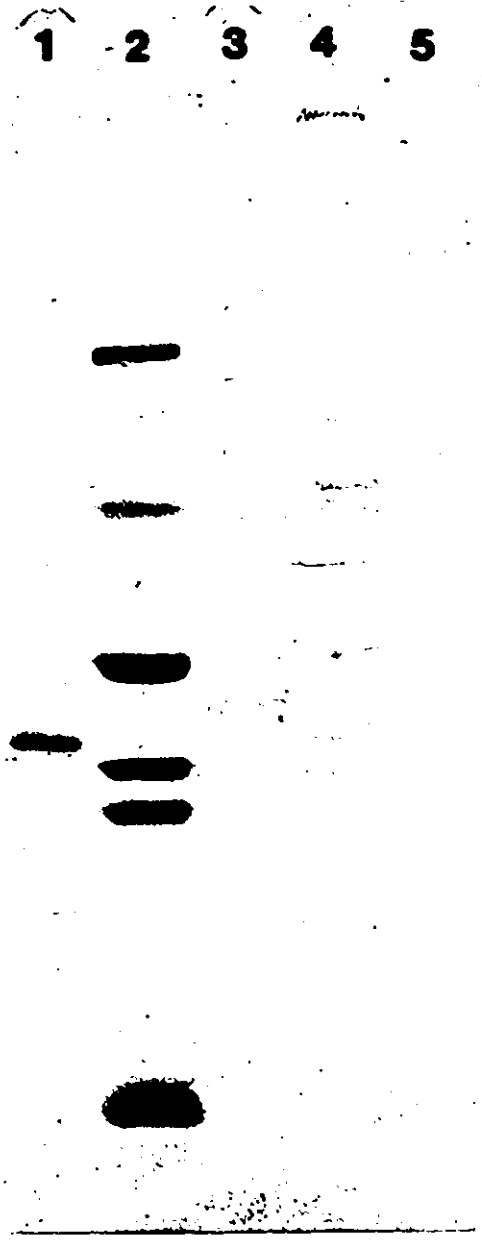
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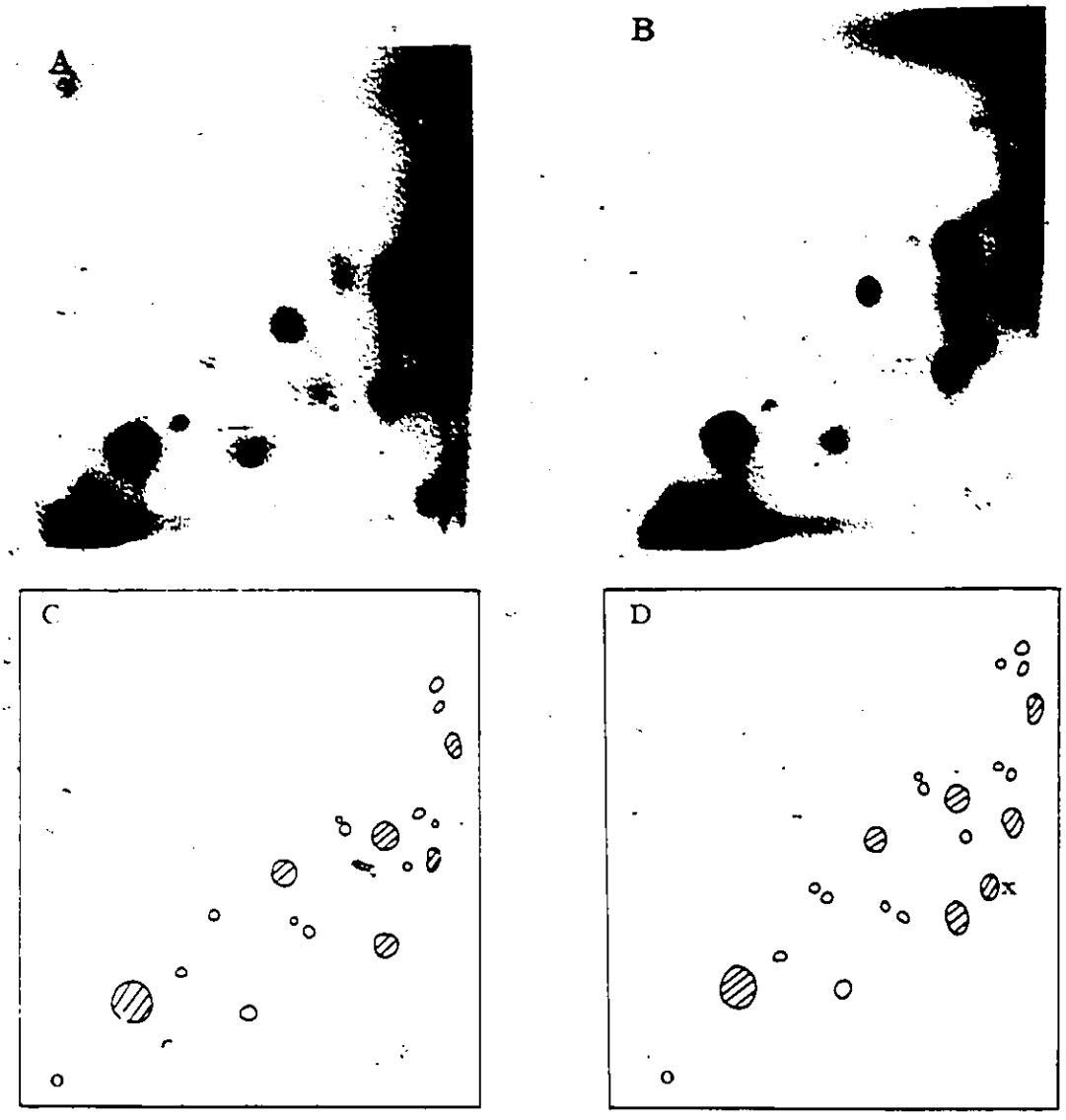
Fig. 26. Two-dimensional peptide maps of the putative precursor of the rat liver adenine nucleotide carrier and the mature protein. The rat liver adenine nucleotide carrier and its newly synthesized form were radio-iodinated and then digested with S. aureus V8 protease within gel slices. The peptides were then analyzed by two-dimensional thin-layer chromatography and visualized by autoradiography. Panel A, autoradiogram of the peptide fragments of the mature carrier; Panel B, autoradiogram of the peptide fragments of the newly synthesized carrier; Panel C (mature carrier) and Panel D (the precursor) are tracings of the autoradiograms indicating probable identical major (lined circles) and minor spots (open circles). The spot marked X in the case of newly synthsized carrier is the only major extra spot present in the peptides of the precursor, but not in the mature protein.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was a distinct spot (X) which was present only in the case of the precursor. It was of some concern that the similar patterns observed may be due to a common contaminating protein present in both samples. However, this possibility was virtually excluded by the fact that different procedures were used in obtaining the two samples. The mature carrier was labeled in a gel slice containing hydroxylapatite-isolated rat liver protein and the putative precursor from a gel slice with the N-antiserum immunoprecipitate of a translation mixture. Furthermore, the same peptide-mapping method was used to confirm the precursor-protein relationship of malate dehydrogenase and its precursor (Fig.2). The peptide patterns obtained in the case of malate dehydrogenase was completely different from that of rat liver adenine nucleotide carrier. These factors were against the possibility that the similar patterns observed in Fig. 26 was an artifact of the method used.

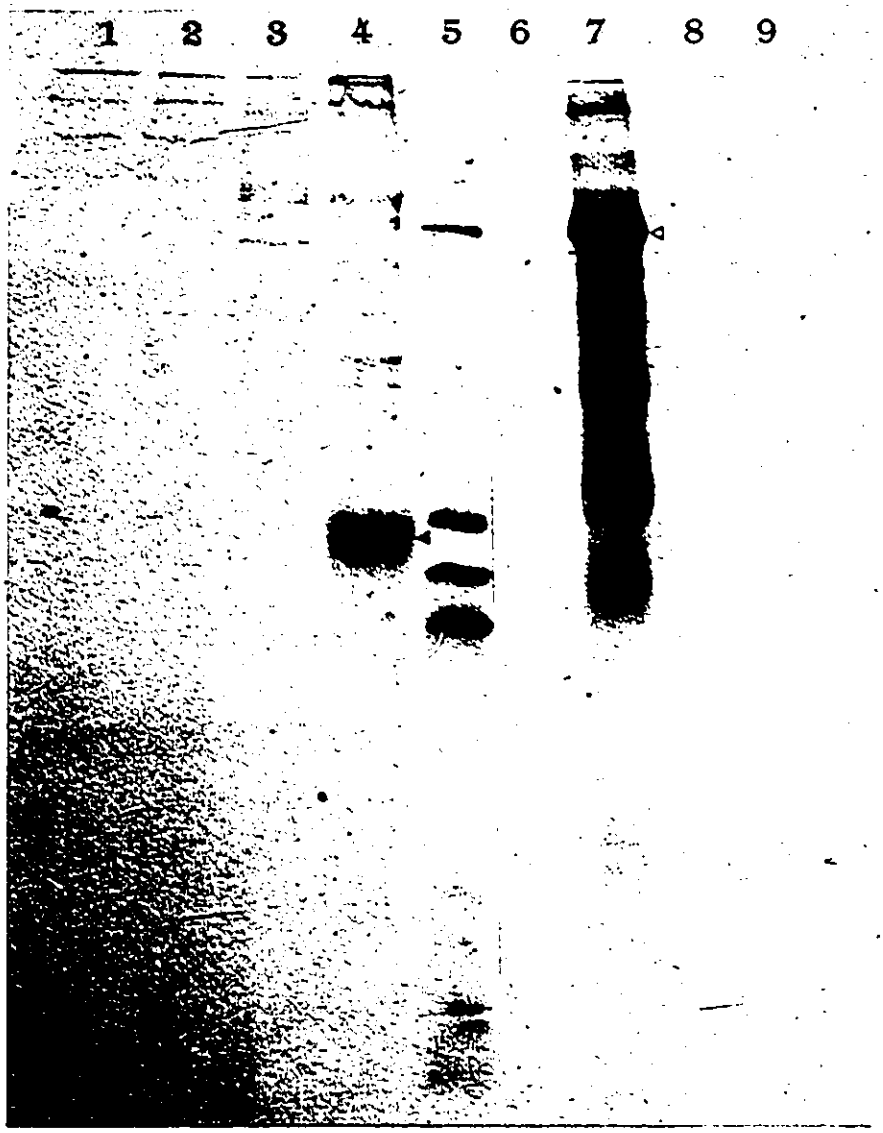
Subcellular Site of Synthesis of the Rat Liver Adenine Nucleotide Carrier--In yeast, some nuclear coded proteins are synthesized on both membrane-free ribosomes as well as ribosomes that are bound to the mitochondrial outer membrane (Kellam and Butow, 1972; Suissa and Schatz, 1982). Heinrich and his co-workers also reported that the subunits IV and V of the rat liver cytochrome c oxidase are synthesized on both free- and membrane-bound polysomes (Northemann et al., 1981; Schmelzer et al., 1982). In Neurospora, the adenine nucleotide carrier is synthesized on membrane-free ribosomes (Zimmermann et al., 1979a). In the experiments discussed above, the precursor of the rat liver adenine nucleotide carrier was immunoprecipitated from free-polysomal products. Fig. 27

shows that the mRNA for the carrier was located exclusively in free polysomes (lane 4, solid pointer) and not in membrane-bound polysomes (lane 9). Albumin, which is synthesized on membrane-bound polysomes was used as an indicator of the relative purity of the free and membrane-bound polysomes preparations (lane 2 and lane 7, open pointer). It was estimated that approximately 0.035% of the total acid-precipitable counts could be immunoprecipitated by 20 ul of the N-antiserum when translation was primed with free polysomes.

Size of the Rat Liver Adenine Nucleotide Carrier in the Post-ribosomal Supernatant Solution—The post-ribosomal supernatant of the translation mixture was fractionated by centrifugation on a sucrose density gradient and also by molecular sieving chromatography on a Sephadex G-100 column. The fractions collected from both procedures were subjected to immunoprecipitation with the N-antiserum. Fig. 28A shows the immunoprecipitates obtained from the sucrose density gradient fractionation with the N-antiserum. The precursor of the adenine nucleotide carrier was distributed among the top 7 of the 12 gradient fractions after centrifugation. The precursor was not immunoprecipitated by the pre-immune serum (Fig. 28B). Most of the precursor was in fractions 3 and 4, where the trimer and tetramer of cross-linked bovine serum albumin (200 to 270 kDa) were located in a parallel gradient. However, if the gradient was in 1% (v/v) Triton X-100, the precursor was found mainly in fractions 2 and 3 (result not shown).

The size of the precursor-containing aggregate or complex was

Fig. 27. Subcellular site of synthesis of the putative precursor of the rat liver adenine nucleotide carrier. Cell-free translations were primed with either rat liver free polysomes (3.8 A_{260} units/100 ul lysate) or with bound polysomes (6.5 A_{260} units/100 ul lysate), which gave the same stimulation of translation. Translation was for 60 min at 25°C. The mixture was centrifuged at 120,000 x g for 30 min. Immunoreaction mixtures contained 100 ul of the supernatant and 20 ul of one of preimmune serum, anti-rat albumin serum and N-antiserum. The immunoprecipitates were analyzed as described in Fig. 25. The solid (lane 4) and open (lane 7) pointers indicate the precursors for the carrier and albumin, respectively. Lanes 1-4, free polysomes; lanes 6-9, bound polysomes, lanes 1, 3, 6 and 8, preimmune serum; lanes 2 and 7, anti-rat albumin serum; lanes 4 and 9, N-antiserum; lane 5, [^{14}C]formaldehyde-labeled molecular mass markers.


1
2
3
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5
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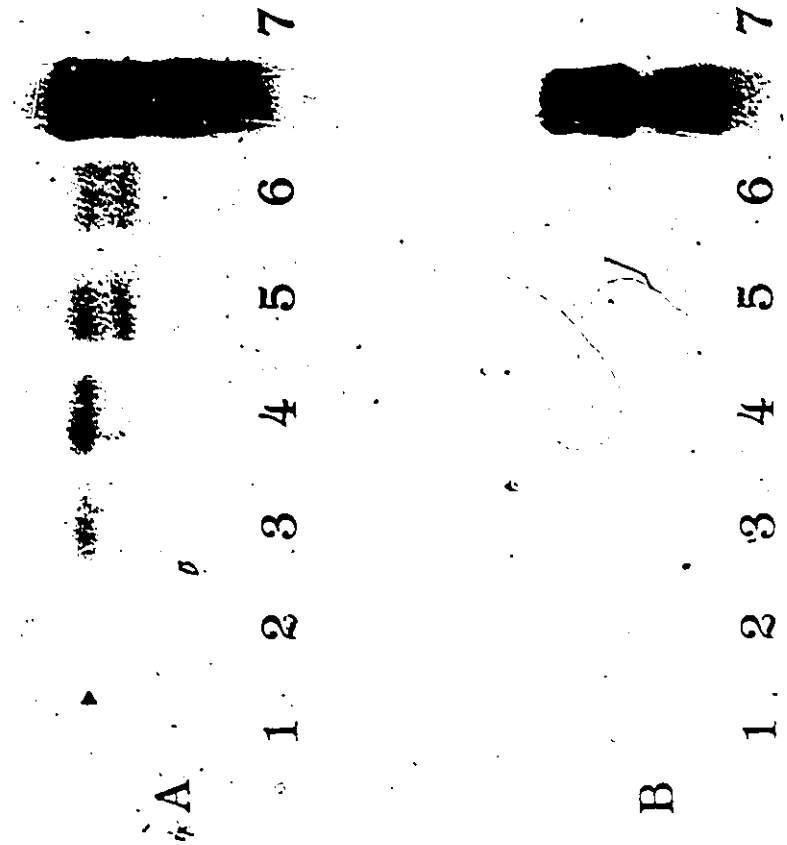
8
9

Fig. 28. Estimation of the size of the newly synthesized adenine nucleotide carrier by sucrose density gradient centrifugation. The post-ribosomal supernatant of a reticulocyte lysate primed with rat liver RNA was adjusted to 0.3 M KCl, and centrifuged on a sucrose density gradient at 160,000 x g for 16 h. Twelve fractions of 1 ml were collected and immunoprecipitated with N-antiserum (A) and preimmune serum (B). The immunoprecipitates were analyzed as described in Fig. 25. The first seven fractions are indicated, with 1 being at the top of the gradient. The solid pointer indicates the precursor of the adenine nucleotide carrier. The gradient was calibrated by the following molecular mass markers: carbonic anhydrase (30 kDa), bovine serum albumin (68 kDa), and the trimer and tetramer of cross-linked bovine serum albumin. Markers and sample were centrifuged on separate gradients at the same time. Fractions of the gradients containing the marker proteins were collected after centrifugation and analyzed by SDS-12.5% polyacrylamide gel electrophoresis. The lane between fractions 6 and 7 gives the positions of molecular mass markers of 34, 30 and 26 kDa.


A
1 2 3 4 5 6 7
B
1 2 3 4 5 6 7

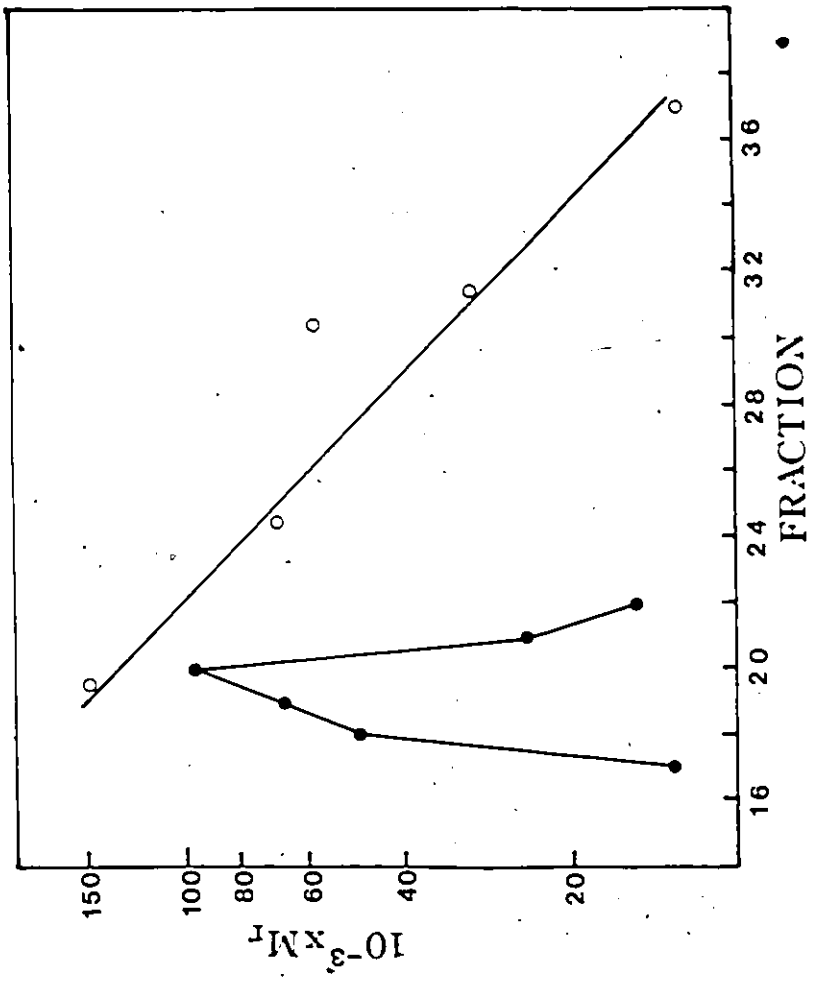
further resolved by fractionation on a Sephadex G-100 column in the absence of Triton X-100. As shown in Fig. 29, most of the precursor was eluted on or before the marker protein aldolase, indicating that it has a molecular mass slightly larger than 150,000 kDa. These results are very similar to those with adenine nucleotide carrier in Neurospora. In the post-ribosomal supernatant, the newly synthesized fungal protein has a size about five times its monomeric form (Zimmermann et al., 1980).

DISCUSSION

Results from the above experiments indicated that the precursor form of the rat liver adenine nucleotide carrier is larger than the mature protein. The relationship between the immunoprecipitated protein and the mature carrier was confirmed by the following three criteria: (1) the precursor was immunoprecipitated by antiserum specific for the protein, but not by preimmune serum. (2) Immunoprecipitation of the precursor can be competed by the presence of excess cold mature protein. (3) The peptide pattern of the precursor was virtually identical to that of the carrier isolated by hydroxylapatite chromatography, indicating that the two proteins have an extensive homology in their primary structures. The finding of a larger precursor for the rat liver adenine nucleotide carrier is supported by a study which showed that the human adenine nucleotide carrier synthesized in tissue culture cells was also larger than its mature counterpart (Webster K.A. and Wallace D.C., personal communication). However, it was reported that in rat hepatoma cells, the adenine nucleotide carrier was synthesized in a form that had

Fig. 29. Estimation of the size of the newly synthesized adenine nucleotide carrier by Sephadex G-100 chromatography. The G-100 column was precalibrated with 0.5 ml of a mixture of the [^{14}C]formaldehyde-labeled proteins, aldolase (150 kDa), bovine serum albumin (68 kDa), and non-radioactive hemoglobin (58 kDa), carbonic anhydrase (30 kDa) and cytochrome c (12.5 kDa). Although the position of hemoglobin was anomolous, it was used as an internal marker to compare the calibration and sample elutions. The post-ribosomal supernatant containing [^{35}S]methionine-labeled proteins was adjusted to 0.3 M KCl and 0.5 ml of the solution was applied to the column. The void volume of the column was 60 ml (fracion 16) and thereafter 2.5 ml fractions were collected. Fractions were subjected to immunoprecipitation and the immunoprecipitates were separated by SDS-12.5% polyacrylamide gel electrophoresis and visualized by fluorography. The amount of precursor in each fraction was estimated by densitometric scanning of the precursor band and then calculating the area under the peaks.

RELATIVE AMOUNT OF pANC (cm^2)



the same size as the mature protein (Hatalova and Kolarov, 1983). At present, the reasons for the discrepancy are not known.

The precursor of the rat liver adenine nucleotide carrier, like the precursor in Neurospora, is synthesized on membrane-free polysomes. Furthermore, both proteins exist as a complex or an aggregate in the post-ribosomal supernatant, which is a few times larger than their mature monomeric forms. However, in both cases, the nature of the complex or aggregate is not clear. As mentioned in the Introduction, the precursors may interact with other cytoplasmic components which facilitate their transport to the mitochondrial outer membrane. On the other hand, the adenine nucleotide carrier is a hydrophobic integral membrane protein, and its precursor polypeptides may form an aggregate with themselves or with other molecules in such a way as to maintain or facilitate the solubility of the precursor in the hydrophilic environment of the experimental solution or the cytoplasm. There is also a possibility that the aggregate formation is an artifact due to the experimental conditions.

The finding in the in vitro studies of a larger precursor for the adenine nucleotide carrier in mammals, but not in Neurospora, is unusual but not unprecedented. The precursor of subunit VI of the cytochrome bc_1 complex in yeast (Cote et al., 1979), but not in Neurospora (Teintzê et al., 1982), is larger than its mature protein. Similarly, the precursor of the carbamoyl phosphate synthetase, which is synthesized with the same size as the mature protein in frog (Mori et

al., 1979), is about 5 kDa larger than its mature counterpart in rat (Shore et al., 1979; Mori et al., 1981; Campbell et al., 1982).

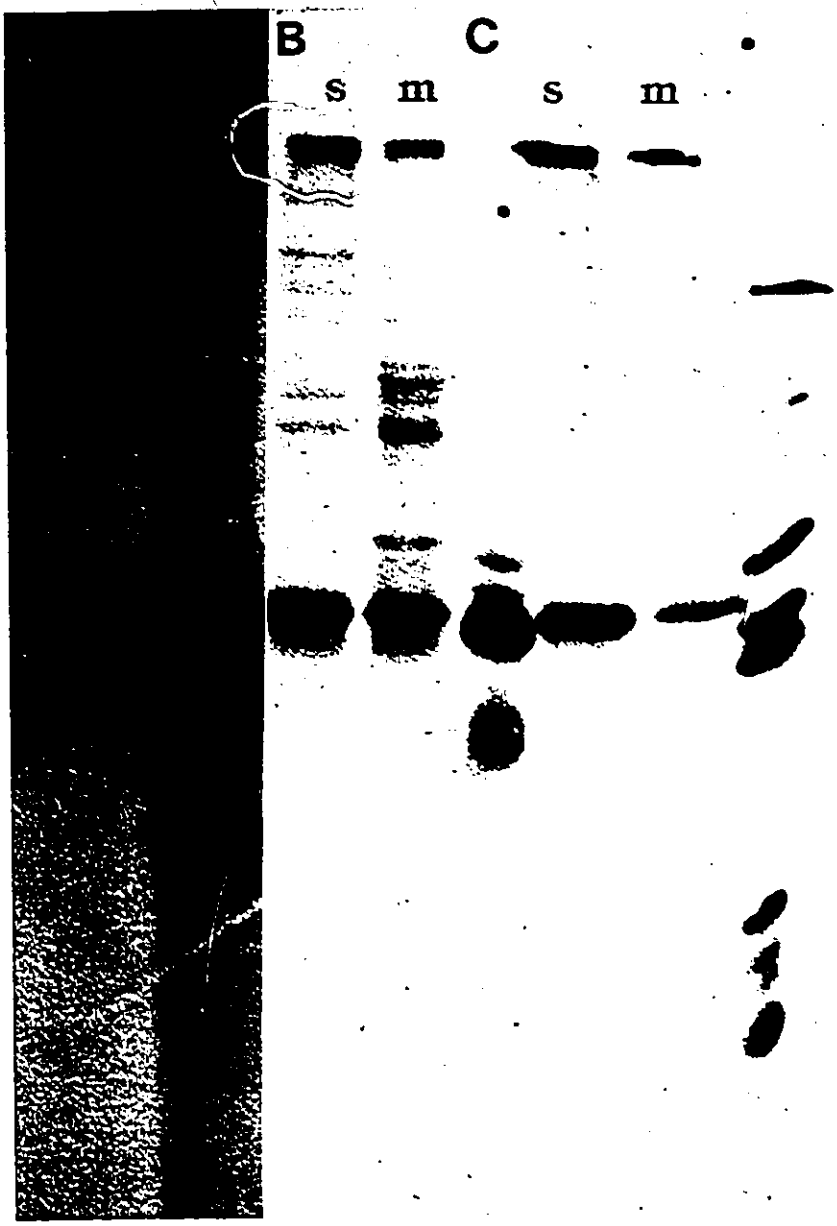
(III) IMPORT OF THE IN VITRO SYNTHESIZED RAT LIVER ADENINE NUCLEOTIDE CARRIER INTO ISOLATED MITOCHONDRIA

In the study of the mechanism of importing the precursor of rat liver malate dehydrogenase into mitochondria, an in vitro import system was developed. Experimental evidence indicated that information obtained from the in vitro system, at least in the case of malate dehydrogenase, reflects the processes that occur in vivo. This in vitro import system was used to study the import of the precursor of the rat liver adenine nucleotide carrier into mitochondria.

RESULTS

In Vitro Import of The Precursor of Rat Liver Adenine Nucleotide Carrier--[^{35}S]Methionine-labeled free polysomal products of rat liver were incubated with mitochondria isolated from CHO cells for 60 min at 30°C. After the incubation, the reaction mixtures were separated into supernatant (s) and mitochondrial (m) fractions. Both fractions were subjected to immunoprecipitation by the N-antiserum. As shown in Fig. 30B, the precursor form of the rat liver adenine nucleotide carrier was immunoprecipitated from both fractions. However, proteolytic processing of the precursor to the mature size was not observed in the mitochondrial fraction. The protease sensitivity of the precursor in the mitochondrial fraction was examined to investigate whether the precursor had been translocated into mitochondria. As shown in Fig. 30A, the precursor in the supernatant was degraded by the externally added

Fig. 30. Import of the precursor of the rat liver adenine nucleotide carrier into isolated mitochondria. [^{35}S]Methionine-labeled proteins from cell-free translation primed with rat liver free polysomes were incubated with mitochondria isolated from CHO cells. Each reaction mixture contained about 400 ug of mitochondrial protein and 3 x 10^7 cpm of radioactive protein. Incubation was for 60 min, and reaction mixtures were separated into supernatant fraction (s) and mitochondrial (m) fractions by centrifugation after the incubation. The fractions were subjected to immunoprecipitation by the N-antiserum. The immunoprecipitates were analyzed by SDS-12.5% polyacrylamide gel electrophoresis with subsequent fluorography. In panel A, the reaction mixture was incubated with trypsin (6 ug/100 ug mitochondrial protein) for 30 min at 0°C. Proteolytic action was stopped with soybean trypsin inhibitor, and the mixture was then separated into supernatant and mitochondrial fractions for immunoprecipitation. In panel B, the reaction mixture was not treated with trypsin. In panel C, import was carried out at 30°C, but in the presence of 10 uM CCCP. (▶), the precursor form of the rat liver adenine nucleotide carrier; (▷), the mature protein. Molecular mass markers are shown on the right-hand-side of panel C.


B
C
s
m
s
m

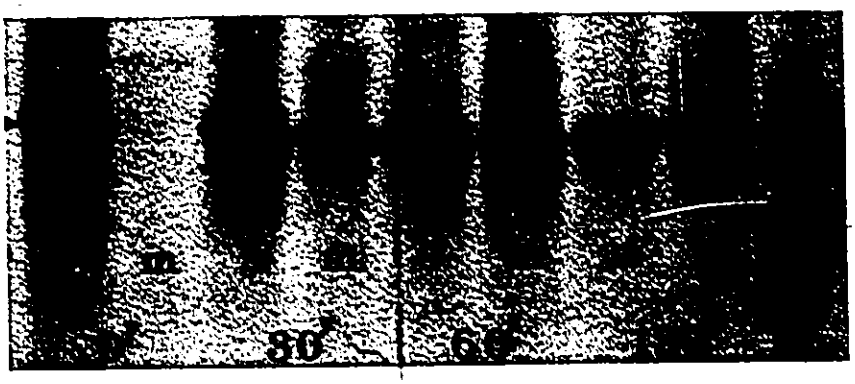
protease. However, the precursor in the mitochondrial fraction was protease insensitive, indicating that it had been translocated past the mitochondrial outer membrane. The effect of uncoupler on the import of the rat liver adenine nucleotide carrier precursor was also studied and the result is shown in Fig. 30C. The amount of the precursor recovered by immunoprecipitation from the mitochondrial fraction incubated in the presence of CCCP was much less than in its absence, suggesting that the binding of the precursor in the incubation solution to mitochondria requires an energized membrane.

Kinetic Study on the Import of the Precursor of the Rat Liver Adenine Nucleotide Carrier—Mitochondria isolated from CHO cells were incubated at 30°C with in vitro synthesized, radiolabeled rat liver proteins for different periods of time. The precursor form of the rat liver adenine nucleotide carrier was immunoprecipitated from both the supernatant and mitochondrial fractions after the reaction mixtures were separated by centrifugation. As shown in Fig. 31, there was a time-dependent transfer of the precursor from the incubation solution to the mitochondria. This result is in sharp contrast to the kinetics of the import of the precursor of rat liver malate dehydrogenase, in which binding of the precursor to mitochondria was completed within 2.5 min and proteolytic processing within 30 min.

DISCUSSION

The demonstration of the protease insensitivity of the precursor

Fig. 31. Time course of import of the precursor of the rat liver adenine nucleotide carrier. Mitochondria were incubated with an equal volume of in vitro synthesized radioactive rat liver protein at 30°C for various periods of time as indicated at the bottom of Fig 31A. Each incubation mixture contained 500 ug of mitochondrial protein and 1.5 x 10^7 cpm of radioactive protein in a final volume of 300 ul. Mitochondria were recovered at the times indicated, and both the supernatant (s) and mitochondrial (m) fractions were subjected to immunoprecipitation by the N-antiserum. The immunoprecipitates were analyzed by SDS-12.5% polyacrylamide gel electrophoresis and fluorography (Fig. 31A). Five percent of each immunoprecipitate was counted and the results were plotted against time (Fig. 31B). (▶), the precursor form of rat liver adenine nucleotide carrier, (▷), the mature protein.


30'
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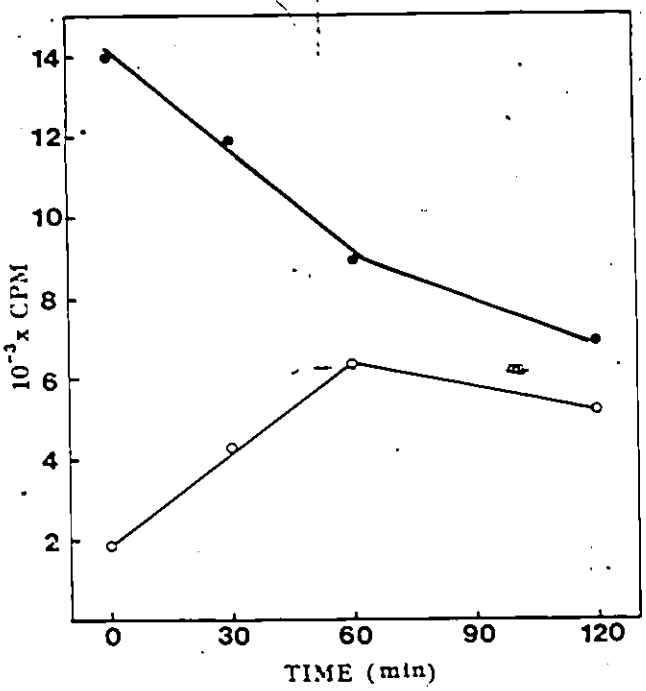
14
12
10
8
6
4
2
10^{-3} x CPM
0
30
60
90
120
TIME (min)
●-Supernatant
○-Mitochondria

of the rat liver adenine nucleotide carrier in the mitochondrial fraction indicates that the precursor had been translocated into mitochondria. In the study on the import of the precursor of rat liver malate dehydrogenase, the binding of the precursor of malate dehydrogenase to isolated mitochondria was not affected by temperature and did not require an electrochemical potential across the inner membrane. However, the addition of CCCP to the in vitro import system greatly reduced the amount of the precursor of the adenine nucleotide carrier recovered in the mitochondrial fraction, suggesting that the binding of the precursor to mitochondria requires an energized membrane. As mentioned previously, the precursors of yeast ATPase subunit 2 and cytochrome b_2, as well as the precursors of subunits I and VII of the Neurospora cytochrome bc_1 complex also bind poorly to mitochondria in the presence of an uncoupler (Daum et al., 1982; Gasser et al., 1982b; Tenitz et al., 1983).

In the import of the precursor of rat liver malate dehydrogenase, the binding of the precursor to the mitochondrial outer membrane was essentially completed within 2.5 min, but the conversion of the bound precursor to the mature size and a protease protected form required 10 to 30 min. Thus, the rate limiting step in the import process is the translocation of the precursor of malate dehydrogenase across the inner membrane and its processing to the mature size. However, Fig. 31 showed that there was a linear increase in the amount of the precursor of the adenine nucleotide carrier recovered in the mitochondrial fraction in the in vitro import system up to 60 min,

indicating different mechanisms may be involved in the import of the two precursor proteins. Furthermore, the finding that the binding of the precursor of rat liver adenine nucleotide carrier to mitochondria may require the presence of an energized membrane suggests that the binding of the adenine nucleotide carrier precursor to mitochondria is tightly coupled to its translocation into mitochondria.

The reason for the absence of proteolytic cleavage of the precursor of the rat liver adenine nucleotide carrier is not known and should be pursued in the future. However, if the precursor was inserted into the mitochondrial membranes without proteolytic cleavage to its mature size, it would suggest that the insertion or translocation of some mitochondrial precursors into or across the inner membrane is not necessarily coupled with the processing of the precursors. Indeed, it was shown that the in vitro translocation of the precursors of cytochrome c peroxidase in yeast (Reid et al., 1982) and of subunit 9 of the F_1F_0-ATPase in Neurospora (Zwizinski & Neupert, 1983) across the mitochondrial inner membrane can occur in the absence of the proteolytic processing of the two precursors. However, Kolarov and Hatalova (1984) reported that, in hepatoma cells, the proteolytic processing of the larger precursor of the β-subunit of the F_1-ATPase is tightly coupled to the translocation step which requires the presence of an energized membrane, but the translocation of the precursor of the adenine nucleotide carrier, which has the same size as the mature protein, can occur in the absence of a membrane potential across the inner membrane. These results in hepatoma cells suggest that translocation of larger

precursors into or across mitochondrial membranes is coupled to the proteolytic processing step. It should be noted that in Neurospora, the precursor of the adenine nucleotide carrier also has the same size as the mature protein, but requires a membrane potential for its insertion into the mitochondrial inner membrane in vitro (Zimmermann et al., 1979, 1980).

One of the possible explanations for the absence of in vitro processing of the precursor of the adenine nucleotide carrier is that the protease responsible for its cleavage is different from the one for converting the precursor of malate dehydrogenase into its mature size, and that the protease is either lost during the isolation of mitochondria from whole cells or is inactive in the in vitro import system. On the other hand, the difference in molecular mass between the rat liver adenine nucleotide carrier and its precursor is based on their electrophoretic mobilities on SDS-polyacrylamide gels. It remains a possibility that the apparent difference in mobility on gels is due to factors other than size. In that case, the definitive answer has to wait for the elucidation of the sequence of the precursor protein. This could be achieved by allowing synthesis in vitro in the presence of radiolabeled amino acids followed by protein sequencing. Alternatively, a cDNA clone of the protein's mRNA would permit determination of the N-terminal sequence of the precursor.

CONCLUSION

Results obtained from this research have led to the elucidation of the biosynthetic pathway of rat liver mitochondrial malate dehydrogenase. The pathway is shown schematically in Fig. 32. The precursor of the enzyme is synthesized on cytoplasmic membrane-free polysomes with a size that is approximately 1.5 to 2.0 kDa larger than the mature monomeric form. The newly synthesized precursor exists in a complex of about 90 kDa in the post-ribosomal supernatant. At present, the nature of the components forming the complex is not clear. However, they may play a role in the transfer of the precursor to mitochondria as discussed previously in the Results and Discussion. At the cytoplasmic surface of the mitochondrial outer membrane, binding of the precursor to its receptor triggers the translocation of the protein across the mitochondrial membranes. Although it is clear that translocation requires a membrane potential across the mitochondrial inner membrane, it is not known how the process is achieved. It may occur at sites of apposition of the two mitochondrial membranes, and involve transmembrane proteinaceous pores for the precursor protein to pass through the two lipid bilayers and into the matrix. Alternatively, it may cross the phospholipid bilayers directly. Translocation is followed by the proteolytic processing of the larger precursor to its mature monomeric size. The processing step occurs in the matrix by a protease requiring divalent cations as cofactor. In the in vitro system, it is difficult to

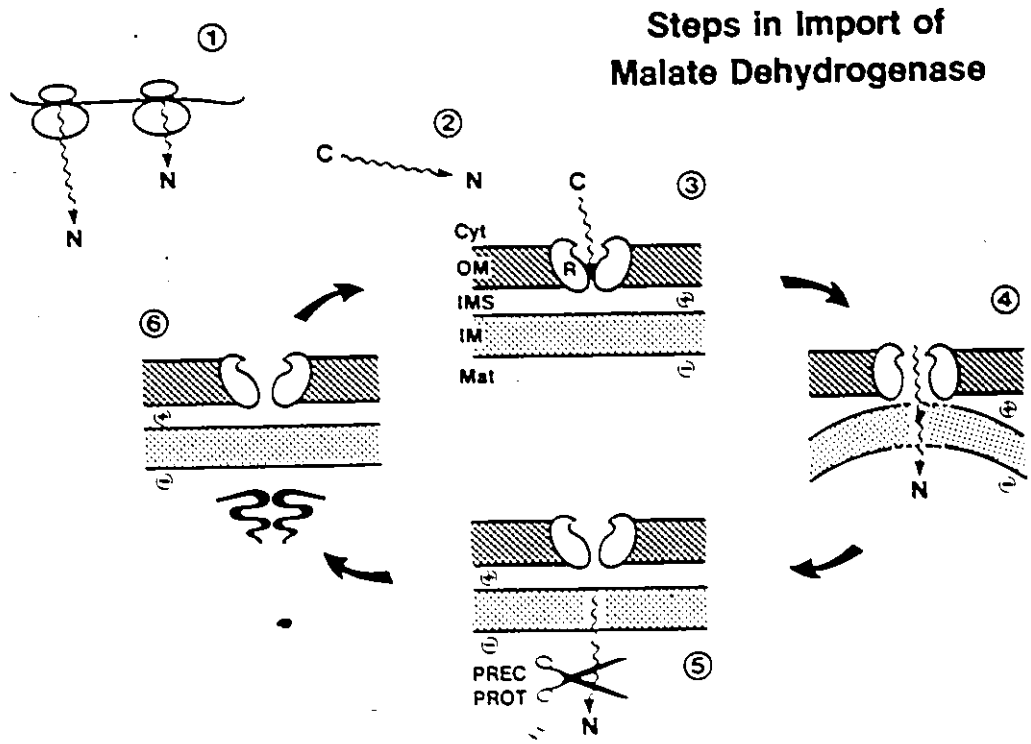

Fig. 32. The Biosynthetic Pathway of Rat Liver Malate Dehydrogenase

distinguish the translocation process from the proteolytic processing step, and it is not known whether the processing occurs before or after the completion of the translocation process. In vitro studies also showed that the conversion to mature size results in the formation of a NAD^+-binding site in the newly processed protein. The NAD^+-binding site is not present in the larger precursor, suggesting that the proteolytic cleavage of the larger precursor is followed by conformational changes which results in the formation of the binding site.

Results also showed that similarities exist between the biosynthetic pathway of the rat liver adenine nucleotide carrier and that of the malate dehydrogenase. The carrier is also synthesized on membrane-free polysomes with a size that is about 1.5 kDa larger than the mature monomeric form and, after synthesis, the precursor exists as a large complex that is about five times its size. A precursor of larger size has also been identified for the human adenine nucleotide carrier (Webster, K.A. and Wallace, D.C., personal communication). However, a larger precursor form was not observed in an in vivo study using rat hepatoma cells (Hartlova and Kolarov, 1983). The discrepancy between the latter study and that of here is not known, and resolution requires the elucidation of the coding sequence of the protein.

Although in vitro processing of the precursor of the rat liver adenine nucleotide carrier was not observed, the protease insensitivity of the precursor recovered in the mitochondrial fraction indicates that,

at least, some of the precursor had been translocated through the mitochondrial outer membrane. If the precursor is indeed larger than the mature form, the result would imply that the translocation step is not obligatorily linked to the proteolytic cleavage of the precursor protein. Similar results have been obtained in yeast and Neurospora (Reid et al., 1982; Zwizinski and Neupert, 1983).

Other differences in the import of the precursors of the rat liver adenine nucleotide carrier and malate dehydrogenase are also observed. As mentioned previously, the rate limiting step in the import of the precursor of malate dehydrogenase is at the translocation and the processing steps. Binding of the precursor to receptor on the mitochondrial outer membrane occurred in less than 2.5 min in vitro. However, the conversion of the bound precursor to mature size required 10 to 30 min in vitro. In the import of the adenine nucleotide carrier, in vitro experiments showed that there was a time-dependent transfer of the precursor from the incubation solution to mitochondria of up to 60 min. Furthermore, the binding of the adenine nucleotide carrier precursor to mitochondria seems to require the presence of an energized inner membrane, suggesting that binding of the precursor to its receptor may be tightly coupled to its translocation across the membrane. These differences in the import of the two proteins seem to be a variation in an otherwise very similar biosynthetic pathway and the present evidence indicates that the site of variation occurs at the binding step, suggesting that different receptors may be involved in the import of the two precursors into mitochondria.

In our laboratory, the import of the uncoupling protein of brown adipose tissue was also investigated. The precursor form of this inner membrane protein has the same size as the mature protein (Freeman et al., 1983; Ricquier et al., 1983). Experiments with the in vitro import system showed that in vitro synthesized precursor form of this tissue specific protein could be imported into mitochondria isolated from CHO cells and converted into a protease resistant form, indicating there is no tissue specificity in the uptake of this protein (Freeman et al., 1983). Similar results were obtained on the import of the precursors of hepatic ornithine transcarbamylase (Argan et al., 1983) and adrenocortical adrenodoxin (Matocha and Waterman, 1984). These results show that mammalian precursor proteins share receptors for their import into mitochondria. However, adrenocortical cytochrome $P\text{-}450_{sec}$ can be imported into adrenocortical, but not heart mitochondria, suggesting a specific receptor for the protein is present on adrenocortical mitochondria (Matocha and Waterman, 1984), and indicates that there are different receptors for importing different precursor proteins. In Neurospora, different receptors for importing mitochondrial proteins are also found (Zimmermann et al., 1981). There is also evidence in both Neurospora and mammals that the receptors on the mitochondrial outer membrane for importing precursor proteins are proteinaceous in nature (Ziwisinski et al., 1984; Argan et al., 1983).

There is very limited information on how the receptor-bound

precursor proteins are translocated into or across the mitochondrial membranes. Does the binding of a precursor protein to its receptor result in the formation of a hydrophilic channel for the passage of the polypeptide across the membranes? If so, what are the components that constitute the channel? For precursor destined for the inner membrane or the matrix compartment, how does the interaction of the precursor with its receptor on the outer membrane result in the translocation across the two lipid bilayers? Do sites of apposition between the inner and outer membranes play a role in the translocation process? These questions are difficult to answer and require the identification and isolation of the receptor as well as better understanding of the receptor-precursor protein interaction.

Recently, research have been concentrated in studying the roles of the amino terminal extension peptides of mitochondrial precursor proteins in the import process. During the writing of this thesis, several reports showed that the extension peptide is essential in targeting proteins into mitochondria. Douglas et al. (1984) showed that a hybrid protein, consisting of the amino-half of the β-subunit of yeast ATPase at its amino terminal end and β-galactosidase at its carboxyl terminal end, was imported into mitochondria, both in vitro and in vivo. Hurt et al. (1984) reported that the fusion of the 25 amino acids extension peptide of the precursor of subunit IV of yeast cytochrome c oxidase to the amino terminus of a mouse cytoplasmic enzyme, dihydrofolate reductase, resulted in the translocation of the cytoplasmic enzyme into mitochondria and with the correct cleavage of

the extension peptide. In fact, only 12 of the 25 amino acids are required for targeting the hybrid protein into mitochondria (Hurt et al., 1985). Similarly, Horwich et al. (1985), using the same approach, showed that the extension peptide of the human ornithine transcarbamylase can direct the mouse dihydrofolate reductase into rat liver mitochondria in vitro and into mitochondria of a CHO cell line expressing the hybrid protein. The above results strongly indicate that the information for directing mitochondrial precursor proteins of larger size into the organelle resides solely in the entire or a portion of the extension peptide. It is not known what serves to direct proteins which are not made as larger precursors into mitochondria. The signal could be located at the N-terminus or in the internal sequence of the mature protein. Alternatively, different receptors may be involved in translocating precursors with and without an extension peptide into mitochondria.

Although mitochondrial outer membrane proteins are usually synthesized without an amino terminal extension peptide, there is an indication that the information for targeting these proteins to the outer membrane may also be located at the amino termini of the proteins. Hase et al. (1984) showed that the 41 amino acids at the amino end of the 70 kDa outer membrane protein of yeast, which accounts for only 7% of the complete polypeptide, is necessary for the targeting and the anchorage of the protein to the outer membrane. A hybrid protein of β-galactosidase with the 41 amino acids at its amino end was incoporated into the mitochondrial outer membrane. Without this peptide,

the hybrid protein remained in the cytoplasm.

At present, it is not clear how imported mitochondrial proteins are assembled into functional enzymes inside the organelle. Many mitochondrial enzymes have a multi-subunit structure with coding sequences in either mitochondrial or nuclear genome. Thus, proper assembly of these enzymes requires a well coordinated effort from both genomes. Furthermore, there is little information on the regulatory mechanisms regarding the import of mitochondrial proteins. How is the rate of protein import adjusted with the physiological demands of a living cell? How much influence does the nucleus have on the import of proteins into mitochondria? Do mitochondria in an intact cell have control over the proteins as well as the amount they are importing? These areas as well as the detail of the translocation process will be the major interests in future studies on the import of mitochondrial proteins.

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2. K.B. Freeman, S.-M. Chien, D. Litchfield and H.V. Patel. Synthesis in vitro of rat brown adipose tissue 32,000 Mr protein. FEBS Lett. 158, 325-330 (1983).

3. S.-M. Chien and K.B. Freeman. Import of rat liver mitochondrial malate dehydrogenase: synthesis, transport, and processing in vitro of its precursor. J. Biol. Chem. 259, 3337-3342 (1984).

4. S.-M. Chien, H.V. Patel and K.B. Freeman. Import of rat liver mitochondrial malate dehydrogenase: binding of the precursor to mitochondria, an intermediate step in import. J. Biol. Chem. 259, 13633-13636 (1984).

5. S.-M. Chien and K.B. Freeman. Import of rat liver mitochondrial malate dehydrogenase: acquisition of an NAD^+-binding site on the in vitro processed precursor protein. Submitted to J. Biol. Chem.

Abstracts

1. S.-M. Chien, K.A. Webster and K.B. Freeman. Proc. Can. Fed. Biol. Soc. 24, 287 (1981).

2. S.-M. Chien and K.B. Freeman. Fed. Proc. 42, 2125 (1983).

3. S.-M. Chien and K.B. Freeman. Proc. Can. Fed. Biol. Soc. 26, 88 (1983).

4. K.B. Freeman, H.V. Patel and S.-M. Chien. Rossiter Res. Conf. 1, 16 (1983).

5. S.-M. Chien and K.B. Freeman. International Congress of Biochemistry, 688 (1985).

6. S.-M. Chien and K.B. Freeman. International Symposium on Achievements and Perspectives in Mitochondrial Research, 118 (1985).

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