

CELL MEMBRANES

Methods and Reviews

Volume 1

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PLENUM PRESS • NEW YORK AND LONDON

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BIOSYNTHESIS AND ASSEMBLY OF MITOCHONDRIAL PROTEINS

Martin Teintze and Walter Neupert

1. INTRODUCTION

Eukaryotic cells are divided into a number of subcellular compartments, each surrounded by one or more membranes. By creating these compartments (i.e., organelles), eukaryotic cells have evolved a host of new capabilities compared to the simpler prokaryotic cells. The creation within the cell of compartments enclosed by membranes allows, for instance, the use of metabolic pathways in opposite directions at the same time, or the storage of substances (such as Ca^{2+} in the sarcoplasmic reticulum) where they can be released when needed to initiate reactions. Proteolytic enzymes can be sequestered where they do not interfere with other cell functions, and ion or proton gradients can be generated across membranes within the cell to drive reactions such as the synthesis of ATP. This increased complexity, however, brings with it a host of new problems for the eukaryotic cell. A major one is that almost all the proteins of the intracellular organelles are synthesized on cytoplasmic ribosomes (Schatz and Mason, 1974; Chua and Schmidt, 1979). Hundreds of different proteins must be directed into their proper organelles during or after translation. The membranes of these organelles, on the other hand, are practically impermeable to macromolecules such as proteins. Therefore mechanisms must exist for specifically transferring newly synthesized proteins into or across the membrane or membranes of the proper organelle.

Most theories that attempt to explain this phenomenon postulate the

existence of complementary structures on the proteins and the organelles, i.e., receptors in the outer membranes of these organelles. Receptor proteins would be able to bind specifically a protein or class of proteins destined for an organelle during or after its synthesis and mediate its transport into or across the membrane.

Although no such receptor proteins have been isolated yet, the evidence suggesting their existence has been growing (Hennig and Neupert, 1981). The transfer of proteins from their site of synthesis to their functional site is irreversible; this requires that the proteins be synthesized as precursors that are in some way different from the mature functional form of the protein. Some of the ways by which this is accomplished will be discussed in the following sections.

Two kinds of organelles, the mitochondria and the chloroplasts, have their own DNA and ribosomes and are able to synthesize a few of their own proteins. A number of those are subunits of enzyme complexes that also contain subunits coded for on nuclear genes and synthesized on cytoplasmic ribosomes. Very little is known to date about how these two synthetic systems are coordinated so that these enzyme complexes are assembled with stoichiometric ratios of subunits.

The study of the molecular mechanisms of intracellular protein transport involves following single, well-defined proteins that can be assigned to one particular organelle over the entire path from the synthetic origin to the functional site. This requires isolation and purification of such proteins and the preparation of specific antibodies with which one can detect the minute quantities of precursors or intermediates that might be involved in the transfer process.

Many studies on intracellular protein transport have focused on mitochondria. A large number of mitochondrial proteins have been well-characterized with respect to structure, localization, and site of synthesis. Mitochondria can easily be separated from the remaining cell components, allowing separation of the synthesis and transfer processes.

2. POST- VS. COTRANSLATIONAL TRANSPORT

There are probably several different mechanisms by which proteins synthesized in the eukaryotic cell reach their final destinations. The proteins

whose intracellular transport has been studied can be divided into two major classes: those that are transported cotranslationally and those that are transported posttranslationally.

Cotranslational transport is observed in the endoplasmic reticulum (ER) and probably the plasma membrane of bacteria (Sabatini *et al.*, 1982). Proteins that are destined to be secreted via the Golgi apparatus or have their functional site in the lumen of the endoplasmic reticulum are synthesized by ribosomes bound to the cytoplasmic face of the ER membrane (Palade, 1975; Blobel and Dobberstein, 1975). The nascent polypeptide chain is discharged through the membrane during protein synthesis, before the polypeptide is fully folded.

Some details of the cotranslational mechanism have recently been elucidated. An 11 S protein composed of six polypeptides was purified from dog pancreas rough microsomes and was shown to bind to polysomes synthesizing secretory proteins. Since it apparently recognizes the amino terminal signal sequence of the nascent polypeptide, it was termed the "signal recognition protein" (SRP) (Walter *et al.*, 1981). This protein complex apparently also mediates the binding of the polysomes synthesizing the secretory protein to microsomal membranes (Walter and Blobel, 1981a) and stops chain elongation until the SRP-ribosome complex binds to the microsomal membrane (Walter and Blobel, 1981b). The receptor for the SRP or "docking protein" has been identified as an integral glycoprotein (M_r 72,000) of the ER membrane (Walter *et al.*, 1979; Meyer and Dobberstein, 1980; Meyer *et al.*, 1982). Furthermore, the rough ER contains ribosome binding sites called ribophorins (Kreibich *et al.*, 1978) that may serve to keep the ribosome attached to the membrane while elongation of the polypeptide is completed. During this second elongation phase the polypeptide traverses the membrane and a "signal peptidase" removes the signal sequence from the nascent chain (Jackson and Blobel, 1977). A "leader peptidase" has also been isolated from *E. coli* inner and outer membranes (Zwizinski and Wickner, 1980).

On the other hand, the intracellular transport of a large number of proteins into mitochondria, as well as chloroplasts, glyoxysomes, and peroxisomes, has been shown to take place by a posttranslational mechanism (Chua and Schmidt, 1979; Neupert and Schatz, 1981). This means that synthesis of the precursor protein is separated in both space and time from the transfer into the organelle rather than being obligatorily coupled as in cotranslational transport.

Two different types of experiments have been employed to follow the biogenesis of a mitochondrial protein. In *in vivo* pulse-labeling studies one

can often detect the precursors, if they differ in molecular weight or immunological properties from the mature proteins, and follow their processing by lysing the cells at different time points and immunoprecipitating the proteins. These experiments show that processing and transfer still occur when protein synthesis has been stopped with cycloheximide, and that labeled precursors appear in the cytosol before the labeled proteins appear in the mitochondrial fraction (Hallermayer *et al.*, 1977; Schatz, 1979; Teintze *et al.*, 1982). It is difficult to tell on what type of ribosome a protein is made, however, or where it is localized in the cell.

Experiments can also be done *in vitro* using precursors synthesized in a homologous or heterologous cell-free system and isolated mitochondria. These can be resuspended in the postribosomal supernatant of the cell-free system after protein synthesis has been completed, and import of precursor proteins into the mitochondria in the absence of protein synthesis can be observed (Korb and Neupert, 1978; Maccicchini *et al.*, 1979a; Zimmermann and Neupert, 1980). In this type of experiment one can distinguish proteins that remain in solution, those that are bound to recognition sites on the mitochondria but still accessible to externally added proteases, and those that have been imported into a protease-resistant and/or functional location in the mitochondria. The results show that as the precursors disappear from the supernatant they are first bound to the outside of the mitochondria and then transferred to their functional site. It is during this second step that they are converted to their mature, functional form.

So far almost all mitochondrial precursor proteins studied are synthesized on free polysomes and are transferred posttranslationally (Table I). However, general rules for transfer into organelles cannot be drawn. In fact, insertion of proteins into ER or plasma membranes appears to obey both mechanisms, depending on the protein (Sabatini *et al.*, 1982). Transfer of proteins into the plasma membrane of bacteria may also occur by both cotranslational and posttranslational pathways (Wickner, 1980).

3. PRECURSOR FORMS OF MITOCHONDRIAL PROTEINS

The mitochondrial proteins that are made on cytoplasmic ribosomes are synthesized as precursors that can be immunoprecipitated after pulse-labeling cells *in vivo* or after synthesis in an *in vitro* cell-free system. Many of these

TABLE I. Mitochondrial Precursor Proteins

Protein	Organism or tissue	Location in mitochondrion	Site of synthesis (polysomes)	Posttranslational transfer shown	Energy-dependent	Precursor MW (approx.)	Mature MW (approx.)	Reference(s)
Porin	<i>Neurospora</i>	Outer membrane	Free	Yes	No	31,000	31,000	Freitag <i>et al.</i> (1982a,b)
Cytochrome <i>b</i> ₂	Yeast	Intermembrane space	N.D. ^a	Yes	Yes	68,000	58,000	Gasser <i>et al.</i> (1982)
Cytochrome <i>c</i> peroxidase	Yeast	Intermembrane space	N.D.	Yes	No	39,500	33,500	Maccecchini <i>et al.</i> (1979b), Nelson and Schatz (1979)
Sulfite oxidase	Rat liver	Intermembrane space	Free	No	N.D.	59,000	55,000	Mihara <i>et al.</i> (1982)
Cytochrome <i>c</i>	<i>Neurospora</i>	Intermembrane space-inner membrane	Free	Yes	No	12,000	12,000	Korb and Neupert (1978), Zimmermann <i>et al.</i> (1979a, 1981)
	Rat liver	Intermembrane space-inner membrane	Free	Yes	No	12,000	12,000	Matsuura <i>et al.</i> (1981)
ADP/ATP carrier	<i>Neurospora</i>	Inner membrane	Free	Yes	Yes	32,000	32,000	Zimmermann <i>et al.</i> (1979b), Zimmermann and Neupert (1980)
ATPase subunit 9	<i>Neurospora</i>	Inner membrane	Free	Yes	Yes	12,000	8,000	Michel <i>et al.</i> (1979), Zimmerman <i>et al.</i> (1981)
Cytochrome <i>bc</i> ₁ complex	<i>Neurospora</i>	Inner membrane	N.D.	Yes	Yes	51,500	50,000	Teintze <i>et al.</i> (1982)
Subunit I			N.D.	Yes	Yes	47,500	45,000	
Subunit II			Free	Yes	Yes	38,000	31,000	
Cytochrome <i>c</i> ₁			N.D.	Yes	Yes	28,000	25,000	
Subunit V			N.D.	No	N.D.	14,000	14,000	
Subunit VI			N.D.	Yes	Yes	12,000	11,500	
Subunit VII			N.D.	No	N.D.	11,600	11,200	
Cytochrome <i>bc</i> ₁ complex	Yeast							Côté <i>et al.</i> (1979), Nelson and Schatz (1979)
Cytochrome <i>c</i> ₁			N.D.	No	Yes	37,000	31,000	
Subunit V			N.D.	No	Yes	27,000	25,000	

(Continued)

TABLE I. (Continued)

Protein	Organism or tissue	Location in mitochondrion	Site of synthesis (polysomes)	Posttranslational transfer shown	Energy-dependent	Precursor MW (approx.)	Mature MW (approx.)	Reference(s)
Cytochrome <i>c</i> oxidase	Rat liver	Inner membrane						Schmelzer and Heinrich (1980), Heinrich (1982)
Subunit IV			Free + bound	No	N.D.	19,500	16,500	
Subunit V			Free + bound	No	N.D.	15,500	12,500	
Cytochrome <i>c</i> oxidase	Yeast	Inner membrane						Lewin <i>et al.</i> (1980), Mihara and Blobel, (1980)
Subunit IV			N.D.	No	N.D.	—	—	
Subunit V			N.D.	Yes	N.D.	—	—	
Subunit VI			N.D.	No	N.D.	20,000	12,500	
Subunit VII			N.D.	No	N.D.			
Cytochrome P450 _{cc}	Bovine adrenal cortex	Inner membrane	N.D.	No	N.D.	54,500	49,000	DuBois <i>et al.</i> (1981)
Cytochrome P450 _{11β}	Bovine adrenal cortex	Inner membrane	Free + bound	No	N.D.	50,000	45,000	Nabi <i>et al.</i> (1980)
F ₁ -ATPase	Yeast	Matrix-inner membrane						Maccacchini <i>et al.</i> (1979a), Nelson and Schatz (1979), Lewin <i>et al.</i> (1980)
α Subunit			N.D.	Yes	Yes	64,000	58,000	
β Subunit			N.D.	Yes	Yes	56,000	54,000	
γ Subunit			N.D.	Yes	Yes	40,000	34,000	
Adrenodoxin	Bovine adrenal cortex	Matrix	Free + bound	Yes	N.D.	20,000	12,000	Nabi and Omura (1980)
δ-Aminolevulinic acid synthetase	Rat liver	Matrix	Free	No	N.D.	51,000	45,000	Yamauchi <i>et al.</i> (1980a,b)
Aspartate aminotransferase	Chicken heart	Matrix	Free	No	N.D.	47,500	44,500	Sonderegger <i>et al.</i> (1980, 1982)
Carbamoyl-phosphate synthetase	Rat liver	Matrix	Free	Yes	N.D.	47,000	45,000	Sakakibara <i>et al.</i> (1980)
	Rat liver	Matrix	Free	Yes	N.D.	165,000	160,000	Mori <i>et al.</i> (1979), Raymond and Shore (1979, 1980), Shore <i>et al.</i> (1979)

Citrate synthase	<i>Neurospora</i>	Matrix	N.D.	No	N.D.	47,000	44,500	Harmey and Neupert (1979)
L-Glutamate dehydrogenase	Rat liver	Matrix	Free	No	N.D.	60,000	54,000	Mihara <i>et al.</i> (1982)
D- β -Hydroxybutyrate dehydrogenase	Rat liver	Matrix	Free	No	N.D.	37,000	32,000	Mihara <i>et al.</i> (1982)
Malate dehydrogenase	Rat liver	Matrix	Free	No	N.D.	38,000	37,000	Mihara <i>et al.</i> (1982)
Ornithine carbamoyl-transferase	Rat liver	Matrix	N.D.	Yes	Yes	39,400 43,000	36,000 39,000	Mori <i>et al.</i> (1980) Conboy and Rosenberg (1981), Morita <i>et al.</i> (1982)
Superoxide dismutase	Yeast	Matrix	N.D.	No	Yes	26,000	24,000	Autor (1982)

^a N.D., not determined .

precursors have an additional sequence that is removed by a proteolytic enzyme during or after the transfer process. Their apparent molecular weights are larger than those of the mature proteins by anywhere from 500 daltons for subunit VII of the *Neurospora* cytochrome bc_1 complex (Teintze *et al.*, 1982) to 10,000 daltons for cytochrome b_2 (Gasser *et al.*, 1982). Other proteins for which this type of transfer with proteolytic processing has been observed are listed in Table I.

For cytochrome c_1 in *Neurospora* (Teintze *et al.*, 1982) and for cytochromes c_1 and b_2 in yeast (Gasser *et al.*, 1982), the proteolytic processing takes place in two separate steps via a form with intermediate molecular weight.

Some mitochondrial proteins, however, are synthesized with the same molecular weight as the mature functional protein and are transferred without proteolytic processing. These include cytochrome c (Zimmermann *et al.*, 1979a), the ADP/ATP carrier (Zimmermann *et al.*, 1979b), subunit VI of the cytochrome bc_1 complex (Teintze *et al.*, 1982), and the outer membrane porin (Freitag *et al.*, 1982b). In these cases there must be some kind of conformation change that allows the proteins to get into or through the membrane and prevents them from leaving their compartment.

The most extensively studied example of this type of transfer process is that of cytochrome c , because it is the only imported protein whose precursor, apocytochrome c , has been prepared in large quantities. Apocytochrome c is transferred across the outer membrane into the intermembrane space in conjunction with the covalent attachment of a heme group to form mature holo-cytochrome c (Hennig and Neupert, 1981). The heme group can be removed from isolated holo-cytochrome c to produce chemical quantities of apocytochrome c , which is identical to the precursor synthesized in the cell. Antibodies prepared against *Neurospora* apo- and holo-cytochrome c do not cross-react (Korb and Neupert, 1978). Excess unlabeled apocytochrome c , but not holo-cytochrome c , can compete with radioactive apocytochrome c for binding and transfer into *Neurospora* mitochondria (Hennig and Neupert, 1981).

Thus, upon addition of the heme group, the protein probably undergoes a conformational change that results in transfer of the holo-cytochrome c into the intermembrane space.

No covalent modifications have been detected so far for any of the other mitochondrial protein precursors that are imported without proteolytic processing. The precursor of the ADP/ATP carrier of *Neurospora* mitochondria synthesized *in vitro* in a heterologous cell-free system has the same apparent

molecular weight on sodium dodecylsulfate gels as the mature protein and was shown not to have an additional sequence at the N terminal by translation in the presence of *N*-formyl[³⁵S]methionyl-tRNA (Zimmermann *et al.*, 1979b). The precursors were found to be present as soluble aggregates with molecular weights in the range of 100,000 to 500,000 (Zimmermann and Neupert, 1980), whereas the mature protein is an insoluble integral inner membrane protein with a molecular weight of about 30,000.

The outer membrane porin of *Neurospora* mitochondria, which is also synthesized with the same molecular weight as the mature protein (Freitag *et al.*, 1982b), has recently been isolated in a water-soluble lipid-free form (H. Freitag and W. Neupert, unpublished observations). This water-soluble form of porin can compete with the *in-vitro*-synthesized precursor for binding and transfer into mitochondria, unlike isolated porin, which contains lipid and can only be solubilized in the presence of detergent.

4. EVIDENCE FOR THE EXISTENCE OF SPECIFIC RECEPTORS

The precursor proteins that are synthesized on free cytoplasmic ribosomes must have a method of recognizing the organelle for which they are intended and a method of entering or crossing a membrane that is normally impermeable to proteins. The most logical mechanism for such a process is to have specific receptors in the outer membrane of the organelle that can both bind the precursor and mediate its transfer.

The evidence for the presence of a specific receptor in the outer mitochondrial membrane is strongest in the case of cytochrome *c*. The presence of specific saturable binding sites is indicated by the following data. Excess unlabeled apocytochrome *c* can compete with labeled apocytochrome *c* and inhibit its transfer into the mitochondria (Hennig and Neupert, 1981). Deuterohemin, an analog of the natural prosthetic group protohemin lacking the vinyl groups necessary for covalent attachment to the apoprotein, inhibits transfer but not binding. In the presence of deuterohemin, radioactive apocytochrome *c* bound to *Neurospora* mitochondria can be displaced by excess unlabeled apocytochrome *c* from *Neurospora* (Hennig and Neupert, 1981). Apocytochrome *cs* from other species compete less effectively in a manner that parallels their sequence homology with the native *Neurospora* protein, with an apocytochrome *c* from a bacterium (*Paracoccus denitrificans*) not

able to compete for the binding sites at all (B. Hennig and W. Neupert, unpublished observations). If mitochondria with radiolabeled apocytochrome *c* bound in the presence of deuterohemin are isolated, washed, and placed in a medium containing protohemin, the natural prosthetic group, the apocytochrome *c* is converted to holocytochrome *c* and transferred into the mitochondria (Hennig and Neupert, 1981). This shows that the apocytochrome *c* is initially bound to a location on the outside of the mitochondria from which it can be displaced by externally added unlabeled apocytochrome *c*, yet it is bound to a location from which it can be transferred into the mitochondria when the inhibition by deuterohemin is relieved.

Detailed binding studies have been carried out using [¹⁴C]apocytochrome *c* prepared by reductive methylation (H. Köhler, B. Hennig and W. Neupert, unpublished observations). The binding of labeled apocytochrome *c* to whole mitochondria gives a biphasic Scatchard plot with about 90 pmoles of high-affinity binding sites ($K_d = 10^{-7}$ M) per mg of mitochondrial protein. This is a very large number considering the low protein content of the outer mitochondrial membrane. In fact, the only outer membrane protein present in sufficient quantity to explain the number of binding sites found is the porin (Freitag *et al.*, 1982a). Studies are now under way to determine whether the porin could be the receptor for apocytochrome *c*.

The outer membrane of mitochondria also does not appear to have enough proteins for there to be a separate receptor for each of the many different cytoplasmically synthesized proteins that must be bound and imported. However, efforts to find another protein that uses the same receptor as cytochrome *c* have proved fruitless. In experiments using *Neurospora* mitochondria and radioactive precursor proteins synthesized in reticulocyte lysates, excess unlabeled apocytochrome *c*, at a concentration sufficient to inhibit the import of radioactive cytochrome *c*, had no effect on the transfer of the ADP/ATP carrier, subunit 9 of the oligomycin-sensitive ATPase, cytochrome *c*₁, subunit V of the cytochrome *bc*₁ complex, or the outer membrane porin (Zimmermann *et al.*, 1981; Teintze *et al.*, 1982; M. Teintze, H. Freitag, and W. Neupert, unpublished observations).

Although studies of binding to putative receptors by precursors of mitochondrial proteins other than cytochrome *c* are hampered by the fact that the precursors cannot be obtained in sufficient quantity to saturate the binding, there is nevertheless some evidence for the existence of receptors that may be involved in the transfer of the ADP/ATP carrier and that of subunit 9 of the oligomycin-sensitive ATPase. Treatment of *Neurospora* mitochondria with

trypsin prior to incubation with the reticulocyte lysate containing the precursors blocks the import of ATPase subunit 9, indicating that a protein in the outer membrane is probably involved in its transfer (B. Schmidt and W. Neupert, unpublished observations). Also, the precursors to both proteins can be bound to mitochondria in the presence of antimycin A and oligomycin, which break down the membrane potential (see Section 5) and thereby prevent transfer. The bound precursors can be degraded by adding a protease such as proteinase K. However, if the mitochondria with the bound precursors are reisolated, washed, and resuspended in a medium containing *N,N,N',N'*-tetramethylphenylenediamine (TMPD) and ascorbic acid, which reenergize the mitochondria, the precursors are transferred to a protease-resistant location in the mitochondria and converted to their mature forms (as determined by carboxyatractyloside (CAT) binding ability for the ADP/ATP carrier and by processing to the size of the mature protein for ATPase subunit 9) (M. Schleyer, B. Schmidt, and W. Neupert, unpublished observations). These results indicate that the precursors were bound to a location on the outside of the mitochondria, probably to a protein, from which they could then be imported when the membrane potential was restored.

The precursor of the outer membrane porin from *Neurospora* synthesized in a reticulocyte lysate in the presence of [³⁵S]methionine binds to mitochondria rapidly at 4°C but is transferred only very slowly. If the mitochondria are reisolated after a short incubation with precursor at 4°C and resuspended in unlabeled reticulocyte lysate at 25°C, the radioactive porin is inserted into the outer membrane and becomes protease-resistant like the mature protein (H. Freitag and W. Neupert, unpublished observations).

5. TRANSFER OF MANY PROTEINS REQUIRES A MEMBRANE POTENTIAL

The posttranslational transfer of many cytoplasmically synthesized proteins into mitochondria is energy dependent. This has been shown for the ADP/ATP carrier and subunit 9 of the oligomycin-sensitive ATPase; cytochrome *b*₂; cytochrome *c*₁; subunits I, II, V, and VII of the cytochrome *bc*₁ complex; and ornithine carbamoyltransferase in *in vitro* transfer experiments (Table I). In addition, the transfer of subunits α , β , and γ of F₁-ATPase, ATPase subunit 9, cytochrome *b*₂, cytochrome *c*₁, subunit V of the cytochrome

bc_1 complex, and superoxide dismutase has been shown to be energy dependent using *in vivo* pulse-labeling experiments (Table I). In most of these experiments, uncouplers or ionophores were used to break down the potential across the mitochondrial inner membrane and this resulted in an inhibition of protein transport and proteolytic processing. The absence of a membrane potential, however, also leads to a depletion of the intramitochondrial ATP because the oligomycin-sensitive ATPase tries to compensate by pumping out protons (Stigall *et al.*, 1979; Scarpa, 1979). This ATPase activity is strongly inhibited by oligomycin (Schleyer *et al.*, 1982). In order to determine whether it is the membrane potential or ATP that is required for the import of these proteins into mitochondria, the transfer has been studied *in vitro* under conditions in which either the membrane potential is broken down but ATP is still present or the membrane potential is maintained but the intramitochondrial ATP is depleted. The transfer of the ADP/ATP carrier and ATPase subunit 9 was studied in a system employing isolated *Neurospora* mitochondria suspended in the postribosomal supernatant of a reticulocyte lysate that had been incubated with *Neurospora* RNA and [35 S]methionine (Schleyer *et al.*, 1982). The uncouplers and protonophores carbonyl cyanide *m*-chlorophenylhydrazine (CCCP) and dinitrophenol were used. Both with and without oligomycin and ATP the transfer of the precursor proteins and the processing of ATPase subunit 9 were inhibited, but binding of the precursors to the mitochondria still occurred. Valinomycin, a K^+ ionophore that breaks down the membrane potential in the presence of high K^+ concentrations like that present in the reticulocyte lysate (90 mM), gave the same results as CCCP. In both of these experiments the intramitochondrial ATP level was high, as demonstrated by the protein synthesis on the mitochondrial ribosomes (Schleyer *et al.*, 1982). This is due to three factors: (1) the inhibition of ATPase by oligomycin, (2) the high ATP concentration in the lysate, and (3) the absence of a membrane potential, which causes the external and internal ATP pools to equilibrate via the ADP/ATP carrier (Heldt *et al.*, 1972). In another experiment carboxyatractyloside, which blocks the ADP/ATP carrier, was added together with oligomycin to deplete the intramitochondrial ATP pool while leaving the membrane potential intact (Schleyer *et al.*, 1982). Under these conditions there was no inhibition of transfer or proteolytic processing. The same experiments have since been carried out on cytochrome c_1 and subunits I, V, and VII of the cytochrome bc_1 complex (Teintze *et al.*, 1982). In each case the same pattern of response to inhibitors was observed. These observations show that the membrane potential, and not ATP, is the source of energy for

the import of precursor proteins into mitochondria. The ionophore nigericin, which exchanges K^+ for H^+ and causes a breakdown of the proton gradient without affecting the membrane potential (Graven *et al.*, 1966; Pressman, 1976), did not inhibit the transfer of the ADP/ATP carrier or the transfer and processing of ATPase subunit 9 (Schleyer *et al.*, 1982). Apparently it is the electrical membrane potential and not just the proton gradient that is required.

The energy-dependent transfer of precursor proteins can also be blocked by a combination of antimycin A, an inhibitor of the electron transport chain, and oligomycin to inhibit the oligomycin-sensitive ATPase, thus blocking both pathways for generating a membrane potential. This inhibition can be relieved by the addition of ascorbate and TMPD, which feed electrons directly into the cytochrome oxidase, i.e., after the site of inhibition of antimycin A, and allow the membrane potential to be reestablished. If precursors are bound in the presence of antimycin A and oligomycin and the mitochondria are reisolated and resuspended in a medium containing TMPD and ascorbate but no additional precursor, the bound precursors are imported and converted to the mature proteins (Schleyer *et al.*, 1982).

Schatz and co-workers studied the transfer of subunits α , β , and γ of F_1 -ATPase, cytochrome c_1 , and subunit V of the cytochrome bc_1 complex *in vivo* and ρ^- mutants of *S. cerevisiae* and came to the conclusion that ATP was required rather than the membrane potential (Nelson and Schatz, 1979). ρ^- mutants have mitochondria deficient in protein synthesis and thus lack both a functional respiratory chain and functional oligomycin-sensitive ATPase (Lloyd, 1974). These mitochondria should therefore not be able to generate a membrane potential by respiration or ATP hydrolysis. Nevertheless, ρ^- cells contain mitochondria that import and process cytoplasmically synthesized proteins. Thus it was concluded that ATP obtained from the cytoplasm via the ADP/ATP carrier (Subik *et al.*, 1974) must be providing the necessary energy (Nelson and Schatz, 1979). Later studies on *in vitro* import of cytochrome b_2 into isolated yeast mitochondria, however, suggested that this protein requires an electrochemical gradient for import (Gasser *et al.*, 1982). The situation in the ρ^- mutants remains unclear; however, one possibility is that the import of ATP coupled to the export of ADP generates a small membrane potential in the mitochondria because it is an electrogenic process (Scarpa, 1979).

Both cytochrome c and the porin of the outer mitochondrial membrane do not require energy for their import (Zimmermann *et al.*, 1981; Freitag *et al.*, 1982b). Both are not proteolytically processed, but neither is the ADP/

ATP carrier, which nevertheless requires energy for import. The reason for the lack of an energy requirement for import of cytochrome *c*, which is located in the intermembrane space, and the outer membrane porin may be the fact that neither needs to be transferred into or across the inner mitochondrial membrane. Cytochrome *b*₂ in yeast, an intermembrane space enzyme that does require an inner membrane potential for import and processing, is thought to cross and recross the inner mitochondrial membrane before reaching its functional site. The same may be true for cytochrome *c*₁, which is located with its major domain on the cytoplasmic side of the inner membrane (Gasser *et al.*, 1982).

6. PROTEOLYTIC PROCESSING ENZYMES

There have been a number of reports recently describing proteolytic enzymes isolated from mitochondria that process precursors of mitochondrial proteins to the apparent molecular weights of the mature proteins. Böhni *et al.* (1980) discovered a protease activity in hypotonic extracts of yeast and rat liver mitochondria that apparently correctly processed the precursor of yeast F₁-ATPase subunits α , β , and γ and cytochrome *c* oxidase subunit V synthesized in an *in vitro* cell-free system. All four of these proteins are synthesized as larger precursors on cytoplasmic ribosomes and then posttranslationally transferred to their functional sites in the mitochondrial matrix or inner membrane (Maccacchini *et al.*, 1979a; Lewin *et al.*, 1980). Both yeast and rat liver processing enzyme activities could be inhibited by the metal chelators *o*-phenanthroline and ethylenediaminetetraacetic acid (EDTA) but not by serine protease inhibitors; the processing activity of the rat liver mitochondria fractionated with marker enzymes of the matrix space (Böhni *et al.*, 1980). The yeast mitochondrial protease apparently also processes the precursors of cytochrome *c*₁ and cytochrome *b*₂, an intermembrane space enzyme, but only to their intermediate forms (Gasser *et al.*, 1982). The proteolytic processing enzyme of the yeast mitochondria was further purified and characterized by McAda and Douglas (1982). They showed that the enzyme that processes subunit 2 (β) of the F₁-ATPase in yeast was a metallo-endoprotease complex of *M_r* 115,000 with a pH optimum between 7 and 8. Its activity was inhibited by EDTA and *o*-phenanthroline, and could be restored with excess Co²⁺ or Mn²⁺.

In studies on rat liver mitochondria, two different groups have isolated a protease from the mitochondrial matrix that processes the precursor of ornithine carbamoyltransferase (Miura *et al.*, 1982; Conboy *et al.*, 1982). The protease characterized by Miura *et al.* (1982) had an M_r of 108,000 and was inhibited by the metal chelators EDTA, *o*-phenanthroline, and zincon. It processed the precursor of ornithine carbamoyltransferase (M_r , 39,400) to an intermediate form (M_r , 37,000) but not to the size of the mature subunit (M_r , 36,000). This processing enzyme was also isolated from the mitochondrial matrix of rat kidney, spleen, heart, and ascites tumor cells, all of which lack ornithine carbamoyltransferase, and therefore probably has a broader specificity. The enzyme isolated by Conboy *et al.* (1982) converts pre-ornithine carbamoyltransferase to the apparent molecular weight of the mature subunit as well as to the intermediate form. It was shown to require Zn^{2+} , Co^{2+} , or Mn^{2+} for activity but still processed the precursor of ornithine carbamoyltransferase to the intermediate form in the absence of these metal ions.

Considering the common characteristics of all the precursor-processing enzymes described above, it would be interesting to know whether there is only one such enzyme, whether this enzyme can process precursors other than those described above, and whether the same enzyme can be found in the mitochondria of other species. Sequencing analyses will be required, however, to show that any such protease actually processes the precursors correctly.

7. DIFFERENT ORGANISMS HAVE CLOSELY RELATED TRANSFER MACHINERIES

Labeled precursors to *Neurospora* mitochondrial proteins synthesized in a reticulocyte lysate cell-free system can be imported into isolated mitochondria from other organisms and are converted to their mature forms. The transfer of the *Neurospora* ADP/ATP carrier and ATPase subunit 9 into rat liver mitochondria requires an electrochemical gradient across the inner mitochondrial membrane just like the corresponding transfer into *Neurospora* mitochondria (Schleyer *et al.*, 1982). Both proteins were transferred into protease-resistant locations and the ATPase subunit 9 was processed to the molecular weight of the mature subunit. Similar results have been obtained with the transfer of these proteins into yeast and guinea pig heart mitochondria (B. Schmidt, M. Schleyer, and W. Neupert, unpublished observations). The *Neu-*

rospora ADP/ATP carrier was shown to be in its functional form after transfer into yeast mitochondria on the basis of its ability to bind CAT, which the precursor does not. The ATPase subunit 9 precursor was processed correctly by the yeast mitochondria during transfer, as shown by radiosequencing analysis (B. Schmidt, B. Hennig, and W. Neupert, unpublished observations). This is especially interesting since the yeast ATPase subunit 9 is encoded on the mitochondrial genome and synthesized inside the mitochondria without an additional sequence (Sebald and Wachter, 1978; Macino and Tzagoloff, 1979; Hensgens *et al.*, 1979). Yeast mitochondria therefore do not have to import and process a cytoplasmically made ATPase subunit 9 precursor *in vivo*, but the mechanism for doing so must be conserved. The precursor of subunit V (Fe/S protein) of the *Neurospora* cytochrome *bc*₁ complex is also imported into yeast mitochondria and processed to the molecular weight of the mature protein when an inner membrane potential is present (Teintze *et al.*, 1982). Recently the *Neurospora* outer membrane porin precursor was also successfully imported into rat liver and yeast mitochondria (H. Freitag and W. Neupert, unpublished observations).

The precursor of rat liver ornithine carbamoyltransferase is also imported and processed to the correct molecular weight by mouse liver and kidney mitochondria (Morita *et al.*, 1982).

Combined with the data discussed in the previous section showing that a protease isolated from rat liver processes yeast precursors (Böhni *et al.*, 1980), these results suggest that the mechanism for importing cytoplasmically synthesized precursors into mitochondria including the proteins mediating recognition, import, and processing has been highly conserved in evolution.

8. FUNCTIONAL ASSEMBLY OF MITOCHONDRIAL ENZYME COMPLEXES

Only a very small number of mitochondrial proteins are encoded on the mitochondrial genome (Tzagoloff *et al.*, 1979). Most of these proteins are subunits of enzyme complexes that also contain subunits synthesized in the cytoplasm, such as the oligomycin-sensitive ATPase, cytochrome *c* oxidase, and the cytochrome *bc*₁ complex. The question of how the synthesis of subunits in the mitochondrial and the cytoplasm is regulated so that stoichiometric

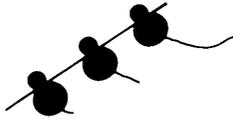
amounts of each subunit are available for assembly into a functional complex remains unanswered. It is known only that inhibition of mitochondrial protein synthesis has no gross effect on the production of cytoplasmically synthesized subunits. For instance, *Neurospora* cells can be grown for many generations in the presence of chloramphenicol, which inhibits mitochondrial but not cytoplasmic protein synthesis. The cells still make mitochondria, and even though they contain very little cytochrome *b* (a mitochondrial gene product), cytochrome *c*₁ is still synthesized in the cytoplasm, imported into the mitochondria in normal quantities, and proteolytically processed to the mature form, although it cannot be assembled into a cytochrome *bc*₁ complex (Weiss and Kolb, 1979).

Another problem that has not been completely solved is to demonstrate that precursors of nuclear-coded subunits synthesized in a cell-free system and then transferred into isolated mitochondria are actually assembled into functional complexes. Following transfer *in vitro* of radiolabeled *Neurospora* precursor into mitochondria, the labeled subunit 9 of the oligomycin-sensitive ATPase can be precipitated with antibodies directed against the F₁ portion of the complex (B. Schmidt and W. Neupert, unpublished observations). These antibodies will not precipitate isolated ATPase subunit 9, so the imported subunits must be assembled with the F₁ subunits, but one cannot rule out a subunit exchange during the immunoprecipitation process.

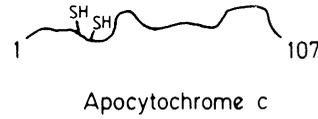
After transfer *in vitro*, the precursor to the ADP/ATP carrier acquires certain characteristics of the dimeric functional carrier (M. Schleyer and W. Neupert, unpublished observations). It binds CAT, as demonstrated by protection against protease activity; CAT is bound by the dimeric carrier in the membrane (Klingenberg *et al.*, 1979) but not by the precursor in the reticulocyte lysate, or the precursor bound to mitochondria in the presence of energy inhibitors. Also, both the mature assembled ADP/ATP carrier and the protein transferred *in vitro* pass through hydroxyapatite columns, whereas the precursor binds.

Assembly *in vitro* has been clearly demonstrated for ribulose-1,5-bisphosphate carboxylase in chloroplasts (Chua and Schmidt, 1978). The small subunit of this enzyme is made on free cytoplasmic ribosomes and transferred into the chloroplast, where it is proteolytically processed and assembled with the large subunit, which is made in the chloroplast. After synthesis and transfer *in vitro* it was shown that 80% of the labeled small subunit had been assembled with the large subunit to form the holoenzyme, as determined by density gradient centrifugation and electrophoresis on nondenaturing gels.

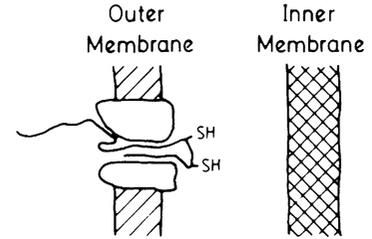
1 Synthesis on Free Ribosomes



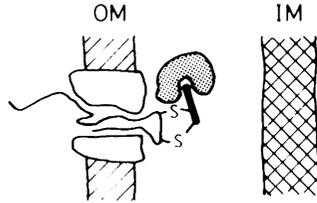
2 Extramitochondrial Precursor



3 Binding to Receptor



4 Attachment of Heme by a Lyase



5 Refolding and Transfer

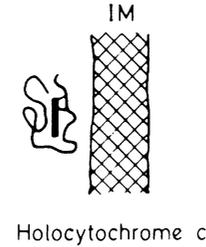


FIGURE 1. Proposed assembly mechanism for cytochrome c.

9. POSSIBLE ASSEMBLY PATHWAYS

The mitochondrial protein whose biogenesis is best understood is cytochrome *c*. This enzyme is synthesized as apocytochrome *c* in the cytoplasm and must cross the outer mitochondrial membrane and gain a covalently attached heme group to reach its functional location in the intermembrane space as holocytochrome *c* bound to the cytoplasmic face of the inner membrane. A schematic representation of the mechanism by which this is thought to occur is shown in Figure 1. Apocytochrome *c* is synthesized on free cytoplasmic ribosomes with the same amino acid sequence as the mature protein (Zimmermann *et al.*, 1979a). It then binds to specific sites on the outside of the mitochondria before being transferred across the outer membrane in conjunction with the covalent attachment of a heme group via two cysteine residues (Hennig and Neupert, 1981). When labeled apocytochrome *c* is bound to its receptor, it is still accessible to the outside since it can be displaced specifically by unlabeled apocytochrome *c*. It must also be accessible to heme and the enzyme that attaches it to the protein. Probably the portion of the apocytochrome *c* to which the heme must be attached extends into the intermembrane space so that the change in conformation induced by attachment of the heme group pulls the rest of the protein into the intermembrane space. This mechanism does not require the presence of any type of "signal" sequence in the protein; the receptor recognizes the conformation of apocytochrome *c* but not holocytochrome *c*, and the change in conformation upon attachment of the heme group is probably responsible for both the transfer across the membrane and for the irreversibility of the import process. The portion of apocytochrome *c* responsible for recognition may be located in the highly conserved sequence around residues 70–80, which is rather hydrophobic. This was concluded from competition experiments with fragments of apocytochrome *c* (Matsuura *et al.*, 1981; B. Hennig and W. Neupert, unpublished observations).

The simplest mechanism in mitochondrial membrane assembly should be the insertion of cytoplasmically synthesized proteins into the outer membrane, since such proteins do not have to cross one or more membranes to reach the functional site. The only well-characterized protein of the outer mitochondrial membrane whose biogenesis has been studied is the porin. The outer membrane porin is synthesized on free cytoplasmic ribosomes as a water-soluble precursor that has the same molecular weight as the mature protein

(Freitag *et al.*, 1982b). Also, in this case, specific binding of the precursor precedes its insertion into the membrane. Isolated detergent-solubilized mature porin will insert into artificial lipid bilayers to form pores (Freitag *et al.*, 1982a). The water-soluble precursor form can be regenerated from the isolated porin by removing all the detergent. This lipid-free form can compete with *in-vitro*-synthesized precursor for binding and transfer into the mitochondrial outer membrane, but it will not insert into artificial lipid bilayers until sterols are added (H. Freitag, R. Benz, and W. Neupert, unpublished observations). This suggests that the composition of the membrane may be important for the recognition or insertion process and that no covalent modification is necessary for the assembly of the porin.

The insertion of proteins into the outer mitochondrial membrane is topologically similar to the insertion of proteins into the plasma membrane or microsomal membranes. Posttranslational transfer into these compartments has also been observed. Cytochrome *b₅* and cytochrome *b₅* reductase are synthesized on free polysomes without any additional sequences (Okada *et al.*, 1982) and the large unglycosylated subunit of Na⁺, K⁺-ATPase is also made on free polysomes before assembly into the plasma membrane, although the small glycosylated subunit is synthesized in the rough endoplasmic reticulum (Sabatini *et al.*, 1982).

The transfer of proteins from the cytoplasm to the inner mitochondrial membrane or the matrix space is more complicated, since it involves crossing the outer membrane, the aqueous intermembrane space, and then the inner membrane. This process may occur via some type of contact site between the inner and outer membranes (Figure 2). Almost all precursors of inner membrane and matrix proteins whose sites of synthesis have been investigated are made on free polysomes (Table I). In most cases a cytoplasmic precursor pool has been observed and the import into mitochondria is posttranslational. The transfer of proteins into the inner membrane or the matrix requires energization of the mitochondria (Table I) and for a number of inner membrane proteins it has been shown that it is the electrochemical gradient across the inner membrane that drives the import and processing (Schleyer *et al.*, 1982; Teintze *et al.*, 1982). Most inner membrane and matrix proteins are made as larger precursors, whose additional sequences are proteolytically cleaved sometime during the transfer and assembly process. A metal-dependent endoprotease that processes some of these precursors to their mature size has been isolated from the mitochondrial matrix by a number of groups. As might

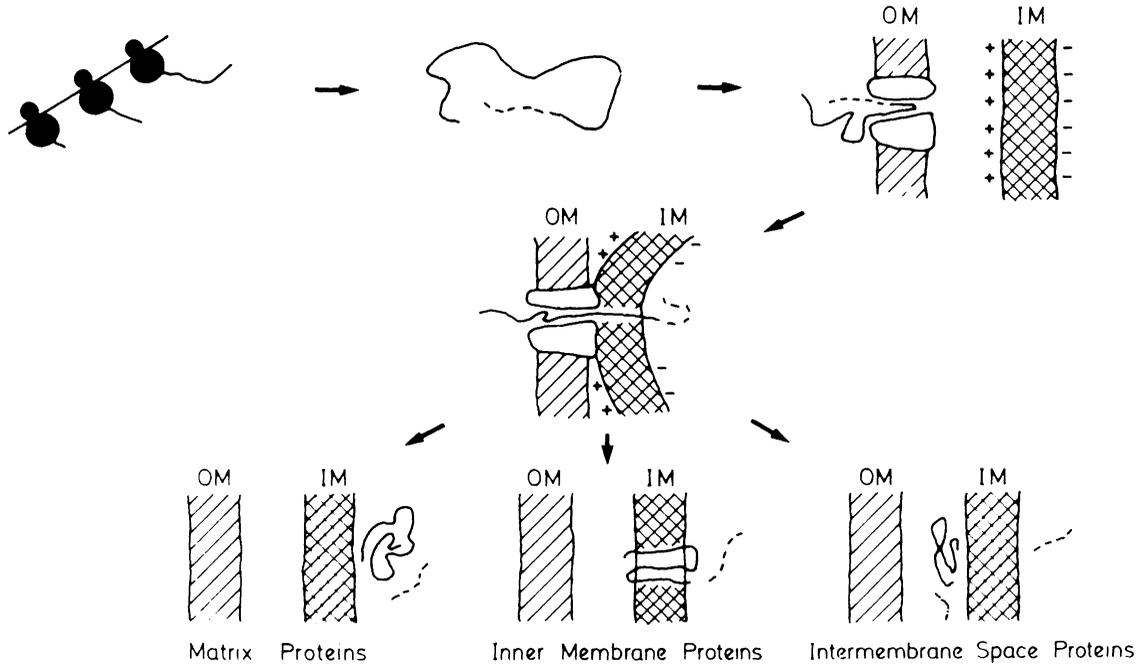


FIGURE 2. Hypothetical pathway for assembly of mitochondrial proteins requiring energy for transfer. The dashed and dotted lines represent portions of the sequence that are removed during transfer. OM, outer membrane; IM, inner membrane.

be expected, the proteins correctly processed by this enzyme are located in the matrix or on the matrix side of the inner membrane.

The function of the additional sequences on the precursors is not altogether clear. They probably serve to keep the precursors in a different (i.e., water soluble) conformation outside the mitochondria, and removal of the extra sequences inside the mitochondria probably makes the assembly process irreversible. The additional sequence may also be important for the recognition of the precursor by a receptor on the mitochondrial surface.

Cytochrome c_1 (located on the cytoplasmic side of the inner membrane) and cytochrome b_2 (located in the intermembrane space), are both processed in two separate steps during transfer. The second processing step requires heme for cytochrome c_1 , which has a covalently linked heme group, but not for cytochrome b_2 , whose heme group is not covalently attached (Gasser *et al.*, 1982). Both precursors are processed to the apparent molecular weight of their intermediate forms by the protease found in the matrix. It was therefore suggested that the polypeptides initially extend into the matrix space during their transfer, so that a portion of the sequence can be removed by the matrix protease. Following this step the intermediate form would return to the intermembrane space side of the inner membrane and be processed again by a different enzyme (Gasser *et al.*, 1982). Both cytochrome c_1 and cytochrome b_2 require a membrane potential for the first part of the transfer process (Teintze *et al.*, 1982; Gasser *et al.*, 1982), and the processing of cytochrome c_1 from the intermediate form to the mature size also requires energy (Teintze *et al.*, 1982). It is possible that other proteins share this two-step processing mechanism, but their intermediates may be so short-lived that they have escaped detection.

Insertion into or transfer across the inner membrane evidently requires an intact membrane potential, even for proteins like the ADP/ATP carrier that are not proteolytically processed (Schleyer *et al.*, 1982). The energy dependence of the import of cytochrome b_2 into the intermembrane space further supports the idea that this enzyme makes a "detour" through the inner membrane to reach its functional location.

The least-understood step in the transfer process at this time is how the proteins get through or into the membrane after recognition by a receptor on the mitochondria. They cannot be "pushed" through by polypeptide chain elongation as they may be in cotranslational transport, so some feature of the protein sequence must result in this translocation. So far, however, no com-

mon structure has been found among the many imported mitochondrial proteins that would suggest how this might occur.

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