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GENES
Structure and Expression

Volume Editor
A. M. Kroon
Laboratory of Physiological Chemistry,
University of Groningen,
Groningen, The Netherlands

Series Editors
E. Quagliariello
Department of Biochemistry,
University of Bari

and

F. Palmieri
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Laboratory of Biochemistry,
University of Bari

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Edited by A. M. Kroon

ASSEMBLY OF MITOCHONDRIAL PROTEINS

Bernd Hennig and Walter Neupert

INTRODUCTION

The eucaryotic cell is organized by a variety of membranes: the plasma membrane which forms the border of the cell and various intracellular membranes which delimit the various organelles. In most cells intracellular membranes greatly exceed the plasma membrane with respect to surface area. The diverse membranes of the cell have properties in common which are the basis for compartmentation: a) membranes possess identity, i.e. with few exceptions each particular membrane of a cell has a unique protein composition; b) membranes have continuity, not only in space but also in time, i.e. membranes are formed by insertion of newly made components into preexistent membranes; c) membranes display selective permeability, i.e. they prevent the free diffusion of most molecules between the two separated spaces.

A membrane gives the compartment which it encloses identity: It determines the protein composition of the luminal or cisternal space ("matrix") of that organelle. This is because the proteins of the various organelles and their membranes - with only very few exceptions - are not synthesized in the same compartments where their functional sites are located. Rather, they are synthesized on cytoplasmic ribosomes and have to be selectively translocated across the diverse membranes. Organelle membranes therefore must not only have devices to specifically recognize proteins which are destined

for the compartment they enclose, but also have mechanisms to translocate these proteins across the lipid bilayer. This is quite remarkable in light of the fact that many of these proteins are not only large but also very hydrophilic. Therefore, translocation of newly formed proteins across membranes and their intracellular sorting are quite puzzling phenomena.

I. A GENERAL VIEW ON MEMBRANE AND ORGANELLE ASSEMBLY

According to our present knowledge, two different mechanisms are involved in the translocation of proteins across membranes and their insertion into membranes (1). Cotranslational mechanisms are employed with proteins which traverse the membrane of the endoplasmic reticulum (ER). In contrast, the import of proteins into organelles such as mitochondria, chloroplasts, and probably peroxisomes and glyoxysomes is apparently the result of posttranslational processes. Some proteins of the ER and the plasma membrane appear to utilize this latter mode of transport as well.

Cotranslational Transport: The Translocation of Proteins Is Coupled to the Elongation of the Nascent Polypeptides.

Many proteins are translated on polysomes tightly associated with the ER. They are cotranslationally inserted into or transferred across the membrane, i.e. this process is concomitant with polypeptide chain elongation. Glycosylation of nascent polypeptide chains has been observed, and it is generally accepted that glycosylation occurs only in the luminal space of the ER (2). Completed polypeptides are never seen on the cytosolic side of the membrane which binds the ribosomes. Cotranslationally transported proteins can be synthesized *in vitro* in cell-free translation systems as complete precursors but they never cross the membrane of vesicles derived

from the ER unless those vesicles are present during translation.

A detailed mechanism by which cotranslationally transported proteins reach their proper positions was first proposed in the "signal hypothesis" and has been extensively modified in response to new data (3). A large body of evidence now supports a mechanism which entails the following steps: Synthesis of the polypeptide begins on free ribosomes. The nascent polypeptide carries an amino-terminal presequence of 15 - 30 amino acid residues representing the "signal sequence". The signal sequence protrudes out of the ribosome when a polypeptide of about 60 - 70 amino acid residues in length has been synthesized. An oligomeric "signal recognition protein" (SRP) binds to the freely accessible signal sequence and arrests further elongation of the polypeptide. The SRP - polysome complex then interacts with an SRP receptor or "docking protein" at the ER membrane (4). This releases the arrest of elongation and results in the nascent polypeptide chain penetrating the membrane.

Recognition in this cotranslational transfer thus seems to involve two specific interactions: the signal sequence interacts with the SRP, and the SRP interacts with its receptor on the membrane. Further proteins such as the "ribophorins" may stabilize the ribosome - membrane interaction (5). The signal sequence is cleaved off the polypeptide before peptide elongation is completed. This is accomplished by the "signal peptidase", an integral membrane protein which is probably located at the luminal side of the ER membrane (6). According to information inherent in the structure of the polypeptide chain, the protein will be transferred either into the lumen of the ER or integrated into the membrane.

Proteins obeying this mechanism are not only destined for integration into the membrane or for the luminal compartment of the ER itself. For a large number of proteins, traversing the ER membrane is only the first step in a complicated process by which they

are allocated to destinations such as the Golgi complex, lysosomes and plasma membrane, or outside of the cell in the case of secreted proteins. They reach these final locations in a transport process involving a flow of membranes, shuttling between several organelles and the plasma membrane (7). The cell must have quite a few mechanisms to correctly sort out these proteins after the initial, common step of traversing the ER membrane. However, our knowledge about the molecular rules organizing this traffic is scarce as yet.

Posttranslational Transport: The Translocation of Completed Precursor Proteins.

In contrast to the cotranslational transport described above, posttranslationally transferred proteins are synthesized essentially on free polysomes (1). They are released from the ribosomes into the cytosol as completed polypeptide chains. The primary translation products differ, as far as we know, in structure and properties from their mature counterparts. In particular, precursors of most proteins carry aminoterminal presequences. The newly synthesized polypeptides enter extraorganellar pools of free precursors from which they are rapidly cleared by uptake into the appropriate organelles. The precursors interact directly with the target membranes, apparently via specific receptor sites. During translocation across the membrane, the precursors are processed to the mature proteins. A specific endopeptidase removes the presequences of larger precursors. Synthesis of the polypeptides on the one hand and translocation of these polypeptides across the membranes and processing to the mature forms on the other hand are clearly separate events. This kind of precursor - product relationship is the most characteristic feature of posttranslational transport.

Assembly of Mitochondria Depends on Two Separate Genetic Systems.

The general features of posttranslational protein transport across membranes described above pertain also to the membranes of mitochondria and chloroplasts. However, these organelles are peculiar in an important respect: Their biosynthesis and maintenance depends on the expression of two different genetic systems in the cell. One of these is the DNA located in the cell nucleus; the other is the small DNA enclosed within mitochondria and chloroplasts themselves (8).

It is estimated that mitochondria contain several hundred different proteins. Some 5 - 7 proteins which are coded by mitochondrial DNA (mtDNA) in diverse species have been identified (cf. section VI). MtDNA apparently codes for a few additional proteins whose functions are not known. For example, human cells may contain up to eight additional proteins coded by mtDNA since complete sequence analysis of human mtDNA has revealed a total of 13 open reading frames (9). This number varies with different species but it is obvious that the majority of mitochondrial proteins are coded and synthesized by the nucleocytoplasmic system. Considering the total protein mass of mitochondria, about 95 % is of cytoplasmic origin and becomes imported into mitochondria.

The processes which interconnect the two genetic systems and coordinate the expression of genes for mitochondrial proteins are largely unknown. An important role may be played by the mitochondrial RNA-polymerase which transcribes the mitochondrial genes and which itself is coded by a nuclear gene. It is formed according to the cellular demands for mitochondrial gene expression (10).

Mitochondrial Proteins are Located in Four Different Submitochondrial Compartments.

It is another important aspect of mitochondria that these

organelles are composed by two strongly differing membranes whereas only single membranes surround most other organelles like peroxisomes, glyoxysomes, and lysosomes. The two mitochondrial membranes allow the division of mitochondria into four different compartments: the outer membrane, the inner membrane, the intermembrane space which lies between the outer and inner membrane, and the matrix which is enclosed by the inner membrane.

The function of the outer membrane is not really understood. In contrast to almost all other cellular membranes, it is permeable in an unspecific manner to molecules with molecular weight up to 2,000 - 6,000 daltons. This unusual permeability is due to large channels formed by the porin, the most abundant protein in the mitochondrial outer membrane (11). Relatively few other proteins have been identified in this membrane. One of these is the mitochondrial cytochrome b_5 which participates in the rotenone-insensitive NADH-oxidase pertinent in the outer membrane. It is closely related to the microsomal cytochrome b_5 present in the membrane of the ER.

The inner membrane, on the other hand, is quite rich in proteins compared to other cellular membranes. It contains a number of carrier systems such as the ADP/ATP carrier as it is a typical biological membrane impermeable to charged and/or polar molecules of low molecular weight. The various carrier systems allow the passage of the multitude of low-molecular-weight metabolites required by enzymes of the mitochondrial matrix. The inner membrane is usually extensively invaginated, thereby providing a large area containing the enzymes and components of cellular respiration (e.g. cytochrome oxidase and the cytochrome bc_1-complex) and oxidative phosphorylation (i.e. the oligomycin-sensitive ATPase) which consist of numerous hydrophobic subunits. The surface area of the inner membrane, i.e. the number and size of invaginations or "cristae", varies among mitochondria of different cells and is affected by the

metabolic state of a cell. As an extreme example, mitochondria of glucose-repressed yeast and promitochondria of anaerobic yeast are practically devoid of cristae (12).

The space between the two mitochondrial membranes, the intermembrane space, contains soluble enzymes, e.g. adenylate kinase, sulfite oxidase (rat liver) or cytochrome b_2 (yeast). The latter two proteins interact with cytochrome c, a protein contained in mitochondria of all species. Cytochrome c is confined to the intermembrane space too, but is loosely attached to the surface of inner membrane where it mediates electron transport between the cytochrome bc_1-complex and cytochrome oxidase.

The innermost space of mitochondria, the matrix, contains a large number of soluble and hydrophilic enzymes, in particular those of the citric acid cycle and the urea cycle. The various proteins engaged in replication and transcription of mtDNA (e.g. mtRNA-polymerase) and intramitochondrial protein biosynthesis (i.e. mitochondrial tRNA-synthetases, subunits of mitochondrial ribosomes, etc.) also reside in this compartment.

Biogenesis of mitochondria occurs by insertion of individual components into preexistent mitochondria, i.e. mitochondria multiply by growth and division (13). The ultrastructure of mitochondria and the protein composition of the different submitochondrial compartments are subject to change depending on the developmental fate of a cell. The transfer of the several hundred mitochondrial proteins into the various submitochondrial compartments is a complex process. Several major questions concerning the mechanism(s) of this assembly may be asked. We will focus on those for which answers have begun to emerge. These include: How do precursors of mitochondrial proteins differ from their mature forms ? What is the evidence that these precursors are posttranslationally imported into mitochondria ? How are the precursors recognized by mitochondria so that

transport is specific ? What are the mechanisms by which these proteins are translocated into the different mitochondrial compartments ?

II. SYNTHESIS AND POSTTRANSLATIONAL TRANSPORT OF MITOCHONDRIAL PRECURSOR PROTEINS

Two different approaches have been used to study synthesis of mitochondrial proteins and their translocation into mitochondria. The first relies on experiments performed in vitro employing cell-free translation and transfer systems. The second is based on experiments performed in vivo, i.e. employing whole cells. The following conclusions have been derived from both kinds of approaches: a) mitochondrial proteins are synthesized as extramitochondrial precursors, b) the precursors of most mitochondrial proteins are larger than the mature forms, and c) the completed precursors are transported into mitochondria.

Most Proteins are Synthesized as Larger Precursors in Cell-free Systems.

Cell-free translation systems are frequently employed to analyse the properties of precursors as they are released from ribosomes. In such systems the mRNA can be from a different organism than the ribosomes and cofactors and mitochondria are absent during protein synthesis. This minimizes the danger that the primary translation products undergo some posttranslational modification which is part of their intracellular biogenetic pathway. Preparations of osmotically lysed reticulocytes or extracts of wheat germs are used for most studies because these systems are standardized and can be efficiently programmed with mRNA from any eucaryotic organism. Efficient translation of mitochondrial proteins, however,

was also achieved using homologous cell-free systems dependent on endogenous mRNA. Mitochondrial proteins are synthesized in the presence of a radioactive amino acid, usually methionine or leucine. The radioactively labelled proteins are collected from the translation mixtures by immunoprecipitation or immunoadsorption onto Staphylococcus protein A using specific antibodies against defined proteins isolated from mitochondria. The immunoprecipitates are analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate (SDS), which dissociates the polypeptide - antibody complexes. This separates the proteins according to the molecular weights of their subunits. Autoradiography (or fluorography) of the dried gels serves to locate the labelled proteins. By this procedure, the protein synthesized in vitro can be compared with the corresponding mature form present in mitochondria.

It has turned out that the majority of mitochondrial proteins, whether membrane bound or soluble polypeptides, are formed as precursors which are larger than the mature proteins by some 500 to 10,000 daltons (Table). Although mobility differences on SDS gels must be interpreted with care since a number of factors do influence this parameter, it was demonstrated in several cases that these larger apparent molecular weights are actually due to additional amino acid sequences. As far as studied, these sequences are located at the aminoterminus. During import into mitochondria they are proteolytically removed (cf. section V).

Several large protein complexes inserted in the mitochondrial inner membrane consist of different subunits most of which are coded for by nuclear genes (e.g. 4 subunits of cytochrome oxidase, some 7 subunits of the cytochrome bc_1-complex, and at least 7 subunits of ATPase). It was investigated whether they are synthesized as polyprotein precursors similarly to certain viral capsid proteins. Evidence from several studies demonstrates that the different subunits

are synthesized as individual precursors (14,15). The presequence of one precursor, that of the subunit 9 of Neurospora ATPase, was deduced from the corresponding DNA sequence (16). This sequence of 66 residues is quite hydrophilic and positively charged, whereas the mature protein (81 amino acid residues) is very hydrophobic and deeply imbedded in the membrane.

Some Precursors are Just as Large as their Mature Forms.

In a few cases, comparison of precursor and mature form does not reveal a molecular weight difference. Two such proteins, namely the ADP/ATP carrier and cytochrome c, were thoroughly investigated and it was shown that the failure to detect a difference in molecular weights between these precursors and their mature proteins was not an artifact of the cell-free systems or of gel electrophoresis. In these experiments radioactively labelled N-formyl-methionyl-tRNA served to selectively label the initiator methionine in reticulocyte lysates. Aminoacylation and N-formylation of Met-tRNA from calf was performed by employing enzymes from E. coli. N-formyl-methionine is not removed by the peptidase which normally cleaves off the unformylated initiator methionine specified by the AUG codon in eucaryotic systems.

The ADP/ATP carrier, a major integral protein of the inner membrane was transported in vitro into mitochondria without losing this radioactive label (17). This shows that import of this particular precursor occurs without removal of any amino acid from its amino terminus. In contrast, the radioactive N-formylmethionine of the larger precursor of ornithine carbamyoltransferase disappears when this protein is processed during import into mitochondria (18).

Cytochrome c is also synthesized without a presequence. Experiments with the N-formylated precursor of cytochrome c led to similar results as described for the ADP/ATP carrier (19). In the case of

TABLE

Cytoplasmic Precursors of Mitochondrial Proteins

Protein	Species	Mature Protein (subunit size) $MW_{app.}$ (kD)	Precursor (extension) $MW_{app.}$ (kD)
OUTER MEMBRANE:			
Porin	Neurospora	32	-
INTERMEMBRANE SPACE:			
Cytochrome c	Neurospora, rat	11.7*	-
Cytochrome b_2	yeast	58	10 (7)**
Sulfite oxidase	rat	55	4
INNER MEMBRANE:			
ADP/ATP carrier	Neurospora	32	-
Cyt. c oxidase,			
subunit IV	yeast, rat	16.5	3
subunit VI	yeast	12.5	7.5
Cyt. bc_1-complex,			
Cytochrome c_1	yeast, Neurospora	31*	7 (4)**
subunit 7	Neurospora	12	0.5
ATPase,			
subunit 2	yeast	54	2
subunit 9	Neurospora	8	6
MATRIX:			
Ornithine carbamoyl-transferase	rat	39	4
Carbamoylphosphate synthetase	rat	160	5
Citrate synthase	Neurospora	45	2
mtRNA-polymerase	yeast	45	2

* Polypeptide without covalently linked heme.

** Data in brackets refer to intermediates during processing.

This table describes only proteins discussed in the text.

cytochrome c from yeast or rat, a comparison of the amino acid sequence of the mature protein with that derived from the nucleotide sequence of the DNA is possible. This comparison confirms that no additional amino acids are present at the aminoterminal or the carboxyterminal end of the primary translation product.

It would appear that while proteolytic cleavage often occurs, it is not an obligatory step in protein transfer across mitochondrial membranes. Additionally, the conclusion can be drawn that the absence of a presequence in precursors is not related to a particular submitochondrial site to which these precursors are transported: porin is translocated into the mitochondrial outer membrane, cytochrome c into the intermembrane space, and the ADP/ATP carrier into the mitochondrial inner membrane.

Properties of Precursors Suggest the Existence of Conformational Differences from their Mature Forms.

Precursors are found in the cytosolic fraction after being released from the ribosomes. Apparently even the most insoluble membrane proteins can exist in some kind of soluble form when they are on their way into the mitochondria. Precursors to insoluble membrane proteins perhaps form protein micelles: Analysis of the precursors of inner membrane proteins, such as the ADP/ATP carrier and the subunit 9 of ATPase, indicates that they occur not as monomers but in an aggregated state in the postribosomal supernatant (20). The precursors of soluble matrix proteins, e.g. carbamoylphosphate synthetase and ornithine carbamoyltransferase from rat liver, were reported to form large aggregates as well (21). This formation of soluble aggregates must have a basis in the peculiar conformations of precursors.

There is further evidence to suggest that precursor proteins differ in conformation from the mature forms. The ADP/ATP carrier,

for example, does not bind to hydroxylapatite after being solubilized from mitochondria with detergents. In contrast, its precursor is firmly bound by hydroxylapatite. With other precursors, differences in their affinities to antibodies against the mature forms are observed. The strongest difference in this respect is exhibited by cytochrome c. This protein is synthesized as apocytochrome c, i.e. the polypeptide without the covalently attached heme group. Antibodies against the mature form, holocytochrome c, were obtained which do not precipitate apocytochrome c synthesized in cell-free systems. On the other hand, antibodies against apocytochrome c which was prepared from holocytochrome c by removal of the heme group recognize the biosynthetic precursor, but do not precipitate holocytochrome c (26). Strong conformational differences between holocytochrome c and chemically prepared apocytochrome c are revealed by physicochemical measurements. Obviously it is the heme group which governs the different folding of the polypeptide chain in apocytochrome c and holocytochrome c.

Free Ribosomes are Engaged in the Synthesis of Mitochondrial Proteins.

The question whether free or membrane-bound ribosomes synthesize cytoplasmic precursors of mitochondrial proteins is of importance, since synthesis on free ribosomes points to a posttranslational mode of transport. Two types of experiments were carried out to answer this question: First, free and membrane-bound polysomes were isolated and synthesis of polypeptide chains was completed in homologous or heterologous postribosomal supernatants in presence of radioactive amino acids. Second, mRNA was isolated from free and membrane-bound polysomes and translated in reticulocyte lysates. With both approaches free polysomes were found to be the major site of synthesis of mitochondrial proteins in several

independently performed studies.

There are, however, observations which cannot be easily reconciled with free ribosomes being the exclusive sites of synthesis of mitochondrial proteins: Yeast mitochondria were found to be associated with cytoplasmic ribosomes. Electron micrographs of yeast spheroplasts treated with cycloheximide to arrest protein synthesis, and of isolated mitochondria derived from such cells revealed ribosomes lined up on the mitochondrial outer membrane (22). They were observed especially at sites where outer membrane and inner membrane were in close apposition. Evidence discussed here make it very unlikely that these ribosomes are engaged in cotranslational transport of mitochondrial proteins. It is, however, possible that the nascent chains of these ribosomes interact with specific receptors on the mitochondrial surface (cf. section III). This could also explain observations that certain mitochondrial proteins (e.g. subunit IV of cytochrome oxidase) are synthesized to a significant proportion on membrane-bound polysomes in yeast (23) or rat liver (24), though this is not obligatorily required for translocation of these proteins into mitochondria.

Precursors are Posttranslationally Transferred into Isolated Mitochondria.

Several independent investigations have demonstrated that precursors of various different proteins are transferred into mitochondria when the postribosomal supernatants of cell-free translation systems after mRNA-directed protein synthesis are incubated together with intact isolated mitochondria. A number of observations suggest that translocation of precursors across the mitochondrial membrane(s) actually takes place in such reconstituted systems: 1) Precursors are processed to the sizes of the mature proteins. For some proteins it is proven that proteolytic cleavage does occur at

the correct sites. 2) In contrast to the precursors present in the supernatant, the transferred proteins are resistant to added proteases. Apparently they have crossed at least the mitochondrial outer membrane. 3) The transferred proteins acquire properties of the mature assembled counterparts. The ATPase subunit 9, for example, which is a subunit of the F_0 part of the F_1F_0 complex, is transferred in vitro into mitochondria and can then be precipitated with antibodies against the F_1-part of the F_1F_0 complex (20). This indicates that transfer in such a reconstituted system can lead to the formation of mature proteins which have the ability to interact with other subunits of the whole, functional complex.

Studies with Whole Cells Confirm the Existence of Precursors and their Posttranslational Import into Mitochondria.

Important information on the transfer mechanism is obtained by studying the kinetics of assembly of mitochondrial proteins in whole cells. For this purpose the following techniques can be employed: Cells are pulse-labelled with a radioactive amino acid and mitochondrial proteins are analysed during a chase period, i.e. after adding excess unlabelled amino acid. This is done by immunoprecipitating defined proteins either from homogenates of whole cells or from subcellular fractions. All results obtained by this experimental approach are only compatible with a posttranslational mechanism of precursor transport into mitochondria: 1) Immunoprecipitation from whole cell extracts leads to the detection of the same larger precursor proteins which are also detected after synthesis in cell-free systems. 2) In most cases these precursor molecules are converted to the mature forms with a rather short half life (1 - 3 min). 3) The appearance of pulse-labelled proteins in the mitochondria shows a characteristic lag phase as compared to the kinetics of labelling of cytosolic proteins. This indicates that the newly synthesized pro-

teins first have to pass through an extramitochondrial precursor pool before they enter the mitochondria. These lag phases differ among various mitochondrial proteins, suggesting different sizes of the extramitochondrial pools. 4) A sudden block of cytoplasmic protein synthesis by cycloheximide leads to an immediate block in the labelling of cytosolic proteins, but labelled proteins continue to appear in mitochondria for a certain period (25). Apparently precursor proteins are imported into mitochondria in the absence of protein synthesis.

The observation that complete precursor molecules occur in vivo is certainly one of the strongest arguments that precursor synthesis and transfer into mitochondria are separate events. These observations convincingly rule out a cotranslational transport of mitochondrial proteins. They fully agree with the conclusions derived from experiments with cell-free systems.

III. INTERACTION OF PRECURSORS WITH MITOCHONDRIAL RECEPTOR SITES

One can expect that the transfer of proteins into mitochondria is a process which involves several distinct steps. In order to understand this process on a molecular basis, experimental systems must be available which allow a resolution of these steps. The establishment of cell-free systems employing precursors synthesized in vitro and their transport into isolated mitochondria facilitates such investigations. The first step in the transfer process is the recognition of mitochondrial precursors by the mitochondrial surface.

Translocation of Precursors into Mitochondria Can be Arrested at their Specific Binding to the Mitochondrial Surface.

To study the interaction between precursors and mitochondria in detail one must be able to inhibit the translocation. Several procedures can be utilized for that purpose. One which is generally applicable to precursors relies on the observation that the binding of precursors to the mitochondria appears to be less dependent on temperature than the translocation through or insertion into the membrane. Another approach takes advantage of the observation that the import of most proteins into mitochondria requires an electrical potential across the inner membrane as will be described in detail below. When the membrane potential is dissipated, transfer is halted at the level of precursors being bound to mitochondria.

However, a major drawback in analysing the recognition step is the availability of precursors in only minute amounts. Almost all precursors can only be obtained by synthesis in cell-free systems and the amounts produced are not sufficiently large to study binding to mitochondrial recognition sites in desirable detail. Fortunately there is one precursor which can be obtained in practically unlimited amounts. This is apocytochrome c. It can be prepared by removal of the covalently bound heme group from holocytochrome c by chemical means. Apocytochrome c prepared in this way can be radioactively labelled by reductive methylation or by iodination and is bound to mitochondria, translocated across the outer membrane, converted to holocytochrome c, and competes in this respect with apocytochrome c synthesized by cell-free translation. The translocation of apocytochrome c into mitochondria is inhibited by the heme analogue deuterohemin (26). Apocytochrome c is tightly bound to the mitochondrial surface under this condition. Thus, interaction of apocytochrome c with the mitochondrial surface can be studied in experiments similar to those carried out to elucidate the inter-

action of peptide hormones with their receptors on cell surfaces.

Precursors are Bound to Mitochondria via Specific Receptor Sites.

The following characteristics of precursor binding to the surface of intact mitochondria have emerged:

Binding is rapid and tight, and the number of binding sites on mitochondria is limited. Precursors bind to mitochondria at a rate which is sufficiently large to account for the rate of transport *in vivo*. Once bound, precursors are not removed when the mitochondria are washed in the medium in which binding has been performed. Binding sites for apocytochrome *c* have been titrated. The Scatchard plot revealed that apocytochrome *c* binds with high affinity (K_A= 2.2 x 10^7 M^{-1} in the case of *Neurospora*) to mitochondria (27). The binding sites are located on the mitochondrial surface, probably at the cytoplasmic face of the outer membrane, since bound precursors are sensitive to added proteases in contrast to precursors transferred into mitochondria. There are about 100 pmol high-affinity binding sites for apocytochrome *c* per mg mitochondrial protein.

Binding is specific and functionally related to transfer of precursors into mitochondria. Apocytochromes *c* from various species (e.g. *Neurospora*, yeast, horse, parsnip) have different affinities to apocytochrome *c* binding sites on mitochondria (27). Non-mitochondrial proteins, such as bacterial apocytochrome *c* (*Paracoccus denitrificans*) or the precursor to glyoxysomal isocitrate lyase do not bind to mitochondria. Also, mature proteins do not interfere with the binding of the precursor forms. Apocytochrome *c* bound to mitochondria in the presence of deuterohemin, which inhibits translocation of this particular precursor, is taken up into mitochondria and converted to holocytochrome *c* when the inhibition due to this heme analogue is relieved by addition of excess protohemin (26). The precursor of the ADP/ATP carrier bound to de-energized mitochondria

becomes internalized when the membrane potential is restored (34). The precursor to ATPase subunit 9 is internalized under these conditions and processed to the size of the mature form .

More than One Kind of Receptor is Present on the Mitochondrial Surface.

The results of the binding studies imply the existence of receptors on the mitochondrial surface. However, no such receptor has been isolated yet and many questions remain open: What is the chemical nature of these receptors ? How many different kinds exist ? How is the binding to a receptor related to the translocation across the membrane(s) ? Although no definite answers can be given, some interesting data are available concerning these points.

When mitochondria are treated with protease (trypsin or proteinase K) before they are employed in in vitro transfer experiments, they lose the ability to bind precursors and to import them. This may mean that the receptors are proteins. This finding and the evidence accumulated in the binding experiments suggest that receptors are exposed at the cytoplasmic face of the outer membrane.

There is information that mitochondria have more than one kind of receptor: Apocytochrome c employed in large amounts saturates its own binding sites but does not interfere with the binding of various other precursors, e.g. porin, ADP/ATP carrier, ATPase subunit 9, and cytochrome c_1 (28). On the other hand, it seems extremely unlikely that each of the several hundred mitochondrial proteins has its own receptor. Two observations suggest the existence of a very limited number of different and evolutionary conserved recognition mechanisms. First, transfer in vitro of most proteins studied so far does not exhibit species specificity, i.e. precursors of mitochondrial proteins from Neurospora can be transferred into mitochondria isolated from yeast, rat liver or guinea pig heart. Second, a precursor

protein from one type of cell, e.g. the ornithine carbamoyltransferase from rat liver, can be imported into the mitochondria isolated from another type of cell, e.g. rat kidney cells, which do not contain this protein *in vivo* (29).

Which Part of a Precursor is Recognized by the Mitochondrial Receptors ?

Mature proteins do not compete with their precursors for transfer and precursors processed *in vitro* are not taken up by mitochondria, perhaps with the controversial exception of the mature form of aspartate aminotransferase (30). However, this failure by no means allows the conclusion that addressing of precursors to mitochondria occurs via the presequences of precursors. Rather, the function of these sequences may be of a quite different nature: They could serve to alter the conformation of a mitochondrial protein in such a way that a part of its structure is exposed which can interact with a receptor. The presequences could also have the function of altering the proteins in such a way that the precursors outside the mitochondria cannot become functionally active, for instance because the catalytic site is changed or because a cofactor cannot be bound by the polypeptide. This would correspond to the well established situation with zymogens whose prosequences shield the catalytic sites and removal of the prosequences accompanied by a conformational change leads to the activation of the enzymes (e.g. the conversion of trypsinogen to trypsin).

Mutual recognition between precursors and receptors must also occur with precursor proteins which do not possess additional sequences, in this case obviously by some part of the sequence present in the mature protein. Cytochrome *c* provides important information in this respect. The mature form, holocytochrome *c*, is not bound by the receptor. Apparently the corresponding binding

domain is accessible at the surface of the molecule in apocytochrome c but not in holocytochrome c. Thus, the strong positive charge of cytochrome c cannot be solely responsible for binding to the receptor since this is practically the same in precursor and mature form.

In contrast to holocytochrome c, the three-dimensional structure of apocytochrome c is not known. However, some indications as to the structural part involved in recognition with its mitochondrial receptor may be obtained from a comparison of the amino acid sequences of the some 90 different cytochromes c so far determined. The N-terminal part preceding the heme-binding region (which is at positions 14 - 17 according to standardized nomenclature) is apparently not involved in receptor binding. Several yeast mutants contain holocytochrome c with amino acid exchanges in this particular region, and even a complete deletion of the first 11 sequence positions was found (31). Hence, the absence of this region in apocytochrome c does not interfere with the import of apocytochrome c into mitochondria and its conversion to holocytochrome c. Recent data indicate that the receptor-binding structure is located in the C-terminal half of the sequence where an extremely conservative and hydrophobic sequence of some 10 amino acids is present (32). However, one has to take into consideration that the binding domain is a three-dimensional structure and different distant parts of the molecule could contribute to it.

IV. TRANSLOCATION OF PRECURSORS INTO THE DIVERSE SUBMITOCHONDRIAL COMPARTMENTS

The step which follows selective binding of precursors to mitochondria, i.e. the translocation into one of the four submitochondrial compartments, is presumably of considerable complexity.

The mechanisms involved in a) the insertion of proteins into the outer membrane, b) their translocation across the outer membrane into the intermembrane space, c) the insertion into the inner membrane, and d) the translocation across both membranes, i.e. into the matrix, are poorly understood. It is, for instance, not clear whether a protein destined for the matrix crosses outer membrane and inner membrane in one, two, or even more distinct steps. It has been repeatedly speculated that the two mitochondrial membranes can come into close contact or may even fuse. "Contact sites" or "fusion sites" were observed upon electron microscopy of sectioned mitochondria (22). It is an open question, however, whether these structures are really related to protein transport. An understanding of the precise transfer mechanism would require exact knowledge of the conformation of a precursor and its changes during interaction with the membrane(s).

A Membrane Potential is Required for Translocation of Most Mitochondrial Proteins.

It has been observed that posttranslational transfer of precursors and their proteolytic processing are blocked when whole cells are exposed to an uncoupler of oxidative phosphorylation such as CCCP (carbonyl cyanide m-chlorophenylhydrazone). Subsequent experiments with isolated mitochondria confirmed that the import of precursors is blocked when the mitochondria are de-energized (e.g. refs. 15,28,33). As already described above, the uncouplers do not inhibit binding of the precursors to the mitochondria but do inhibit their transfer across the outer membrane. However, these observations do not allow one to discriminate whether the electrical membrane potential, the proton motive force, or ATP is the primary source of energy involved in protein translocation: Uncoupling of mitochondria not only dissipates the membrane potential but also

results in the induction of intramitochondrial ATPase activity, i.e. the reversed action of ATP synthase, thus lowering the level of ATP in the matrix.

The following experiments employing transfer in vitro (34) identified the membrane potential as the primary source of energy for translocation of extramitochondrial precursors across the mitochondrial membranes:

Conditions were created under which the mitochondrial membrane potential was low, but the level of ATP in the mitochondria was high. The membrane potential was dissipated with protonophores (CCCP or dinitrophenol) or with an ionophore (valinomycin plus K^+), and oligomycin was added to inhibit ATP degradation by the oligomycin-sensitive ATPase. Furthermore, ATP was added in high concentration (5 mM) to the mixture. It is known that ATP is readily imported into the matrix via the ADP/ATP carrier when mitochondria are uncoupled. This does not occur in mitochondria which have a normal membrane potential (150 - 200 mV) since the ADP/ATP carrier is electrogenic and a potential positive outside favours the export of ATP from mitochondria in exchange against ADP. Therefore, in the presence of uncoupler and oligomycin, higher ATP levels than in respiring coupled mitochondria can be obtained. With all the precursors tested, import into mitochondria was blocked under such conditions and the precursors remained at the mitochondrial surface.

On the other hand, conditions were created under which mitochondria maintain a membrane potential but the matrix ATP level is far below normal. Since the direct determination of mitochondrial matrix ATP is difficult in the complex in vitro system, the ATP level was measured indirectly by following a reaction requiring ATP in the matrix, namely mitochondrial protein synthesis. ATP within the mitochondria is derived from two sources: phosphorylation of ADP by the ATP synthase and import from the cytosol via the ADP/ATP

carrier. There are specific inhibitors for both processes, i.e. oligomycin and carboxyatractyloside. Simultaneous addition of the two inhibitors to mitochondria had no effect on protein import into mitochondria, it did however inhibit intramitochondrial protein synthesis.

Nigericin, an ionophore which exchanges H^+ versus K^+ and therefore does not affect the membrane potential but dissipates the proton gradient, did not interfere with the transfer of precursors into mitochondria. Thus it is apparently the electrical membrane potential that is required for protein import. A membrane potential can be generated in mitochondria in two ways: by electron transport and by the reversed action of ATP synthase, i.e. ATP hydrolysis by the oligomycin-sensitive ATPase. Hence, it is easily explained that inhibitors of respiration alone do not (or only weakly) inhibit protein transfer and are effective only in combination with oligomycin.

Which Role Does the Membrane Potential Play in the Translocation of Precursors ?

The translocation of many mitochondrial proteins into mitochondria was found to depend on the energization of the inner membrane. Does this apply to all precursors ? At least the translocation of two proteins is independent from a membrane potential. One example is the porin of the outer membrane (35). This protein is inserted into mitochondria without passing through a membrane. In this context it is interesting that the microsomal cytochrome b_5, which is closely related to the mitochondrial cytochrome b_5 present in the outer membrane, is also synthesized on free ribosomes without a presequence and is posttranslationally inserted into the membrane of the ER (36). Insertion of these proteins into their respective membranes may occur by self-assembly with subunits of these proteins

preexistent in the target membrane, and it might be expected that all proteins destined for insertion into the mitochondrial outer membrane obey this particular mechanism of posttranslational transport. If this suggestion would prove to be correct it would be needless to postulate a special receptor for the assembly of newly formed receptors in the mitochondrial outer membrane.

The second protein whose import is independent from energization of mitochondria is cytochrome c (28). In this case, one could argue that there is no reason for such a dependence since cytochrome c must only be translocated across the outer membrane, whereas the membrane potential is confined to the inner membrane. However, other intermembrane proteins such as cytochrome b_2 in yeast and sulfite oxidase in rat liver are imported only into energized mitochondria (37, 38). Substantial evidence has been presented in favour of an import pathway of cytochrome b_2 which involves a 'detour' of the precursor into the inner membrane. This apparently rather complex pathway involves proteolytic processing of the precursor in two separate steps (cf. section V).

What is the role of the membrane potential in the assembly of those proteins which are inserted into the inner membrane either transiently or permanently ? One possibility is that the membrane potential provides the energy for translocation. The precursor - receptor complex or a complex between precursor and a hypothetical "translocator" protein could respond to the membrane potential in such a way that the precursor is transferred across the membrane(s). Another possibility is that the energy for transmembrane transfer is provided primarily by refolding of the polypeptide chain of the precursor and that the membrane potential serves to trigger such refolding events (39). In this context one should remember that a membrane potential is also required in the export of periplasmic proteins across the plasma membrane of gram-negative bacteria (40).

On the other hand, transport of precursor proteins into chloroplasts was reported to depend on ATP in the chloroplast stroma space (41). It remains to be determined whether this observed difference reflects a genuine difference in the assembly mechanism of mitochondrial and chloroplast proteins.

Translocation of Cytochrome c is Coupled to Heme Attachment to the Precursor.

In contrast to most other mitochondrial proteins, neither a membrane potential nor proteolytic processing is required for import of cytochrome c into mitochondria. How is apocytochrome c translocated across the outer membrane ?

The following view concerning assembly of cytochrome c can be proposed from experimental results (Fig.1): Apocytochrome c is bound to its receptor at the outer surface of mitochondria in such a way that the thiol groups of its heme-binding cysteine residues become exposed at the intermembrane face of the outer membrane. The heme group becomes linked to these cysteines via thioether bonds aided by an enzyme contained in the intermembrane space. The covalent attachment of the heme group forces the polypeptide chain to refold and by this refolding the polypeptide is pulled through the outer membrane. The properly folded holocytochrome c is trapped in the intermembrane space. It associates with its functional binding sites on the outer face of the inner membrane, where it mediates electron transport as a component of the mitochondrial respiratory chain.

The evidence for such a pathway can be summarized as follows: The covalent attachment of heme to apocytochrome c is apparently mediated by an enzyme. Protohemin, but not protoporphyrin IX, is linked to apocytochrome c in a stereospecific reaction (26). This reaction is inhibited by certain heme analogues (e.g. deuterohemin, mesohemin) but not by others (e.g. hematohemin). The converting

enzyme, cytochrome c heme lyase, is presumably contained in the intermembrane space since neither the cytosol nor the isolated mitochondrial outer membrane or inner membrane appears to contain an activity converting apocytochrome c to holocytochrome c. Inhibition of heme attachment causes inhibition of the translocation of apocytochrome c across the mitochondrial outer membrane and leads to accumulation of apocytochrome c at the mitochondrial surface (cf. section III). This inhibition can be releaved by excess protohemin. The covalently linked heme group strongly affects the conformation of the polypeptide (42). Denatured holocytochrome c rapidly resumes the native conformation when the denaturing conditions have been abandoned. Finally, holocytochrome c cannot penetrate the mitochondrial outer membrane (43). Extraction of holocytochrome c from mitochondria and insertion of exogenous holocytochrome c into the mitochondria is possible only after rupture of the mitochondrial outer membrane.

As already mentioned and as will be dicussed in the next section, the assembly of other mitochondrial heme proteins such as cytochrome b_2 or cytochrome c_1, the latter of which also contains a covalently attached heme, follow a different and much more complicated mechanism than the assembly of cytochrome c.

V. PROTEOLYTIC PROCESSING OF PRECURSORS

We have discussed above that most mitochondrial precursor proteins are formed as larger precursors (cf. Table). Hence, they are assembled in mitochondria with concomitant proteolytic removal of their presequences. Where in the mitochondria does this processing occur, and which particular protease is involved ? What is the role of this cleavage in the translocation ?

FIGURE 1: Assembly of Cytochrome c
(Proposed Mechanism)

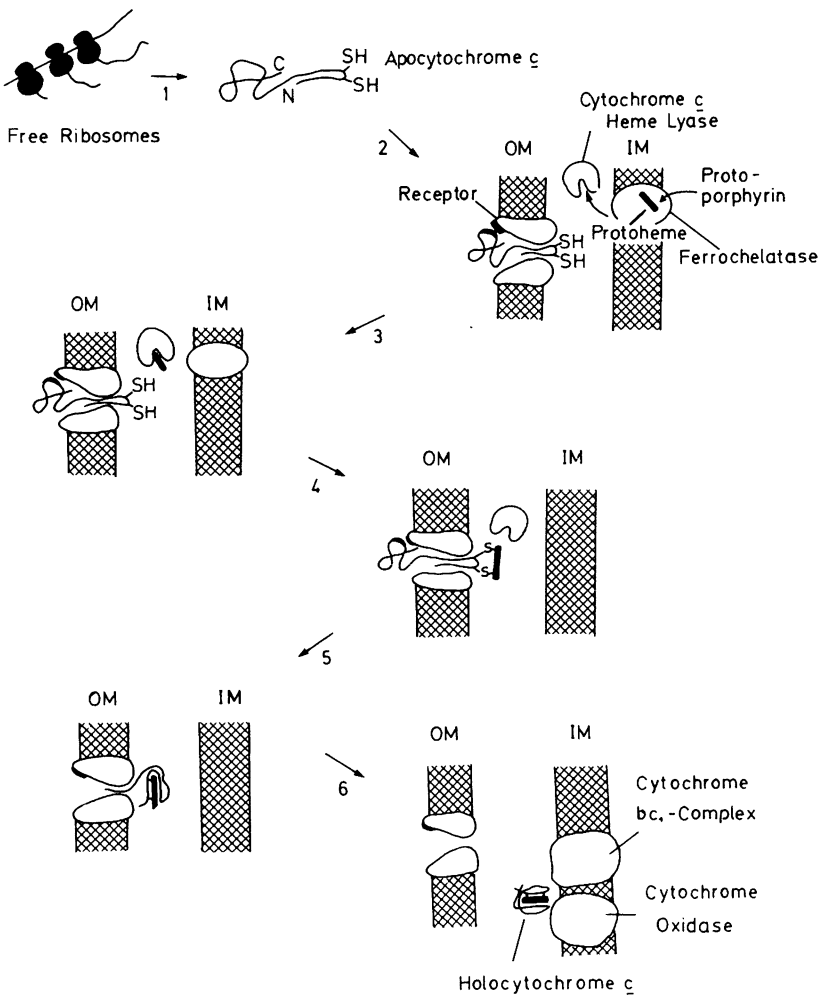
STEP 1: Cytochrome c is synthesized as a precursor, apocytochrome c, on free ribosomes and released into the cytosol.

STEP 2: Apocytochrome c is bound and properly arranged at the surface of the mitochondrial outer membrane (OM) by a specific receptor which either itself might form a pore in the outer membrane or is associated with such a pore. The receptor-bound apocytochrome c exposes the heme-binding cysteine residues through this pore in the intermembrane space.

STEPS 3 and 4: Cytochrome c heme lyase, an intermembrane enzyme, accepts protoheme provided by the ferrochelatase and attaches it in a stereospecific reaction to the apocytochrome c.

STEP 5: The covalently linked heme forces the polypeptide chain of cytochrome c to wrap around the heme, thereby pulling the polypeptide completely through the membrane.

STEP 6: The mature protein, holocytochrome c, is entrapped in the intermembrane space and binds at the surface of the inner membrane (IM), associating with the pertinent components of the respiratory chain.


Free Ribosomes
1
C
N
SH
SH
Apocytochrome c
2
OM
IM
Cytochrome c
Heme Lyase
Receptor
Proto-
porphyrin
Protoheme
Ferrochelatase
SH
SH
3
OM
IM
SH
SH
4
OM
IM
5
OM
IM
6
OM
IM
Cytochrome
bc1-Complex
Cytochrome
Oxidase
Holocytochrome c

An Enzyme Specifically Processing Mitochondrial Precursor Proteins Resides in the Mitochondrial Matrix.

In a number of studies a protease was extracted from mitochondria which cleaved precursors of mitochondrial proteins to the sizes of their mature forms. This enzyme meets the criteria of a true processing protease: a) It processes specifically mitochondrial precursors but not precursors of other proteins such as those secreted across the ER. b) It processes a number of different mitochondrial precursors. c) It does not degrade precursors further than to the sizes of their mature forms.

The processing enzyme is a soluble protein and thus differs from the "signal peptidases" of the endoplasmic reticulum and of bacteria which are integral membrane proteins. Subfractionation of mitochondria has revealed that the enzyme is located in the matrix (44). In order to be active it requires Zn^{++} or certain other divalent metal ions and it is blocked by metal ion chelators such as EDTA or o-phenanthroline, but not by the non-chelating m-phenanthroline. Various protease inhibitors, e.g. phenylmethylsulfonyl fluoride, pepstatin, and chymostatin, which inhibit a variety of intracellular proteases including those of lysosomal origin, do not affect the mitochondrial processing enzyme (45). However, normal processing is inhibited by leupeptin and p-aminobenzamidine. Attempts to purify the enzyme have led to a considerable enrichement. An apparent molecular weight of about 108,000 daltons was determined but a pure enzyme has not been obtained so far. Thus it is not clear whether only a single enzyme is responsible for all mitochondrial precursor proteins or whether the processing activity represents a mixture of different, yet closely related enzymes.

Some Precursors Undergo a Two-Step Processing.

The occurence of the processing enzyme in the matrix has important implications. Apparently any precursor requiring proteolytic processing must be transferred into the matrix or at least inserted into the inner membrane in such a way that the presequence is exposed to the matrix side before proteolytic processing can occur. This is 'en route' for precursors destined for the matrix itself such as ornithine carbamoyltransferase (29) or citrate synthase (46). However, a complex picture emerged for cytochrome b_2 and cytochrome c_1. The former protein is a soluble intermembrane enzyme, the latter one an integral membrane protein which faces the intermembrane space. These two cytochromes are apparently processed by two successive proteolytic events, since an intermediate form between the original precursor and the mature form is transiently generated (15, 37).

It is quite likely that they represent true intermediates in the assembly pathway since they are detected both in vivo and in vitro. The first step which leads to the intermediate forms requires energization of the inner membrane. In the case of cytochrome c_1, the first proteolytic processing step precedes the covalent attachment of the heme group. Heme deficiency leads to accumulation of the intermediate form. The submitochondrial location of the second processing step remains to be determined. The protease which is involved in this second step is apparently different from the matrix protease utilized in the first step. The intermediates, but not the mature proteins, are formed when the precursors are incubated solely with the processing protease prepared from mitochondrial matrix.

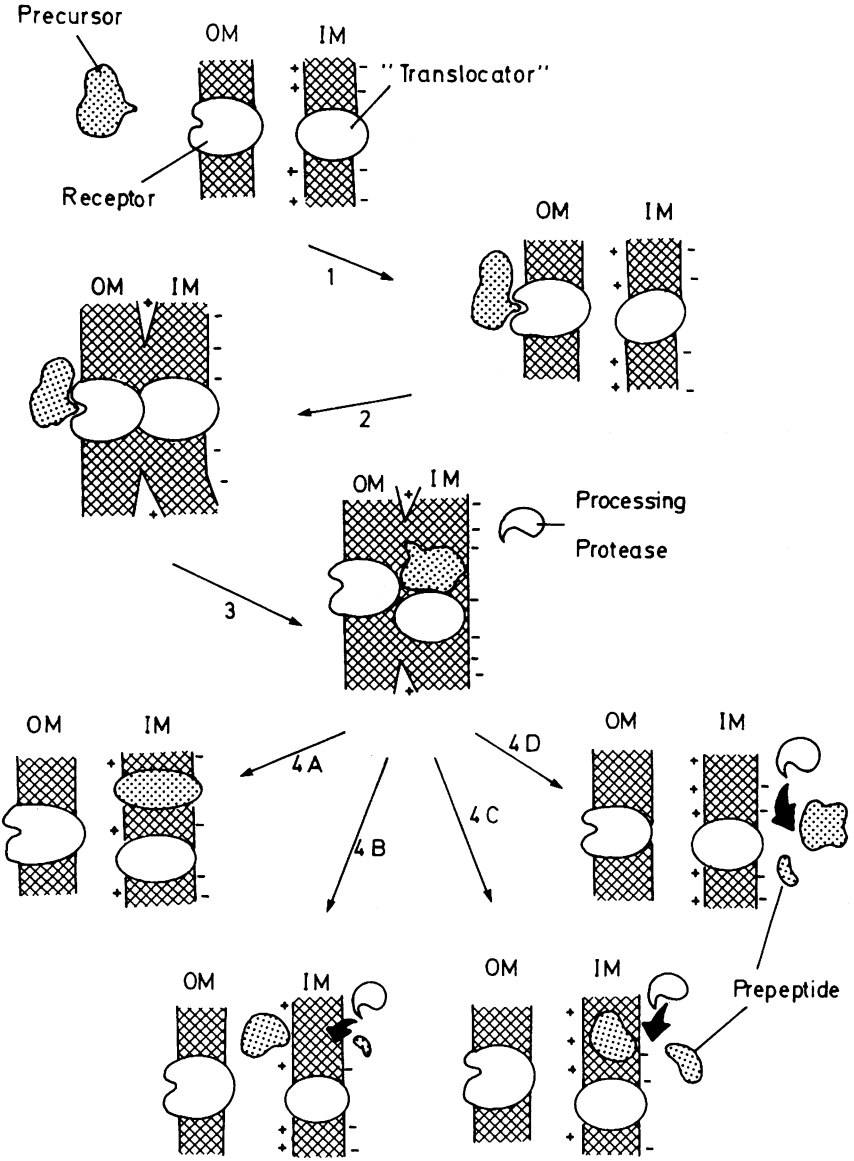
A hypothetical mechanism for the transfer of precursors into mitochondria by the various discussed pathways is presented in Fig. 2.

FIGURE 2: Mechanisms Involved in Transfer of Various Precursors into Mitochondria (Hypothetical Sequence of Events)

STEP 1: Extramitochondrial precursors are recognized by specific receptors at the mitochondrial surface.

STEPS 2 and 3: The outer membrane (OM) and the inner membrane (IM) of mitochondria come into contact at certain sites and form "fusion sites". The precursor - receptor complex reorients in this area, perhaps aided by an hypothetical "translocator" protein. Either the formation of the "fusion sites" or the reorienting of the protein complexes in the fused membrane areas (or both events) depend on the electrical potential across the inner membrane.

STEPS 4 A-D: Precursors are (transiently or permanently) inserted in the inner membrane, processed, and allocated to their final destinations according to their particular properties. A: The precursor refolds and is inserted in the inner membrane without proteolytic processing (e.g. the ADP/ATP carrier). B: The precursor is attacked by a processing enzyme contained in the matrix. After removal of the presequence the protein is relocated into the intermembrane space, a step which may entail a second processing event (e.g. cytochrome b_2). C: The proteolytically processed precursor occupies its final topological position in the inner membrane (e.g. subunit 9 of the ATPase). D: The proteolytically processed precursor is discharged into the matrix (e.g. citrate synthase).


Precursor
OM
IM
"Translocator"
Receptor
1
2
3
Processing
Protease
4A
4B
4C
4D
Prepeptide

VI. ASSEMBLY OF PROTEINS SYNTHESIZED WITHIN THE MITOCHONDRIA

The intramitochondrial genetic system uses a codon language slightly different from that used by the nucleocytoplasmic system (47). Therefore, no simple exchange of translatable information is possible between the two systems, neither *in vivo* nor *in vitro*. Structural genes on mtDNA code for a few proteins of the inner membrane, i.e. for three subunits of cytochrome c oxidase (subunits I, II, and III), one or two subunits of the oligomycin-sensitive ATPase (subunit 6; in yeast also subunit 9), and one subunit of the bc_1- complex (subunit 3, i.e. cytochrome b). Furthermore, one protein of the small subunit of mitochondrial ribosomes is coded on mtDNA at least in yeast and *Neurospora*. Since the amino acid sequences can be deduced from the known nucleotide sequences of several mtDNAs (i.e. man, bovine, mouse, and in part yeast) the complete structures of the primary translation products are known.

Intramitochondrially synthesized proteins may be formed as larger precursors as well.

How are the intramitochondrially synthesized proteins assembled ? The three subunits of cytochrome c oxidase coded for by mitochondrial genes are formed as separate translation products. Subunit II of cytochrome c oxidase from bovine is apparently not formed as a larger precursor because the mature protein retains the N-terminal formylmethionine, i.e. the amino acid by which the mitochondrial system initiates translation. In contrast, a larger precursor of subunit II of cytochrome c oxidase from yeast is observed after translation in isolated mitochondria (48). According to the sequence of the structural gene, the presequence (about 1.5 kD) of the precursor protein does not display exceptionally high

apolarity nor does it display other unusual features. A similarly confusing result is obtained comparing subunit I of cytochrome c oxidase from beef heart and Neurospora (49): A larger precursor is apparently formed in the mold but not in beef heart. No simple explanation is available for this heterogenous picture. It is also not known wich protease is involved in the processing of the larger precursors.

Cytochrome b is present as the apoprotein in mitochondria from heme-deficient yeast cells. The accumulated apoprotein has the same apparent size as the mature protein (50), but it is not known whether a proteolytic processing precedes its accumulation. Although the nucleotide sequence of the mitochondrial gene specifying the amino acid sequence of cytochrome b from yeast has been determined, the presence of a presequence in the primary translation product remains an open question since the aminoterminal sequence of the mature protein is not known yet.

Attachment of mitochondrial ribosomes at the surface of the inner membrane was observed and on the basis of genetic data it has been suggested that this interaction is functionally important (51). However, it is not known whether the intramitochondrially synthesized proteins are integrated into the inner membrane by cotranslational or by posttranslational events, or whether both modes of transport coexist.

How is the Assembly of Mitochondrially and Cytoplasmatically Synthesized Proteins Interconnected ?

Many mitochondrial proteins are assembled not as separate entities but as components of large protein complexes. The two genetic systems of the cell contribute proteins to complexes of the respiratory chain and the ATPase, which are assembled in the inner membrane, and to the ribosomes, which are assembled in the matrix.

Assembling the various subunits destined for a particular complex must be a cooperative process since the subunits pertinent in a complex are present in stoichiometric amounts.

One example is the ribosomal protein which is coded for by a mitochondrial gene (varl) in yeast and which is part of the small subunit (37 S) of mitochondrial ribosomes. This protein is apparently indispensable for the correct assembly of the other ribosomal proteins, all of which are imported from the cytoplasm. When this mitochondrially synthesized protein is defective or absent this leads to an arrest in the final assembly of the small ribosomal subunit at the stage of a 30 S ribonucleoprotein particle (52) which not only lacks the varl protein but also certain cytoplasmically synthesized proteins.

An especially interesting example of the interrelationship between the two genetic systems in the assembly of mitochondria is the biogenesis of subunit 9 of the oligomycin-sensitive ATPase. This protein is coded on nuclear DNA in all species studied so far, with the exception of yeast where it is coded on mtDNA. In Neurospora a nuclear as well as a mitochondrial gene are present but the mitochondrial one appears to be silent (53). The nuclear gene product, which is translated on cytoplasmic ribosomes and posttranslationally imported into mitochondria, is formed as a precursor carrying a transient presequence which is roughly as large as the mature protein itself (14). In yeast, where this protein is synthesized in the mitochondrial matrix, the primary translation product has no presequence (54). Yet, in both cases the protein is transported to the same destination, namely inserted into the mitochondrial inner membrane. Why is it formed as a larger precursor here, but in the same size as the mature form there ? We do not know the answer. This is all the more remarkable since the larger extramitochondrial precursor of ATPase subunit 9 from Neurospora, which has been

synthesized in a cell-free translation system, can be translocated into isolated mitochondria from yeast and correctly processed to the mature size (55).

VII. CONCLUSIONS

The biogenesis of some fourty cytoplasmically synthesized mitochondrial proteins has been investigated so far. These studies have established that these proteins are formed as extramitochondrial precursors and that they are posttranslationally imported into mitochondria. Their uptake into mitochondria requires specific interaction with the mitochondrial surface. This apparently involves receptors. The mechanisms by which precursors are posttranslationally translocated across the membranes are not clearly understood yet. Obviously there is no uniform pathway for the translocation process. Rather, the details of translocation vary considerably with different proteins: Most precursors contain presequences of various length which are proteolytically processed in either one or two steps, but a few precursors lack a presequence. Import of most precursors into mitochondria depends on the electrical potential of the inner membrane, but a few precursors which are destined for the outer membrane or the intermembrane space do not require a membrane potential for assembly. Far less is known about the events in the assembly of intramitochondrially synthesized proteins.

As far as one can judge presently, no correlation exists between transfer of precursors into a particular submitochondrial compartment and any special sequence of events during translocation. As a common theme, however, in all cases irreversible steps such as proteolytic processing, covalent modification, or substantial refolding occur during translocation in order to trap the proteins in their proper submitochondrial locations.

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