MEMBRANES IN GROWTH AND DEVELOPMENT

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Preface

The central role that membranes play in cellular processes is becoming increasingly apparent. The present symposium contains contributions on various aspects of involvement of membranes in growth and development. These include genetic determinants and the synthesis and assembly of membrane constituents such as transport proteins and receptors during cell differentiation and cell growth. Neuromuscular and epithelial development together with intracellular organelles are considered. The interplay between various membrane systems and their modulation by cellular control mechanisms are a constant theme of the various chapters. Each contributor considers the historical perspectives and the current status of that particular field. The subjects are timely, and it is hoped that others will be stimulated by the new concepts of this emerging field. It should also be mentioned that the contributions in this volume were presented at the biennial International Conference on Biological Membranes held at Crans-sur-Sierre, Switzerland, June 15–19, 1981.

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Membranes in Growth and Development, pages 37–47

Biogenesis of Mitochondrial Membrane Proteins

Martin Teintze, Bernd Hennig, Manfred Schleyer, Bernd Schmidt, and Walter Neupert

INTRODUCTION

Eukaryotic cells, in contrast to prokaryotic cells, are divided into a number of compartments. By creating these compartments (ie, organelles), the eukaryotic cell gains a host of new properties and capabilities. The presence of specific reaction vessels enclosed by membranes within the cell allows, for instance, that metabolic pathways can be used simultaneously in opposite directions or that substances (such as Ca^{++}) can be sequestered within the cell and released when needed to initiate reactions. Ion or proton gradients can also be generated across the membranes of the organelles to drive the synthesis of substances such as ATP. However, this increase in capabilities brings with it a series of new problems for the eukaryotic cell. A major one is that the proteins of the intracellular organelles are, with few exceptions, synthesized on cytoplasmic ribosomes [Chua and Schmidt, 1979]. They must be transported into the organelles during or after translation. This immediately raises a series of questions. The membranes of the organelles are impermeable to most low molecular weight compounds and practically completely impermeable to macromolecules such as proteins. On the other hand, cytoplasmic ribosomes synthesize proteins for a number of different organelles in the cell. The cell must therefore have mechanisms that specifically insert newly synthesized proteins into the proper organelle.

An explanation of the molecular mechanisms of intracellular protein transport can only be achieved if single, well-defined proteins that can be assigned to one particular cell compartment are followed over the entire path from the synthetic origin to the functional site. This requires the isolation and purification of these proteins and the preparation of specific antibodies, so that the very small quantities of precursors or intermediates involved in the transfer process can be found.

POST-TRANSLATIONAL TRANSFER OF PROTEINS INTO MITOCHONDRIA

The intracellular transport of quite a few proteins into mitochondria and chloroplasts has been studied in this manner [Chua and Schmidt, 1979; Neupert and Schatz, 1981]. Some work has also been done recently on glyoxysomal and peroxisomal proteins [Zimmermann and Neupert, 1980; Frevert et al, 1980; Roberts and Lord, 1981; Goldman and Blobel, 1978; Lazarow, 1980]. The results have shown that the transport into these organelles is posttranslational [Hallermeyer et al, 1977; Harmey et al, 1977; Korb and Neupert, 1978; Maccechini et al, 1979; Raymond and Shore, 1979; Morita et al, 1981; Conboy and Rosenberg, 1981]. This is in contrast to the cotranslational transfer that takes place in the endoplasmic reticulum (ER) to insert proteins into ER membranes and into plasma membrane, and to transfer secretory proteins into the inner of the ER [Palade, 1975; Blobel and Dobberstein, 1975].

There is not one unique mechanism by which proteins are inserted into mitochondria after synthesis on free ribosomes; different proteins, even if they are subunits of the same enzyme complex, will often use a somewhat different mechanism to insert into the mitochondria. There are, however, several unifying features of the transport pathway that have been observed. First of all, every protein that has been studied thus far is synthesized as a precursor that is in some way different from the mature functional protein. This is of course a necessary feature, both for membrane proteins, which must change from a form soluble in the cytosol to one soluble in the membrane, and for proteins solubilized inside the organelle, which must be irreversibly altered to prevent them from leaving their compartment.

IMPORT OF PROTEINS INTO MITOCHONDRIA OCCURS WITH AND WITHOUT PROTEOLYTIC PROCESSING

Many of the proteins whose transport has been studied are synthesized as larger precursors. Their apparent molecular weights exceed those of the mature proteins by anywhere from 500 daltons for subunit VII of the Neurospora cytochrome bc_1 complex to 6,000 daltons for cytochrome c_1 and some of the mitochondrial ATPase subunits. This additional sequence is then removed by a proteolytic enzyme during or after the transfer process. This form of posttranslational transfer with proteolytic processing has been observed, for instance, for ribulose-1,5-bisphosphate carboxylase in chloroplasts [Dobberstein et al, 1977] and for subunit 9 of the mitochondrial oligomycin sensitive ATPase (OS-ATPase) [Michel et al, 1979; Zimmermann et al, 1981] as well as many of the subunits of the cytochrome bc_1 complex [Nelson and Schatz, 1979; Cote et al, 1979] (Teintze et al, unpublished) and subunits of cytochrome oxidase [Lewin et al, 1980; Mihara and Blobel, 1980; Schmelzer and Heinrich,

1980]. In the case of cytochrome c_1, the proteolytic processing seems to take place in two separate steps via a form of intermediate molecular weight that is already located somewhere within the mitochondria (Teintze et al, unpublished; Ohashi and Schatz, personal communication). On the other hand, subunit VI of the cytochrome bc_1 complex appears not to be synthesized as a larger precursor (Teintze et al, unpublished).

For proteins that do not have a precursor with a larger molecular weight such as cytochrome c, ADP/ATP carrier, and mitochondrial porin [Korb and Neupert, 1978; Zimmermann et al, 1979a,b] (Freitag and Neupert, unpublished), something else must trigger a conformational change. In the most extensively studied example, cytochrome c, it is the attachment of the heme group to apocytochrome c that results in a conformational change when the protein transverses the outer mitochondrial membrane [Hennig and Neupert, 1981] (Fig. 1). The fact that apocytochrome c changes its conformation upon attachment of the heme group to form holocytochrome c is demonstrated by the absence of cross-reactivity between antibodies prepared against apo- and holocytochrome c in our laboratory [Korb and Neupert, 1978] and by the fact that cold excess holocytochrome c cannot compete with radioactive apocytochrome c for binding to and transfer into Neurospora mitochondria [Hennig and Neupert, 1981]. In the cases of the ADP/ATP carrier and ATPase subunit 9, the precursors synthesized in vitro in a heterologous cell-free system were found to be present as soluble aggregates with molecular weights in the range of 100,000 to 500,000 [Zimmermann and Neupert, 1980b] (Schmidt and Neupert, unpublished). The mature proteins are soluble only in the presence of detergents and cannot be used to inhibit the transfer of the precursors into mitochondria.

MUTUAL RECOGNITION OF PRECURSORS AND ORGANELLES IS MEDIATED BY RECEPTORS ON THE MITOCHONDRIAL SURFACE

The precursor proteins, which are synthesized on free cytoplasmic ribosomes, must have a method of recognizing the organelle for which they are intended and a method of entering into or transversing a membrane which is normally impermeable to proteins. The most logical mechanism for such a process is the presence of a specific receptor in the outer membrane of the organelle. There is, in fact, considerable evidence to support the existence of such receptors (although none have been isolated thus far). Neurospora proteins can be synthesized in vitro and the postribosomal supernatant incubated with Neurospora mitochondria under conditions where the precursors to the mitochondrial proteins are transferred into the mitochondria. When the mitochondria are then reisolated, washed, and lysed, and antibodies to a specific protein are added, precursors bound to the mitochondria are immunoprecipi-

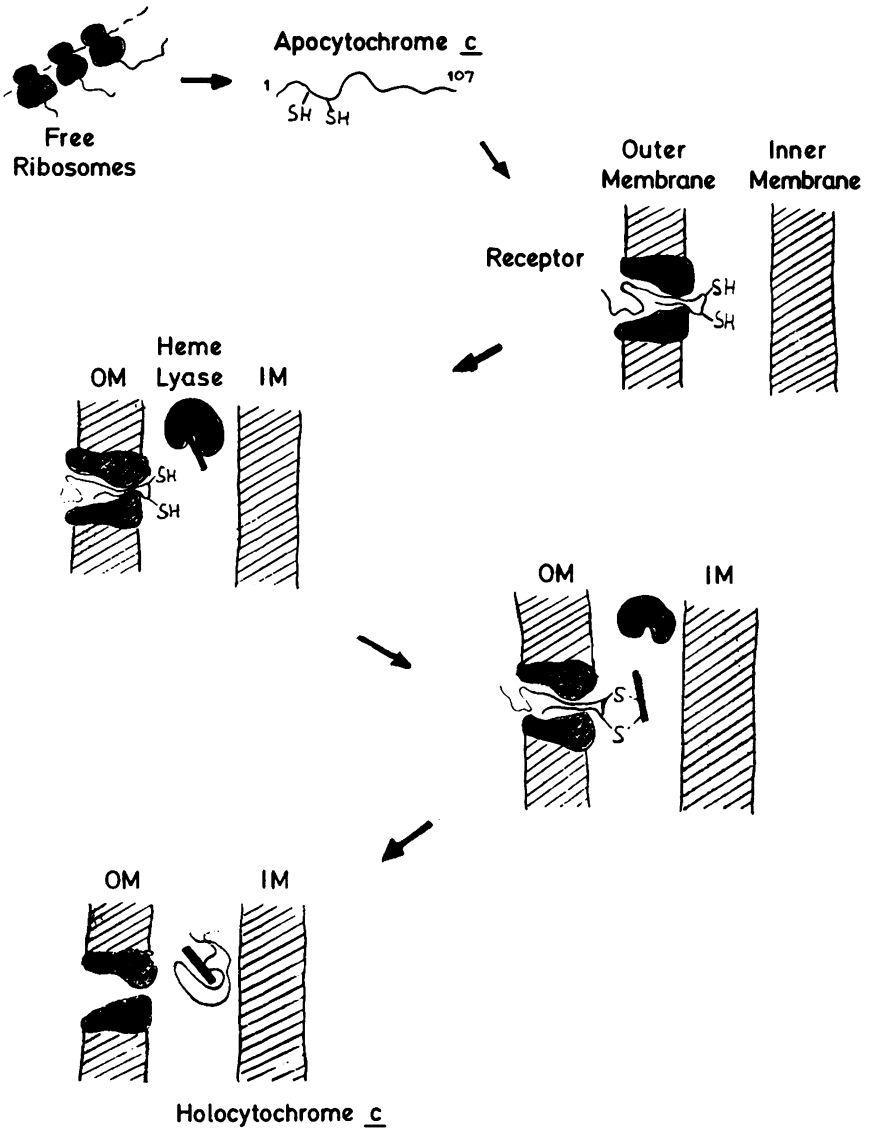

Fig. 1. A possible mechanism for the assembly of cytochrome c. Apocytochrome c is synthesized on free cytoplasmic ribosomes and then bound to a receptor in the outer mitochondrial membrane in such a way that the heme group can be attached by a heme lyase in the intermembrane space. Attachment of the heme group causes a conformational change that completes the transfer and results in mature, functional holocytochrome c.

tated along with the mature protein (Fig. 2). If the mitochondria are treated with proteinase K prior to lysis, the precursors are degraded whereas the mature proteins are not. The preferential sensitivity of precursor proteins to added proteases enables one to distinguish the bound precursor from the mature protein when both have the same molecular weight, as in the case of the ADP/ATP carrier. If the transfer in the reconstituted system is blocked by the presence of energy inhibitors such as valinomycin and carbonyl cyanide m-chlorophenyl hydrazone (CCCP), only the bound precursor is found [Zimmermann et al, 1981].

This suggests that the precursors are bound by some type of receptor in the outer membrane and that the transfer is energy-dependent, whereas the binding of the precursor is not. It is possible that in vivo a precursor may be recognized by its receptor before synthesis is completed [Ades and Butow, 1980], but the transfer appears to be always posttranslational. In the case of cytochrome c, the presence of specific saturable binding sites can be shown because excess unlabeled apocytochrome c, but not holocytochrome c, will compete with labeled apocytochrome c and inhibit its transfer into the mitochondria [Hennig and Neupert, 1981]. The transfer of apocytochrome c does not seem to be energy-dependent, but it can be inhibited by deuterohemin, which cannot be covalently attached to the protein because it lacks the vinyl groups of the natural prosthetic group protoheme. In the presence of excess deuterohemin, bound apocytochrome c accumulates on the mitochondria. If these are then reisolated and resuspended in a medium containing protohemin, but no additional precursor, the bound apocytochrome c is transferred into the mitochondria and converted to holocytochrome c [Hennig and Neupert, 1981] (Table I).

THE TRANSPORT OF MANY PROTEINS INTO MITOCHONDRIA IS DEPENDENT ON THE MEMBRANE POTENTIAL

The import of many cytoplasmically synthesized proteins into mitochondria is energy-dependent. This was shown for the ADP/ATP carrier and subunit 9 of the OS-ATPase [Zimmermann et al, 1981] and for cytochrome c_1 and subunit V of the cytochrome bc_1 complex (Teintze et al, unpublished) in in vitro transfer experiments using Neurospora mitochondria. In addition, the energy dependence of the transfer of subunits α and β of F_1-ATPase, cytochrome c_1, and subunit V of the cytochrome bc_1 complex has been investigated using pulse-chase experiments in vivo with yeast cells, and it was concluded that processing of the precursor proteins was dependent on the ATP level in the mitochondria, rather than on the membrane potential [Nelson and Schatz, 1979]. All these proteins are located within or on the matrix side of the inner mitochondrial membrane. The energy dependence of the transfer of the ADP/ATP

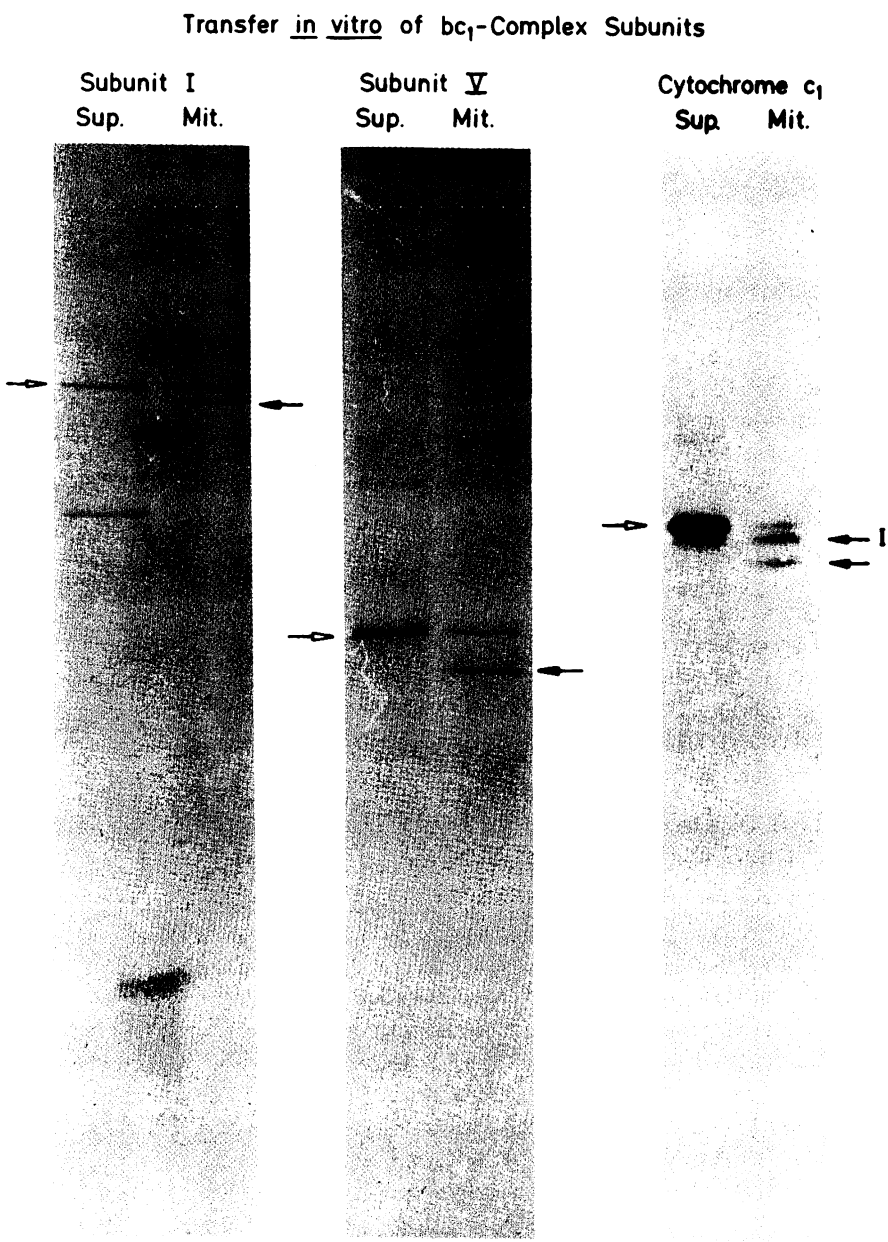
Transfer in vitro of bc1-Complex Subunits
Subunit I
Sup.
Mit.
Subunit V
Sup.
Mit.
Cytochrome c1
Sup.
Mit.
I

TABLE I. Binding of Apocytochrome c to Receptor Sites on Mitochondria[a]

Additions	Cytochrome c bound to mitochondria			
	Counts/min per mitochondrial protein		% Total ^{3}H-cytochrome c of control	
	Apocytochrome c	Holocytochrome c	Apocytochrome c	Holocytochrome c
None (control)	682	4168	14	86
+ Deuterohemin (10 nmol/ml)	4330	1185	89	24
Deuterohemin (10 nmol/ml), then mitochondria washed with 0.44 M sucrose, 1 mM EDTA, 10 mM Tris/HCl, pH 7.4	3752	1120	77	23
+ Deuterohemin (10 nmol/ml), then incubation with protohemin (10 nmol/ml)	1917	2934	40	60
+ Deuterohemin (10 nmol/ml), then incubation with apocytochrome c of Neurospora crassa (10 nmol/ml)	710	1160	15	24
+ Deuterohemin (10 nmol/ml), then incubation with holocytochrome c of Neurospora crassa (10 nmol/ml)	3742	1200	77	25

[a]Apocytochrome c was synthesized in a cell-free system of Neurospora crassa in the presence of ^{3}H-leucine. A postribosomal supernatant was prepared which contained the ^{3}H-labeled apocytochrome c. Mitochondria were isolated from Neurospora cells and incubated with the postribosomal supernatant in the absence or presence of deuterohemin for 15 min at 25°C. Then mitochondria were reisolated. When indicated they were resuspended in fresh unlabeled postribosomal supernatant. Then protohemin, apocytochrome c, or holocytochrome c was added and incubation was continued for 15 min. Mitochondria were then reisolated. From one-half of each of the various mitochondrial samples apocytochrome c was immunoprecipitated, and from the other half holocytochrome c, employing specific antibodies. Immunoprecipitates were analyzed by SDS gel electrophoresis, and radioactivity in the cytochrome c peak was determined. The sum of the radioactivities in apocytochrome c and holocytochrome c in the sample without any addition was set at 100%.

Fig. 2. Transfer in vitro and proteolytic processing of cytochrome bc_1 complex subunits. Radioactive precursors to cytochrome bc_1 complex subunits were synthesized in a reticulocyte lysate cell-free system, and the postribosomal supernatant was incubated with Neurospora mitochondria for 1 h at 25°C. The mitochondria were then separated from the supernatant by centrifugation and both fractions were adjusted to 1% Triton, 0.3 M NaCl. Immunoprecipitation was then carried out using antibodies against the individual subunits, followed by SDS gel electrophoresis and autoradiography. The antibodies precipitated both the precursors (open arrows) and the mature proteins (black arrows); the arrow labeled I points to the intermediate form of cytochrome c_1 (see text).

carrier (Fig. 3) and of subunit 9 of the OS-ATPase was studied in detail in a reconstituted system employing isolated Neurospora mitochondria and the postribosomal supernatant of a rabbit reticulocyte lysate incubated with Neurospora RNA and (^{35}S)methionine (Schleyer et al, unpublished). In these experiments, the uncoupler CCCP and the ionophore valinomycin (which break

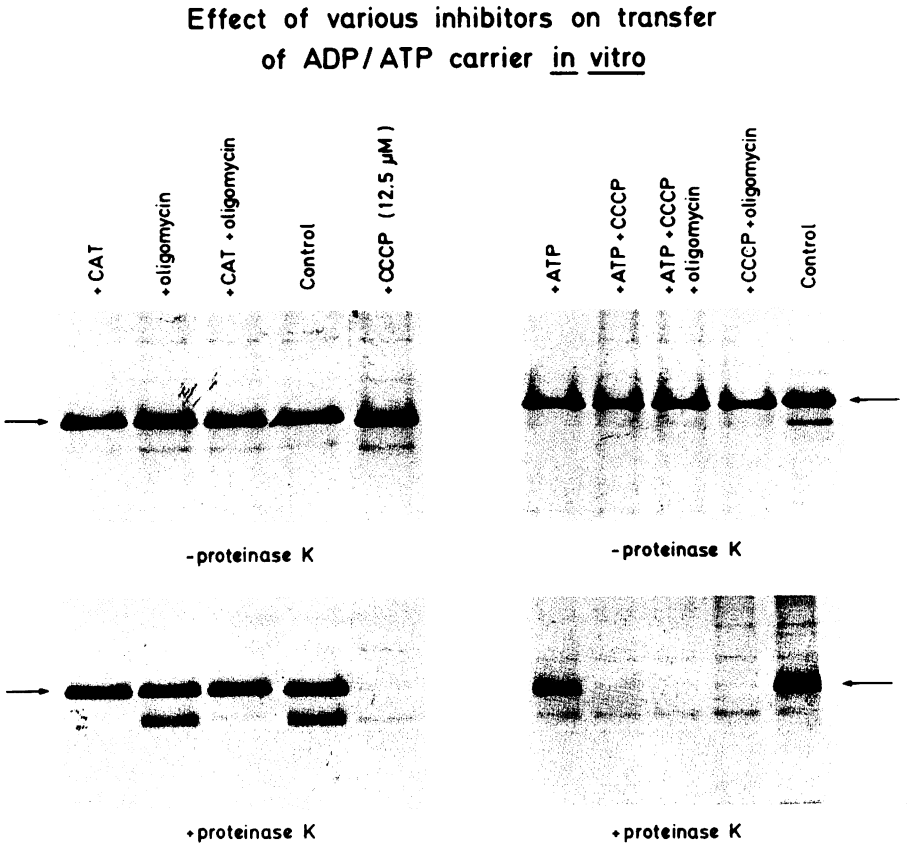

Fig. 3. The transport of the ADP/ATP carrier is dependent on the membrane potential. Labeled precursors were synthesized in a reticulocyte lysate cell-free system, and the postribosomal supernatant was incubated with Neurospora mitochondria for 1 h at 25°C. The mitochondria were reisolated and then lysed in 1% Triton, 0.3 M KCl. The ADP/ATP carrier was immunoprecipitated, electrophoresed on SDS gels, and autoradiographed. One or more of the following were present during the incubation when indicated: 2.6 mM ATP, 2.4 µM carboxyatractyloside (CAT), 12.5 µM CCCP, 5 µM oligomycin. Proteinase K (1 mg/ml, 30 min, 25°C) was used to digest the precursor bound to the mitochondria. The mature protein after transfer is protease-resistant.

down the membrane potential) inhibited the transfer of these proteins into the mitochondria. When oligomycin (which blocks the OS-ATPase) was used together with valinomycin to ensure that the ATP in the mitochondria was not being hydrolyzed, the transfer was still blocked. CCCP together with oligomycin and ATP also had the same effect, showing that even when ATP is present at a high level in the mitochondrial matrix, the transfer will not take place in the absence of a membrane potential [Heldt et al, 1972]. Carboxyatractyloside (which blocks the ADP/ATP carrier) together with oligomycin cannot inhibit the transfer, although the mitochondria should be depleted of ATP [Klingenberg, 1976]. This confirms that it is the membrane potential, rather than ATP, that is required for the import of the proteins into the mitochondria. Cytochrome c, located in the intermembrane space on the cytoplasmic side of the inner membrane, and the outer membrane porin both do not seem to require energy for their import [Zimmermann et al, 1981] (Freitag and Neupert, unpublished). This is to be expected, since they are located outside the potential across the inner membrane.

TRANSFER OF NEUROSPORA PROTEINS INTO RAT LIVER MITOCHONDRIA

When labeled precursors to Neurospora proteins (in the postribosomal supernatant of a rabbit reticulocyte lysate incubated with Neurospora RNA) were incubated with isolated rat liver mitochondria, the results were similar to those obtained with Neurospora mitochondria. The precursors of the ADP/ATP carrier and the mitochondrial porin were transferred to a protease-resistant location in the mitochondria (Freitag and Neupert, unpublished; Schleyer et al, unpublished). The precursor of subunit 9 of the OS-ATPase and subunits I and V of the cytochrome bc_1 complex were processed to the molecular weights of the mature proteins (Teintze et al, unpublished; Schleyer et al, unpublished); the precursor to cytochrome c_1 seems to have been processed only to the molecular weight of the intermediate form (see above) (Teintze et al, unpublished).

The energy dependence of the import into rat liver mitochondria also appeared to be similar to that in Neurospora. These results indicate that the structures of the receptors and proteases involved in the transfer of proteins into mitochondria, as well as the proteins themselves, must be highly conserved.

CONCLUSION

In summary, proteins appear to be transported into mitochondria by the following mechanism. First, the protein is synthesized on free cytoplasmic ribosomes as a precursor with or without an additional sequence, but definitely

with a conformation different from that of the mature functional protein. Next, the precursor protein is bound to a receptor on the outer mitochondrial membrane. The protein is then translocated across the membrane into the intermembrane space, or possibly into the inner membrane or the matrix by way of a contact site between the two membranes. If the protein enters or crosses the inner mitochondrial membrane, this process is dependent upon the potential across this membrane and may also involve an additional protein in the membrane to catalyze the translocation. During or after the transfer from the outer membrane receptor to the functional site, any additional sequences are removed from the precursor and prosthetic groups are attached, if required. The components of this mechanism seem to be common to mitochondria from species as varied as yeast, Neurospora, and rat.

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