

# Hormones and Cell Regulation

## Hormones et Régulation Cellulaire

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## Protein transport into mitochondria : a multi-step process

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### ABSTRACT

The transport of precursor proteins from the cytoplasm into mitochondria can be resolved into several steps. Precursor proteins can be reversibly accumulated at distinct stages of the import pathway: at the level of binding to the mitochondrial surface, in contact sites between outer and inner mitochondrial membranes, and in the mitochondrial matrix. Import intermediates can be trapped by a number of methods: lowering the temperatures or the levels of nucleoside triphosphates in the import reactions, dissipation of the mitochondrial membrane potential, and inhibition of the matrix-located processing peptidase. The following conclusions may be drawn (i) precursors specifically bind to receptor proteins on the mitochondrial surface; (ii) import occurs at translocation contact sites; (iii) proteolytic processing is not required for import into the matrix; and (iv) several precursors destined for the intermembrane space are first completely imported into the matrix and are then retranslocated across the inner membrane.

### KEY WORDS

Mitochondria, protein import, receptors, contact sites, protein sorting

### INTRODUCTION

The translocation of precursor proteins from the cytoplasm into mitochondria is a multi-step process (for review see Ref. 1). Many precursor proteins contain amino-terminal presequences which carry specific information for targeting to mitochondria (for review see Ref. 2). Nucleoside triphosphates are required to keep cytosolic precursors in an unfolded conformation (3, 4). The precursors specifically interact with proteinaceous binding sites on the mitochondrial surface (5-9). Import into or across the inner membrane occurs at contact sites between both membranes (8, 10-12) and requires a membrane potential  $\Delta\Psi$  across the inner membrane (13-16). Presequences are proteolytically removed by the processing peptidase located in the mitochondrial matrix (17-20). The proteins are sorted to their final intramitochondrial location and are often assembled into multi-subunit complexes.

How can the complex process of mitochondrial protein import be studied in detail? We decided to dissect the import reaction into several distinct steps. Precursor proteins are accumulated at distinct stages of their import pathway and are further imported after release of the block. Thus, translocation intermediates are generated. This allows us to investigate defined steps of protein import.

#### METHODS TO GENERATE TRANSLOCATION INTERMEDIATES

The principle method for obtaining import intermediates is to slow down or to prevent distinct reactions which occur on the import pathway of a precursor. This block should be reversible, as it is necessary that it can be relieved and that the precursors then can move further on the import pathway. This demonstrates that the trapped intermediate is an authentic import intermediate, i.e. it is on the correct assembly pathway. In most experiments, mitochondria isolated from the fungus *Neurospora crassa* and precursor proteins synthesized in rabbit reticulocyte lysates are used. Several of the described translocation intermediates were also demonstrated in *in vivo* experiments.

One important method is to lower the temperature of the import reaction. The half-times of many reactions are thereby prolonged and thus the import is slowed down. This allows the transient trapping of precursors in contact sites between outer and inner membranes (10).

Nucleoside triphosphates (NTPs) are required for unfolding cytosolic precursors. With lowered levels of NTPs, precursors are only partially unfolded and thereby can be accumulated at the stage of specific binding or in translocation contact sites (3).

The membrane potential  $\Delta\psi$  is required for the initial entrance of precursors into the inner membrane. In the absence of a membrane potential several precursors can still specifically bind to the outer mitochondrial membrane. Upon reestablishing  $\Delta\psi$ , the precursors are imported from their binding sites (5, 21).

Finally, the processing peptidase in the mitochondrial matrix is dependent on divalent cations. Membrane-permeable chelators, such as ortho-phenanthroline, inhibit the processing activity in intact mitochondria. Precursors can thus be imported into the mitochondrial matrix without the removal of their amino-terminal presequences (11, 22).

#### IMPORT INTERMEDIATES AT THE LEVEL OF SPECIFIC BINDING

At low temperatures (0° to 4°C), the precursor of the outer membrane protein porin specifically binds to the mitochondrial surface. The binding sites are saturable, and the affinity constant has been determined. The precursor can be completely imported and assembled after raising the temperature to 25°C. Furthermore, the binding step is inhibited by a mild protease-pretreatment of the mitochondria (9). These data strongly suggest the involvement of a proteinaceous high affinity binding site (receptor) in the import of porin into the outer membrane.

After dissipation of the membrane potential, the precursor of the inner membrane protein ADP/ATP carrier still binds to the outer mitochondrial membrane. Upon reestablishment of  $\Delta\psi$ , the ADP/ATP carrier is imported from the

binding sites without prior release from the mitochondrial membranes (5). When binding is performed at 25°C, the precursor inserts into saturable proteinaceous sites in the outer membrane, which are beyond the initial protease-sensitive binding sites. However, when the dissipation of  $\Delta\Psi$  is combined with a lowering of the temperature (to 2°C) or of the levels of ATP, the precursor remains on the surface of the mitochondria accessible to externally added proteases. Raising the temperature or the levels of ATP leads to insertion of the precursor into the second binding sites described above (3, 8). These studies show the existence of two subsequent binding sites on the import pathway of ADP/ATP carrier.

Similarly, the precursor of F<sub>1</sub>-ATPase subunit 9 (an inner membrane protein) specifically binds to mitochondria in the absence of  $\Delta\Psi$ , and can be imported, from the binding sites, in the presence of  $\Delta\Psi$ . The same result is obtained for a fusion protein between the complete subunit 9 precursor and cytosolic dihydrofolate reductase (DHFR). Two fusion proteins between the presequence of subunit 9 and DHFR are imported in the presence of  $\Delta\Psi$ . In the absence of  $\Delta\Psi$ , however, their specific binding to the mitochondrial surface is very weak. Furthermore, the import kinetics of the two precursors lacking the (hydrophobic) mature part of subunit 9 are slower than those of the two precursors containing the mature part. Upon studying the efficiency of specific binding and the kinetics of import of other precursor proteins, we arrived at the following proposal: mitochondrial precursor proteins can be grouped into at least two different classes. Class I precursors contain a hydrophobic stretch of about 20 amino acid residues, show a strong interaction with binding sites on the mitochondrial surface and a rapid import. Class II precursors lack a long hydrophobic stretch, show only a weak interaction with binding sites and a slow import (21). These studies suggest that (hydrophobic) 'assistant' sequences increase the affinity of binding to receptor sites and support a rapid import of precursors.

#### IMPORT INTERMEDIATES IN TRANSLOCATION CONTACT SITES

Sites of close contact between mitochondrial outer and inner membranes were described many years ago (23). The accumulation of precursor proteins in contact sites assigned, for the first time, a biochemical function to these sites by demonstrating their involvement in mitochondrial protein import (10). Protein import was performed at low temperatures (2° to 12°C) or at low levels of nucleoside triphosphates. Consequently, the amino-terminal portions of precursor proteins were translocated into the matrix, where the presequences were cleaved off by the processing peptidase. Major portions of the precursors, however, were still outside the outer membrane as they were accessible to proteases or to antibodies added to isolated mitochondria. Thus, the precursor proteins were reaching from the outside of the outer membrane into the matrix thereby spanning sites of close contact between both membranes (translocation contact sites) (3, 8, 10-12). The translocation intermediates with bound antibodies were decorated with protein-A-gold particles. Electron microscopy allowed their visualization and served to confirm that the contact sites previously described morphologically represented the contact sites now defined biochemically (12). The translocation intermediates in contact sites are accessible to aqueous perturbants, such as alkali or urea. Thus, protein import does not seem to directly occur through the lipid bilayer, but rather through a hydrophilic (probably proteinaceous) environment (24).

Only the initial entrance of precursors into contact sites requires the membrane potential across the inner membrane; the completion of protein transport into or across the inner membrane does not require the membrane

potential (10). Furthermore, it was demonstrated that only the electrical component  $\Delta\psi$  of the total protonmotive force was necessary for protein import (15). These results led us to propose that the membrane potential  $\Delta\psi$  exerts an electrophoretic effect on positively charged regions of the precursor proteins (such as the presequences).

#### IMPORT INTERMEDIATES IN THE MITOCHONDRIAL MATRIX

Upon import into mitochondria, the amino-terminal presequences of precursor proteins are cleaved off by the metal-dependent processing peptidase in the matrix. Proteolytic processing, however, is not a prerequisite for translocation of precursors across the mitochondrial membranes (22). When processing activity is blocked by metal chelators, precursors of subunits 2 and 9 of  $F_1F_0$ -ATPase are still imported *in vitro* into a position protected against externally added protease.

Complete import in the absence of proteolytic processing can also be shown for precursors destined for the mitochondrial intermembrane space. The ability to block the processing peptidase is used as a tool to dissect the import pathway of these proteins into intermediate steps. Such experiments with the Rieske Fe/S-protein of the  $bc_1$ -complex, a protein located at the outer surface of the inner membrane, had a surprising result (11, 25). Following inhibition of the processing peptidase precursor of Fe/S-protein is accumulated as a soluble species in the mitochondrial matrix, as revealed by digitonin fractionation of mitochondria. Upon reactivation of the processing peptidase by divalent cations, the precursor is processed to the mature subunit which is correctly sorted into the intermembrane space. The Fe/S-protein, thus, during import and sorting, crosses the inner membrane twice. Interestingly, proteolytic processing of the precursor occurs in two steps. The first cleavage is performed by the processing peptidase and results in the formation of an intermediate-sized species with an eight amino acid residue amino-terminal extension. The second processing step also occurs inside the inner membrane and is very likely dependent on the attachment of the 2Fe-2S cluster to the molecule (Hartl and Neupert, unpublished). Addition of the strong iron chelator ortho-phenanthroline during import causes accumulation of intermediate-sized Fe/S-protein in the matrix.

Similar results are obtained for the import of cytochrome  $b_2$  and cytochrome  $c_1$  (26). Cytochrome  $b_2$  is a soluble component of the intermembrane space while cytochrome  $c_1$  is anchored to the inner membrane but faces the intermembrane space with a large hydrophilic domain. The presequences of their precursors can be divided into two parts. The amino-terminal part contains several positive charges and exhibits the typical features of mitochondrial targeting sequences, however, the carboxy-terminal parts (in contrast to the precursor of Fe/S-protein) contain a hydrophobic stretch of about 20 amino acid residues. Precursors of both cytochrome  $b_2$  and cytochrome  $c_1$  are first imported via translocation contact sites into the matrix of mitochondria. There the action of processing peptidase generates the respective intermediate-sized forms where the amino-terminal peptide extensions still contain the hydrophobic part of the presequence. These intermediate-sized species are subsequently translocated back across the inner membrane into the intermembrane space. Unlike the Fe/S-protein, processing to the mature-sized forms occurs at the outer surface of the inner membrane. Targeting information for the reverse translocation very likely resides in the hydrophobic prepiece of the intermediates.

We suggest that the mechanism of the export from the mitochondrial matrix shares a common origin with the mechanism of protein export from the cytoplasm to the periplasmic space in procaryotes. In light of the hypothesis for the endosymbiotic origin of mitochondria, one may assume that gene transfer from the endosymbiont to the nucleus of the host cell has occurred during development to mitochondria. Import via translocation contact sites brings precursors back onto their ancestral assembly pathway which is still followed with regard to export from the mitochondrial matrix (corresponding to the bacterial cytoplasm) across the inner membrane (corresponding to the bacterial plasma membrane) ("Conservative sorting hypothesis").

## PERSPECTIVES

Translocation intermediates of precursor proteins accumulated at distinct stages of mitochondrial protein import are very useful not only for elucidating functional characteristics of protein transport, but are also a prerequisite for the identification and purification of components of the import machinery. Import intermediates trapped at the level of binding serve as the test system for identifying receptors, either via cross-linking reagents or by extracting the binding sites with mild detergents and reconstituting them into liposomes (9). Translocation intermediates in contact sites are the marker proteins for enriching contact site components either by subfractionation of mitochondria (sonication (12) or use of mild detergents) or by cross-linking. Thus, translocation intermediates are powerful tools for investigating mitochondrial protein import at the molecular level.

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## Résumé

Il est possible de distinguer plusieurs étapes du transport des protéines précurseurs du cytoplasme dans la mitochondrie. Les protéines précurseurs peuvent être accumulées à diverses étapes des voies d'importation: au niveau de la liaison sur la surface mitochondriale, des sites de contact entre membrane externe et interne mitochondriale et dans la matrice mitochondriale. Des intermédiaires d'importation peuvent être bloqués par plusieurs méthodes: diminution de la température ou des niveaux de nucléosides triphosphates dans les réactions d'importation, dissipation du potentiel de membrane mitochondriale et inhibition de la peptidase responsable de la transformation qui est localisée dans la matrice. Les conclusions suivantes peuvent être déduites: 1) les précurseurs se lient spécifiquement aux protéines réceptrices sur la surface mitochondriale; 2) l'importation intervient sur des sites de contact de translocation; 3) une transformation protéolytique n'est pas nécessaire pour que l'importation dans la matrice puisse intervenir; 4) plusieurs précurseurs dont la destination est l'espace intermembranaire sont d'abord complètement importés dans la matrice pour être ensuite retransloqués à travers la membrane interne.