Protein Translocation Across Mitochondrial Membranes

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Summary

Protein translocation across biological membranes is of fundamental importance for the biogenesis of organelles and in protein secretion. We will give an overview of the recent achievements in the understanding of protein translocation across mitochondrial membranes[1-5]. In particular we will focus on recently identified components of the mitochondrial import apparatus.

Introduction

The mitochondrial compartment occupies a substantial fraction of the cytoplasm of almost all eucaryotic cells. Most cellular oxidations are carried out in mitochondria and produce the major proportion of the cell's ATP. Mitochondria are organized in a complex way, consisting of: (i) two distinct membraneous subcompartments, the outer membrane and the inner membrane, and (ii) two distinct aqueous subcompartments, the intermembrane space and the matrix (see Fig. 1). There are many hundreds of proteins within the mitochondrial subcompartments but only few of them are encoded by mitochondrial genes. Most mitochondrial proteins are specified by nuclear genes, synthesized on cytosolic polysomes and continuously imported into growing and dividing organelles. The translocation process requires: (i) signals on the newly synthesized proteins (precursor proteins) and (ii) machinery to decode these signals and to translocate the precursors across one or two membranes. In most cases, precursors become proteolytically processed and

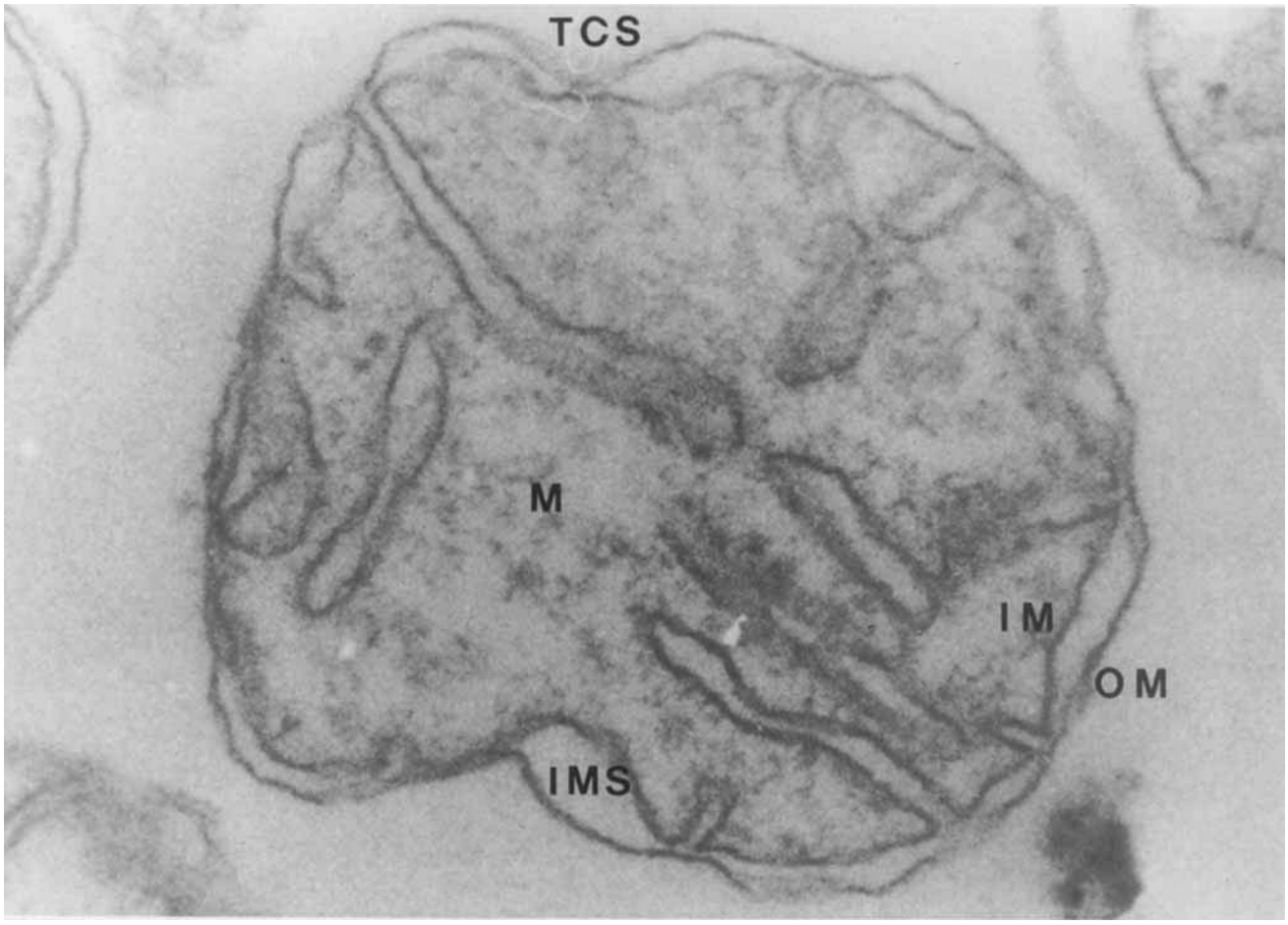

Fig. 1. Electron micrograph of a *Neurospora crassa* mitochondrion. *Neurospora crassa* mitochondria were fixed in 0.5% glutaraldehyde, embedded in Epon and thin sections were examined by electron microscopy as described[36]. A *Neurospora crassa* mitochondrion is shown with the appropriate compartments marked. OM, outer membrane; TCS, translocation contact sites; IMS, intermembrane space; IM, inner membrane; M, matrix space.

eventually are folded to a native conformation. Since the mitochondria are complex organelles, the import pathways cannot be expected to be uniform. However the majority of proteins follow a "general import pathway". Many proteins are known to branch off at earlier stages, others use additional steps.

Signals on mitochondrial precursor proteins have been studied in some detail[2]. Most precursors have amino-terminal peptide extensions of about 15-70 amino acid residues with a clear preponderance of basic and hydroxylated amino acids. There is, however, no sequence consensus. These extension peptides were found to be necessary and sufficient for targeting of precursors. On the other hand, a considerable number of precursors do not possess extensions, rather the information for targeting is contained within the mature sequence.

Precursors interact with receptor-like molecules on the surface of mitochondria and are then inserted into the outer membrane[5]. Further translocation across the inner membrane occurs at translocation contact sites where the two membranes are in close contact[6]. Translocation of the positively-charged targeting sequence requires an electrical membrane potential $\Delta\Psi$ (negative inside); the mature protein part follows without a need for $\Delta\Psi$. ATP is required both for the initial insertion into the outer membrane and for translocation[7]. Extensive unfolding of the polypeptide chains during translocation appears to be an obligatory step; this is followed by (re-)folding in the matrix[8-10]. Proteolytic processing occurs in the mitochondrial matrix[11]. In the following we will discuss recent results of a search for protein components involved in this complex transfer reaction. A continued biochemical and genetic approach has led to the identification of a number of such components (Table 1).

Components in the Cytosol Required for Translocation

It has been demonstrated recently that purified precursor proteins can be imported into isolated

Table 1. *Components involved in protein translocation across mitochondrial membranes*

Name	Molecular mass (kDa)	Function	Refs
Cytosol			
PBF (Presequence binding factor)	50 (rabbit reticulocyte lysate, rat heart and liver)	Stabilization of precursors in translocation competent forms	14, 15
ct-Hsp70 (SSA1-4) (Cytosolic cofactor)	70 (yeast)	Stabilization of precursors in translocation competent forms	16
Outer membrane			
MOM19	19 (*N. crassa*)	Binding protein for several precursors with amino-terminal targeting sequences and for porin	23
MOM72 (MAS70)	72 (*N. crassa*) 70 (yeast)	Binding protein for the presursor of the ADP/ATP-carrier	24, 26 25
MOM22	22 (*N. crassa*)	Component of the receptor complex with unknown function	27
P32	32 (yeast)	Putative binding protein at translocation contact sites	29, 30
GIP/MOM38 (ISP42) (General insertion protein)	38 (*N. crassa*) 42 (yeast)	Insertion of precursors into the outer membrane	27 31, 32
Matrix			
mt-Hsp70 (SSC1)	70 (yeast)	Translocation and folding of precursors	41-43
Hsp60 (MIF4)	58 (*N. crassa*) 60 (yeast)	ATP dependent folding of imported proteins; maintenance of translocation competent forms for re-export to the intermembrane space	44 45
MPP (MIF2/MAS2) (Mitochondrial processing peptidase)	57 (*N. crassa*) 52 (yeast) 55 (rat liver)	Cleavage of NH_2-terminal presequences of imported precursors	46 47, 48 49
PEP (MIF1/MAS1) (Processing enhancing protein)	52 (*N. crassa*) 48 (yeast) 52 (rat liver)	Cooperation in presequence cleavage with MPP	46 47, 48 49
Inner membrane			
IMP1 (Inner membrane protease 1)		Cleavage of the export signal of the precursors of cytochrome b_2 and of cytochrome oxidase subunit II	56, 57
Intermembrane space			
CCHL (Cytochrome c heme lyase)	38 (*N. crassa*) 32 (yeast)	Covalent attachment of heme; translocation of apocytochrome c	60, 61 62, 63

mitochondria without addition of cytosolic components[9,12,13]. This, however, does not exclude the participation of cytosolic factors *in vivo*. Presequence binding proteins that enhance import of purified precursors have been described[14,15]. So far, the role of such proteins in the intact cell remains to be demonstrated. Several lines of evidence, on the other hand, have strongly suggested an important function for cytosolic heat shock proteins (ct-hsps) in protein import, at least in yeast cells. A mutant defective in three of the four genes for cytosolic hsp70s (ssa1-4) showed a reduced import into mitochondria accompanied by the accumulation in the cytosol of precursors to ATPase subunit β ($F_1\beta$) and Rieske Fe/S protein (Fe/S)[16]. On the other hand, cytosolic hsp70s stimulated protein transport into mitochondria *in vitro*[17]. Since not only intracellular transport of mitochondrial proteins was affected, but also transport into the endoplasmic reticulum, ct-hsp70s appear to have a general role in intracellular protein traffic[18].

Heat shock proteins of the hsp70 class are known to interact with proteins that are not in their native conformation[19]. Which structural elements, hydrophobic regions or extended sequences, are recognized are unknown. In any case, such elements seem to be abundantly exposed in newly synthesized proteins. Possible functions of hsp70 binding are: (i) maintenance of a loosely folded structure of precursor proteins to support unfolding during translocation, (ii) prevention of aggregation of precursors due to exposure of hydrophobic surfaces, and (iii) minimization of misfolding of precursors that would render them incompetent for initiation of translocation, e.g. by preventing exposure of the presequence. Hsp70s are known to exert their function in a reaction involving ATP hydrolysis[20,21]. Indeed, import of most precursors is dependent on ATP in the cytosol. Lack of dependence on cytosolic ATP was found with precursors that had been unfolded by treatment with high urea concentrations and with artificial precursors carrying "cytosolic passenger proteins" that were folded into the native ("mature") conformation[7]. In the latter case there is apparently no binding of hsps and therefore no need for ATP hydrolysis to trigger their release[9]. As will be discussed, ATP is also required at later steps of the import pathway in the mitochondrial matrix.

Recognition and Initial Insertion of Precursors

Analysis of import of precursors into isolated mitochondria led to dissection of the overall process into a series of intermediate steps. This analysis suggested the existence of proteinaceous binding sites on the surface of mitochondria and of a further component in the outer membrane which is required for insertion. Transfer of precursors to this latter component from the binding sites results in a partial insertion at least into the outer membrane. Functional studies indicated the existence of multiple surface receptors for precursor proteins and a common insertion site, termed "general insertion protein" (GIP). Precursors could be halted at the level of binding to receptors by lowering concentration of ATP (probably due to prevention of the release of cytosolic factors)[22]. Arrest of precursors at the level of GIP was achieved by dissipating the electrical membrane potential ($\Delta\Psi$) (see Fig. 2).

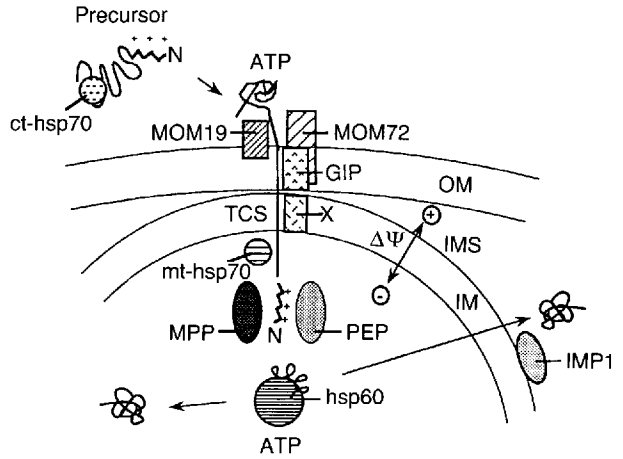

Fig. 2. Model of the general import pathway. The import pathway of a precursor carrying a positively-charged targeting sequence from the cytosol via contact sites into the matrix space of mitochondria is shown (see text for details). OM, outer membrane; TCS, translocation contact sites; IMS, intermembrane space; IM, inner membrane; $\Delta\Psi$, membrane potential across the inner membrane; ct-hsp70, cytosolic heat shock protein of 70 kDa; MOM19, mitochondrial outer membrane protein of 19 kDa in *N. crassa* with receptor function; MOM72, mitochondrial outer membrane protein of 72 kDa in *N. crassa* with receptor function; GIP, general insertion protein; X, unidentified component(s) of contact sites in the inner membrane; mt-hSP70, mitochondrial heat shock protein of 70 kDa; MPP, mitochondrial processing peptidase; PEP, processing enhancing protein; hsp60, heat shock protein of 60 kDa; IMP1, inner membrane protease 1.

So far, two proteins of the outer mitochondrial membrane have been identified as receptors or parts of a receptor complex. Antibodies were raised against about 20 individual outer membrane proteins and tested for their ability to interfere with binding, insertion and import. The mitochondrial outer membrane protein MOM19 (apparent molecular mass 19 kDa) was found to be involved in the import of a large number of precursor proteins that are sorted to the outer membrane, inner membrane, matrix and intermembrane space[23].

MOM72 was detected as a receptor mainly for the ADP/ATP carrier protein (AAC) of the inner membrane[24]. Both MOM19 and MOM72 have putative membrane anchors close to the amino-terminus. Topological studies confirmed that both proteins expose a large hydrophilic domain to the cytosol. MOM72 was initially detected in *Neurospora*, and a homologous protein was also found in yeast[24,25].

Removal of MOM72 by limited proteolysis or its blockage with antibodies in isolated mitochondria led to a substantial reduction, but not complete impairment, of AAC import. Deletion of the gene for MOM72 in yeast resulted in a reduction of the rate of import; however, the mutant cells were still capable of accumulating nearly normal amounts of AAC. Import of some other precursors was also found to be reduced[25,26]. Thus, the AAC precursor appears to be "promiscuous" in its use of receptors. It can be recognized both by MOM72 and MOM19. Direct interaction of precursor proteins with MOM72 and MOM19 was demonstrated by co-immunoprecipitation of MOM72 with bound precursor or crosslinking of precursor with MOM19[27,28].

A 32 kDa protein of yeast mitochondria (P32) was identified as a putative protein import receptor by employing anti-idiotypic antibodies that were raised against a synthetic mitochondrial presequence[29]. Surprisingly the primary sequence of P32 was found to be identical to that of the yeast phosphate carrier[30].

Immunoprecipitation from digitonin extracts of mitochondria using antibodies against MOM19 and MOM72 led to the discovery of a receptor complex in *Neurospora* that is comprised of MOM19, MOM72 and two further outer membrane components MOM38 and MOM22. MOM38 was analysed in more detail. It is tightly integrated into the outer membrane, although its overall character as judged from the primary sequence is rather hydrophilic. According to all available criteria, MOM38 is the equivalent of GIP[27]. An independent approach led to the identification of this component in the import machinery in yeast. A translocation intermediate spanning contact sites was crosslinked using a bifunctional reagent to a 42 kDa component of the outer membrane[31]. This yeast ISP42 (for "import site protein" of 42 kDa) shows 67% sequence similarity (including isofunctional replacements) to *Neurospora* MOM38[27,32]. MOM38/GIP and ISP42 are obviously equivalent proteins, since *Neurospora* MOM19 and MOM72 imported into yeast assemble with the 42 kDa protein. At the same time these data show that precursors remain associated with GIP even when translocation across the inner membrane has commenced. Precisely how precursors interact with receptors, and how they are transferred to GIP, remains unclear. Since the many hundreds of different precursors differ widely in primary structure of their targeting signals, recognition of secondary structure motifs rather than recognition of specific amino acid side chains may prevail.

Translocation Contact Sites

In previous studies, zones of close contact between outer and inner mitochondrial membranes were characterized by electron microscopy. They were initially proposed to be involved in protein import, since cytosolic polysomes were observed tightly attached to the outer membrane[33]. The functional analysis of translocation through mitochondrial contact sites was greatly advanced by reversibly arresting precursor proteins in transit across the two membranes. Completion of translocation was blocked by inducing tight folding of carboxyl-terminal regions of precursors. This caused accumulation of precursors in sites of close contact between outer and inner membranes; their amino-terminal presequences were extended far enough into the matrix to be proteolytically processed by the matrix-localized mitochondrial enzyme[8,34,35]. By immuno electron microscopy it was shown that the accumulated precursors were localized preferentially to sites of close contact of the two mitochondrial membranes[36]. In a recent study, the minimal length of the membrane-spanning portion of a precursor protein in transit was determined to be as short as 50 amino acids, implying a rather extended conformation[10]. It was concluded that precursor proteins undergo extensive unfolding upon translocation across contact sites.

Translocation contact sites occur in limited number. The amount of spanning intermediates required for saturation was estimated to be roughly 40-70 pmol/mg mitochondrial protein, corresponding to a few thousand polypeptide chains per single mitochondrion[35,37]. In competition experiments using intact cells, different precursor proteins were found to share the same translocation machinery. This observation demonstrates the general function of contact sites as entry gates into mitochondria[38]. Accumulation of large amounts of spanning intermediates in mitochondrial contact sites *in vitro* and *in vivo* did not cause de-energization of the mitochondria[35,38]. It was suggested that components of the contact sites (probably proteins) seal the membrane around amino acid side groups of spanning polypeptide chains and thereby prevent leakage of small ions. The initial insertion of precursors into the inner membrane is dependent on $\Delta\Psi$; once the targeting peptide has permeated the inner membrane, the remaining part of the protein follows in a fashion independent of $\Delta\Psi$[8,39]. It was proposed that $\Delta\Psi$ has an electrophoretic effect or that $\Delta\Psi$-dependent opening of a channel supports transfer of presequences.

Contact sites were found to be rather stable structures that survive even lengthy manipulations (e.g. subfractionation) of the mitochondria[36,40]. Precursor proteins spanning both mitochondrial membranes can be extracted with aqueous perturbants, perhaps indicating the existence of a hydrophilic channel[39]. Proteinaceous components of such a channel are yet unidentified.

Components in the Matrix Required for Translocation

The identification of components involved in translocation across mitochondrial membranes was possible by analysing conditional lethal mutations in yeast that result in the accumulation of mitochondrial precursors

in the cytosol. Transfer of precursors into mitochondria was found to be defective in yeast cells which have an altered mt-hsp70. Isolated mitochondria of this mutant also showed a deficiency in import, and precursor proteins were found to be arrested in translocation contact sites. As the import defect could be overcome by urea denaturation of precursors prior to import, it was argued that mt-hsp70 is needed for unfolding of the entire polypeptide chain and complete translocation of precursors after their insertion into mitochondrial contact sites[41]. A tight interaction between mt-hsp70 and a precursor arrested in translocation contact sites was recently found upon co-immunoprecipitation of detergent-lysed mitochondria, as well as by crosslinking studies[41-43]. Furthermore, it was found that precursors imported after urea denaturation remained in an unfolded state in tight association with the mutated mt-hsp70[41]. Apparently mt-hsp70 has a dual function and is also involved in folding of newly imported proteins.

Intermediates associated with mt-hsp70 are believed to be transferred to hsp60. Hsp60 is a tetradecameric complex that belongs to the chaperonin class of stress proteins. ATP-dependent folding and oligomeric assembly of imported proteins catalysed by hsp60 are essential for the viability of yeast cells, but are not directly involved in protein translocation[44,45].

Once translocated across mitochondrial membranes, the amino-terminal presequences of precursors are cleaved off by a highly specific metal-dependent endoprotease in the matrix[11]. Presequence cleavage was found to be an essential function in yeast. Proteolytic processing is not coupled to membrane translocation, but is required for proper assembly of the imported proteins. Cleavage is carried out by the cooperative action of the two components of the matrix-processing peptidase that are structurally related but carry out different functions[46-49]. The catalytic component is the mitochondrial processing peptidase (MPP), which is sensitive to sulfhydryl-modifying reagents, requires divalent metal ions such as Mn^{2+} or Zn^{2+} for activity and appears to belong to a new class of proteases. There is no apparent consensus sequence at the cleavage site of the various precursors, indicating that secondary motifs rather than recognition of amino acid side chains may confer the high specificity of cleavage[2].

The processing enhancing protein (PEP) strongly enhances the catalytic activity of MPP. MPP and PEP, together with the core 1 and core 2 proteins of the respiratory complex III, form a new protein family[50]. It was proposed that PEP interacts with the incoming precursor and thereby exposes the cleavage site to MPP. The precise interaction between precursors, PEP and MPP remains to be elucidated.

Components Involved in Protein Sorting to the Intermembrane Space

Transport of a number of proteins to the inner membrane and to the intermembrane space occurs by translocation first into the matrix[51,52]. Then they enter an export pathway that appears to be functionally and evolutionarily related to protein secretion into the periplasmic space in bacteria[4]. This pathway was therefore termed "conservative sorting". The precursor forms of these proteins carry bipartite amino-terminal targeting sequences that are cleaved in two steps. MPP/PEP produces an intermediate-sized species that still contains the carboxyl-terminal part of the original presequence, the hydrophobic export signal. These sequences have the structural features of the leader or signal sequences of secretory proteins; they are proteolytically removed by a membrane bound peptidase equivalent to the bacterial leader peptidase[51].

Re-export from the matrix to the intermembrane space was found to be defective in yeast cells that have an altered hsp60 in the mitochondrial matrix[45]. Hsp60 is homologous to *E.coli* groEL[53], which has also been described as having chaperonin function in bacterial protein export[54]. Furthermore, transport to the intermembrane space could not be completed at reduced levels of ATP. As the release of proteins bound to hsp60 requires ATP hydrolysis, it was argued that export occurs via binding to hsp60. The physical interaction between an intermediate-sized intermembrane space protein and hsp60 could be directly demonstrated. Proteins bound to hsp60 were found to be in an incompletely folded conformation, indicating that export occurs from a loosely folded state which is preserved by the interaction with hsp60. The presence of the hydrophobic export signal appears to stabilize the binding to hsp60 and thereby an export-competent conformation of the imported intermembrane space proteins in the matrix[55].

In a recent study, translocation intermediates that span three membranes could be arrested. Retranslocation to the intermembrane space had already commenced when a tightly folded carboxyl-terminal domain of the precursor protein was still in the cytosol. Thus the sites of protein import into the matrix and of export into the intermembrane space may be both located at zones of close contact between the mitochondrial membranes[55].

Once translocated across the inner membrane to the intermembrane space, the hydrophobic export signal is removed by membrane bound peptidases. Recently, a yeast mutant was identified in which the activity of one of these proteases termed inner membrane protease 1 (IMP1) appeared to be temperature sensitive, resulting in the accumulation of intermediate-sized cytochrome b_2 and of the precursor form of the mitochondrial-encoded subunit II of cytochrome oxidase[56]. IMP1 was found to have regions of similarity to the leader peptidase of *E.coli*[57]. The two step-processing of other intermembrane space proteins, e.g. cytochrome c_1, was found not to be affected in this mutant, indicating the existence of at least two proteases involved in

proteolytic cleavage of proteins in the intermembrane space.

An entirely different sorting pathway to the intermembrane space is followed by holocytochrome c. This protein is transported to the intermembrane space across the outer membrane directly from the cytosol, without using the receptor/GIP system. The precursor of cytochrome c (apocytochrome c) is believed to have an intrinsic activity for spontaneous insertion into the outer membrane[58,59]. Once translocated across the outer membrane, heme is covalently bound to the amino-terminal part of the precursor by the cytochrome c heme lyase (CCHL). Thereby apocytochrome c is converted to the mature form, holocytochrome c. Completion of translocation is tightly coupled to heme addition[60-63].

Perspectives

Recent progress in the field of mitochondrial protein translocation led to the identification and characterization of several of the components of the import apparatus: presequence binding factor, 70 kDa heat shock protein in the cytosol, two receptors on the mitochondrial surface, outer membrane proteins of 22 kDa and of 42 kDa, mitochondrial processing peptidase, processing enhancing protein, 70 kDa and 60 kDa heat shock proteins in the matrix, inner membrane protease 1 in the inner membrane, as well as cytochrome c heme lyase. Future studies will focus on the isolation and characterization of further components of the import machinery including additional cytosolic factors, components of the translocation contact sites, factors that cooperate with the heat shock proteins of 70 kDa and 60 kDa in the matrix, components involved in translocation across the inner mitochondrial membrane and several enzymes for the modification and processing of precursor proteins. The reconstitution of the whole chain of reactions leading to translocation and assembly of mitochondrial precursor proteins will require much further work.

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