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Topical Review

Mitochondrial Protein Import: Specific Recognition and Membrane Translocation of Preproteins

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1. Introduction

Mitochondria are double-membrane bounded, energy-converting organelles within eukaryotic cells. They synthesize ATP by oxidative phosphorylation. Important progress in understanding their function was made when an isolation procedure for intact mitochondria (Hogeboom, Schneider & Palade, 1948) was developed. Time-lapse microcinematography (Johnson, Walsh & Chen, 1980; Tzagoloff, 1982) revealed that these organelles are remarkably mobile and able to constantly change their shape and are continuously dividing and fusing. Their motility is apparently due to an association with the cytoskeleton which may determine the unique orientation and distribution of mitochondria in various cell types.

Mitochondria are not synthesized de novo; they arise by growth and division of pre-existing organelles (Luck, 1963, 1965). Mitochondria contain DNA (Schatz, Haslbrunner & Tuppy, 1964) and a complete system to carry out DNA replication, transcription, and protein synthesis (McLean et al., 1958; Tzagoloff, 1982); however, only a small number of proteins are encoded by mitochondrial DNA, namely a few subunits of the respiratory chain and the ATP synthase, and depending on the organism, one or a few proteins required for the expression of mitochondrial genes. All other mitochondrial proteins are encoded by nuclear genes, and thus have to be imported into the mitochondria and sorted to the four subcompartments: the outer and the inner membranes, the intermembrane space and the matrix (Attardi & Schatz, 1988; Hartl et al., 1989).

Precursor forms of mitochondrial nuclear encoded proteins contain targeting and sorting signals that are instrumental for directing them to these various subcompartments. Targeting signals can either be present as cleavable amino-terminal signal sequences or signals present within the mature parts of precursor proteins. For all those precursors destined for the outer membrane and many of the inner membrane, the signals are not cleaved but are present in the mature protein sequence. Submitochondrial sorting signals are often found in amino-terminal presequences containing a stretch of hydrophobic amino acids of variable length.

On the other hand, mitochondria contain components which recognize mitochondrial targeting signals in a specific manner and facilitate the translocation of the precursors across the mitochondrial membranes. Thus, targeting of newly synthesized proteins to mitochondria seems to follow a general principle of intracellular protein traffic, that is the presence of signal sequences on the proteins to be transported across membranes and the existence of complementary structures on the surface of organelles which recognize the preproteins belonging to the respective cellular subcompartment (Milstein et al., 1972; Blobel & Dobberstein, 1975).

We will first summarize the principal pathways of mitochondrial preproteins from the cytosol to their final destination where they become assembled into functional complexes (Figs. 1 and 2) and then we will focus on specific aspects of recognition and transmembrane transfer of precursors.

Precursor proteins are stabilized in the cytosol

Key words: Mitochondrial receptor complex—General insertion pore (GIP)—ATP-dependent translocation—MOM22—Topology of the components of the receptor complex

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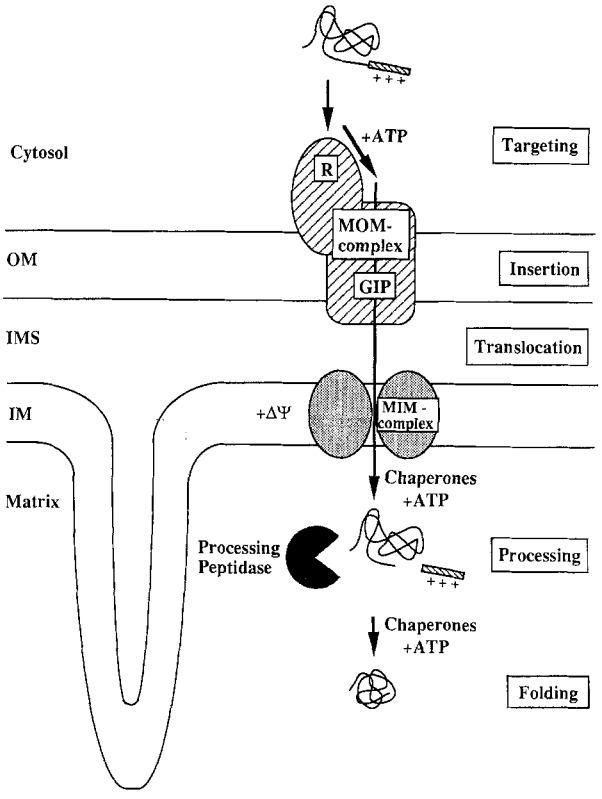


Fig. 1. Schematic overview on import of preproteins into mitochondria. Precursor proteins are recognized by the mitochondrial import receptors (*R*) and subsequently inserted into the outer membrane (*OM*) by the general insertion pore (*GIP*). Then precursors are transferred across the intermembrane space (*IMS*) to the translocation complex (*MIM*) of the inner membrane (*IM*) and enter the matrix.

in an import-competent, unfolded state by heat-shock proteins of the hsp70 class in an ATP-dependent process (for review, *see* Rothman, 1989). Other factors interacting with the presequences appear to be also important. The presequences are recognized by receptors on the surface of the outer membrane. Precursor proteins then become inserted into a transport pore in the outer membrane (Pfanner et al., 1987*a*). Since competition studies revealed that many precursor proteins use the same pore for insertion into the outer membrane, this was termed *general insertion pore* GIP (Pfaller et al., 1988; Pfanner, Hartl & Neupert, 1988).

Precursor proteins destined for the inner membrane, for the matrix and some precursors destined for the intermembrane space, have to be translocated through or inserted into the inner membrane. This occurs at translocation contact sites where the outer and the inner membrane are in close contact (Pfanner et al., 1992). *In vivo,* more than 90% of the outer membrane appears to be in close contact with a specific part of the inner membrane, termed inner boundary membrane (van der Klei, Veenhuis & Neupert, 1992).

Translocation of preproteins across the mitochondrial inner membrane is dependent on a membrane potential $\Delta\Psi$ across the inner membrane and on ATP in the matrix. The precursor proteins in transit interact with mitochondrial heat-shock proteins of the hsp70 class (mt-hsp70) in the matrix in an ATP-dependent manner (Kang et al., 1990). It is assumed that binding and subsequent ATP-dependent release of hsp70 from the precursor provides at least part of the driving force for the translocation of preproteins through the two mitochondrial membranes. The mitochondrial processing peptidase consisting of the two related components α-MPP and β-MPP removes the presequences (Böhni et al., 1980; Yaffe & Schatz, 1984; Hawlitschek et al., 1988; Jensen & Yaffe, 1988; Witte et al., 1988; Pollock et al., 1988; Schulte et al., 1989). Proteins finally located in the matrix are transferred from mt-hsp70 to the chaperonin hsp60 (Manning-Krieg, Scherer & Schatz, 1991), the mitochondrial homologue of the *Escherichia coli* GroEL, which mediates ATP-dependent folding and assembly (Cheng et al., 1989; Ostermann et al., 1989; Cheng, Hartl & Horwich, 1990). Hsp70 and hsp60 may act in concert with some helper proteins such as the recently discovered hsp10 (the GroES homologue; Hartman et al., 1990, 1992, 1993; Lubben et al., 1990) or not yet identified homologues of *E. coli* DnaJ and GrpE.

A number of mitochondrial precursors are known to become processed in a two-step mechanism. With the precursor of F_0-ATPase subunit 9, the second cleavage is also performed by MPP (Schmidt et al., 1984). For several other precursors, cleavage by MPP is followed by the removal of an octapeptide by an octapeptidyl-peptidase or mitochondrial intermediate processing peptidase (MIP) which is located in the matrix (Kalousek, Hendrick & Rosenberg, 1988; Hendrick, Hodges & Rosenberg, 1989; Isaya et al., 1991; Isaya, Kalousek & Rosenberg, 1992*a,b*; Kalousek, Isaya & Rosenberg, 1992). The sorting of some preproteins to the intermembrane space involves another proteolytic step in addition to the one catalyzed by MPP. This processing is performed in the intermembrane space by the membrane-bound protease termed *inner membrane peptidase 1* (IMP1, van Loon, Brändli & Schatz, 1986; Hartl et al., 1987; Behrens, Michaelis & Pratje, 1991; A. Schneider et al., 1991). Interestingly, this protease shares sequence similarity with the *E. coli* leader peptidase supporting the endosymbiontic theory. Some of the intermembrane space precursor proteins contain a bipartite signal at their amino-terminus (Gasser et al., 1982; Kaput, Goltz

& Blobel, 1982; Teintze et al., 1982; Hurt & van Loon, 1986; Hartl et al., 1989). This signal initially consists of a positively charged matrix-targeting sequence, followed by a hydrophobic sorting signal which bears some resemblance to bacterial leader sequences (Randall & Hardy, 1989). The sorting of these precursor proteins destined for the intermembrane space can be explained in different ways. According to the "conservative sorting" model (Hartl et al., 1987; Koll et al., 1992; Schwarz et al., 1993) the preproteins are imported across both membranes into the matrix and are simultaneously or subsequently exported across the inner membrane directed by the hydrophobic sorting signal acting as an export signal. In the alternative "stop-transfer" model (van Loon & Schatz, 1987; Glick et al., 1992) the hydrophobic part of the bipartite presequence acts as an anchor which arrests preproteins upon import through the inner membrane, subsequent sorting occurs by a selective translocation across the outer membrane into the intermembrane space.

In this review, we wish to discuss the recent progress which has been made in understanding the mechanisms of how the specific recognition and membrane translocation of preproteins occur. We will focus on the identification and functional characterization of the components of the mitochondrial receptor complex and its dynamic behavior. In addition, the role of the membrane potential $\Delta\Psi$ and hsp70 for translocation of preproteins across the inner membrane will be discussed in detail.

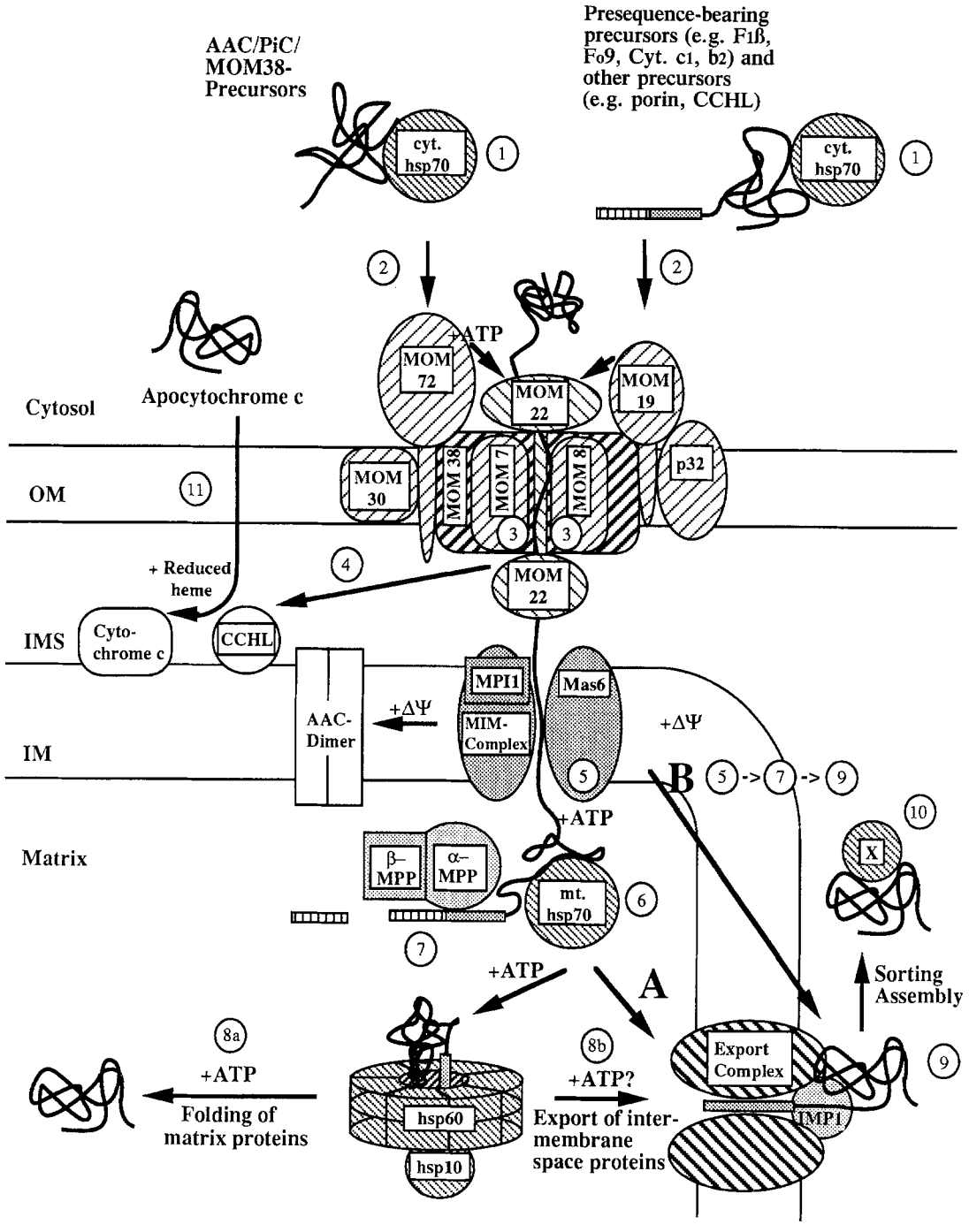
2. Mitochondrial Preproteins are Recognized by Receptors on the Mitochondrial Surface

Proteins destined to mitochondria carry targeting sequences which are recognized by the mitochondrial protein import apparatus. In most cases, the targeting sequence consists of an amino-terminal presequence which is cleaved off upon arrival of the protein in the mitochondrial matrix. Mitochondrial presequences are characterized by an abundance of positive charges and frequently contain a prevalence of hydroxylated amino acids (von Heijne, 1986). Additionally, they have a tendency to form amphipathic helices in a hydrophobic environment. Internal uncleaved targeting sequences (Pfanner et al., 1987*b*; Smagula & Douglas, 1988; Mahlke et al., 1990) have not been characterized so far. In particular, proteins of the outer membrane and also many proteins of the inner membrane are targeted to their final destination by internal signals.

How are the precursor proteins recognized by the mitochondrial import apparatus? For a long time, proteinaceous import receptors have been postulated to exist in the mitochondrial outer membrane (Zwisinski, Schleyer & Neupert, 1983, 1984). In mitochondria of *Neurospora crassa* two outer membrane proteins have now been identified as import receptors: MOM19 and MOM72. Antibodies to MOM19 inhibited import of most mitochondrial precursor proteins studied so far (Söllner et al., 1989). Furthermore, a fusion protein containing the amino-terminal moiety of cytochrome b_2 when arrested during translocation could be coimmunoprecipitated with antibodies to MOM19 (Kiebler et al., 1990). These results suggest a direct interaction between precursors and the receptor proteins.

The precursor of the ADP/ATP carrier (AAC), which contains internal targeting sequences (Pfanner et al., 1987*b*; Mahlke et al., 1990) was shown to primarily use MOM72 as a receptor (Söllner et al., 1990; Hines et al., 1990). A complex between the AAC and MOM72 could be immunoprecipitated using antibodies to MOM72. When the AAC was arrested at the surface of the mitochondria and prevented from getting further imported by reducing the level of cytosolic ATP and by dissipating the membrane potential, it could be crosslinked to MOM72 and to a minor extent to MOM19, suggesting that a direct interaction was taking place (Söller et al., 1992). The interaction of the AAC with MOM19 was observed *in vitro,* albeit with a lower efficiency (Steger et al., 1990). When the equivalent of *Neurospora* MOM72 in yeast, MAS70, was disrupted, the AAC was still targeted to mitochondria supporting the view that both MOM72 and MOM19 can function as a receptor for the AAC. The nature of the specificity of this receptor is not entirely clear, yet. In *N. crassa,* the AAC and to a lower extent cytochrome c_1 use MOM72 as receptor, whereas in *Saccharomyces cerevisiae* also $F_1\beta$ was reported to bind to Mas70 (Hines et al., 1990). Recent data using urea-denatured precursor proteins even suggested that Mas70 is the import receptor for most, if not all, authentic mitochondrial precursor proteins (Hines & Schatz, 1993). Future studies have to show which precursor proteins can actually bind to the purified receptors.

The role of the presequences in recognition by the mitochondrial receptor complex was addressed in experiments using a fusion protein containing the matrix-targeting sequence of cytochrome b_2 fused to dihydrofolate reductase (DHFR). DHFR alone was not imported into mitochondria unless a mitochondrial targeting sequence was fused to it. Import of this fusion protein was inhibited when mitochondria were pretreated with antibodies against MOM19 (Becker et al., 1992). These results suggest that the presequence alone is sufficient for translocation into



A: Conservative sorting of inner membrane (IM) and intermembrane space (IMS) precursors

B: Sorting of IM and IMS precursors according to the stop-transfer model

Components belonging to the same class in the translocation apparatus are represented by the same symbols:

- Members of the heat-shock family
- Receptor-GIP-complex in the mitochondrial outer membrane
- Mitochondrial inner membrane translocation complex
- Processing peptidases:
  - MPP: mitochondrial processing peptidase; subunits α and ß
  - IMP1: Inner membrane protease 1
- Export complex in the mitochondrial inner membrane

Import step	Inhibition
(1) Stabilization of precursors in an import-competent state by cytosolic hsp70 and other factors	Depletion of factors Mutants: *ssa1-4*
(2) Binding of precursor proteins to import receptors (ATP-dependent in the case of MOM72)	Blocking of the receptors by antibodies Removal of receptors by proteases Mutant: *Δmom72 , mir1*
(3) Release of hsp70 and insertion into the outer membrane at the GIP-site	Removal of cytosolic ATP or low temperature Mutant: *isp42*
(4) Folding and assembly of intermembrane space proteins without cleavable presequences	
(5) Membrane potential-dependent insertion into the inner membrane	Dissipation of $\Delta\Psi$
(6) ATP-dependent interaction with the mitochondrial hsp70 (Ssc1p) and completion of translocation	ATP-depletion of the matrix Low temperature Mutant: *ssc1/2*
(7) Cleavage of the matrix-targeting sequence by α– and ß-MPP	Inhibition of α and ß-MPP by metal chelators Mutant: *mif1/mas1 mif2/mas2*
(8a) ATP-dependent interaction with and folding of matrix proteins on hsp60 and helper proteins	Partial depletion of matrix ATP Mutant: *mif4*
(8b) Translocation of precursors with a bipartite presequence from the matrix into and across the inner membrane	
(5) -> (7) -> (9) Sorting of innermembrane and intermembrane space precursors according to the "stop-transfer" model	
(9) Second proteolytic cleavage of the bipartite presequences by IMP1	Mutant: *imp1*
(10) Interaction with a putative intermembrane space chaperone	Mutant: *sco1* ?
(11) Import of cytochrome c independent of the receptor complex and the membrane potential	Mutants: *cyt2-1; cyc3* (CCHL mutants)

Fig. 2. Working model of mitochondrial protein import and strategies for arresting precursor proteins at distinct steps of its pathways into mitochondria. The following abbreviations are used: AAC, ADP/ATP carrier; PiC, phosphate carrier; F1β, β-subunit of the F_1-ATPase; Fo9, subunit 9 of the Fo-ATPase; cyt. hsp70, cytosolic hsp70 (SSA1-4gene products); p32, import receptor in yeast, gene product of MIR1 (Murakami et al., 1990; Pain et al., 1990); CCHL, cytochrome c heme lyase; MIM complex, mitochondrial inner membrane translocation complex; MPI1, Mitochondrial protein import component 1 (Maarse et al., 1992; Scherer et al., 1992); Mas6, yeast inner membrane protein, required for protein import, essential for viability (R. Jensen et al., 1993); $\Delta\Psi$, membrane potential; α- and β-MPP, subunits α and β of the mitochondrial processing peptidase (*mif1/2* or *mas1/2*); mt. hsp70, mitochondrial hsp70 (SSC1 gene product; IMP1, inner membrane protease 1; Sco1p, yeast protein involved in assembly of cytochrome oxidase (Schulze & Rödel, 1988); IC, intermembrane space chaperone (hypothetical). For details, *see text.*

mitochondria and, in particular, for specific recognition of the precursor by MOM19.

In yeast, a mitochondrial integral membrane protein (p32) has been reported to function as a putative import receptor (Pain, Murakami & Blobel, 1990). Anti-idiotypic antibodies, mimicking a chemically synthesized signal peptide of the precursor of subunit IV of cytochrome c oxidase (pCOXIV) recognized this protein. Fab fragments against the purified p32 inhibited import of precursor proteins into mitochondria, indicating that the protein is accessible from outside in intact mitochondria. In addition,

p32 was localized to the outer membrane at contact sites by immunoelectron microscopy (Pain et al., 1990). When p32 was disrupted in yeast, haploid cells were unable to grow in nonfermentable carbon sources indicating that p32 is important but not essential for mitochondrial function and biogenesis. The mutant mitochondria showed a diminished import efficiency for precursor proteins. Unexpectedly, the primary sequence of p32 (Murakami, Blobel & Pain, 1990) was identical to a protein identified as the mitochondrial phosphate carrier, an inner membrane protein (Guérin et al., 1990; Phelps, Schobert & Wohlrab, 1991). More recently, it was shown that purified p32 binds to mitochondrial presequences, but not to signal sequences required for protein translocation into chloroplasts or endoplasmatic reticulum (Murakami, Blobel & Pain, 1993). It remains to be investigated whether p32 is exclusively located in the inner membrane or if it has a dual localization in both the inner and the outer membrane and also, if p32 functions as a signal-sequence binding subunit of a protein-conducting channel in the outer membrane or as a phosphate translocator in the inner membrane.

To identify putative signal-sequence receptors, synthetic peptides derived from presequences of precursor proteins were used. These were either radiolabeled and cross-linked to mitochondrial proteins or used as ligands on an affinity chromatography column. By these procedures, several presequence-binding proteins were identified. In one report, a 28-kD protein of mitochondria from rabbit heart, rat liver, bovine adrenal cortex and *S. pombe* could be crosslinked (Font et al., 1991). This protein was suggested to be located at the outer side of the inner membrane. In other reports, two proteins of 29 and 52 kD could be purified from rat liver mitochondria, and antibodies raised against these proteins inhibited import of precursor proteins into mitochondria (Ono & Tuboi, 1990*a*, 1991). Future studies have to show what the precise role of these signal sequence receptors is in protein translocation across the mitochondrial membranes.

Mitochondrial import receptors can be proteolytically removed from the surface of mitochondria by treatment with proteases. Import into these mitochondria could still occur albeit with low efficiency (Pfaller, Pfanner & Neupert, 1989). This "bypass import" showed the characteristics of authentic mitochondrial protein import except for receptor specificity. Import of a chloroplast preprotein into mitochondria, which occurred with a low efficiency in intact mitochondria, was not inhibited by trypsinizing mitochondria prior to import. These results suggested that the function of the mitochondrial protein import receptors may be explained by conferring specificity and increasing the efficiency of the process.

An example of a protein that enters the mitochondrial import pathway at a post-receptor stage is the precursor to subunit Va of the yeast cytochrome oxidase (Miller & Cumsky, 1991). Its import occurs independently of protease-sensitive receptors in the outer membrane. It is not known with which component it interacts first, but it has been claimed that the protein enters at the stage of GIP. COXVa further proceeds into the mitochondrial matrix at the translocation contact sites. It will be interesting to see which property enables COXVa to bypass the protease-sensitive receptors.

The movement of the AAC from a receptor-bound stage to the GIP requires ATP (Pfanner & Neupert, 1986; 1987). A recent study provided some information on the molecular nature of this ATP requirement by using the ATP analogue 8-azido-ATP which can be photocrosslinked to ATP-binding proteins. Interestingly, irradiation of mitochondria in the presence of 8-azido-ATP resulted in a strongly reduced insertion of the AAC into the outer membrane (M. Kiebler et al., *submitted for publication*). The major target of ATP photocrosslinking was MOM72, whereas the other components of the receptor complex were not labeled. Under the conditions used, mitochondrial hsp70 was not labeled, excluding that interference with hsp70 caused the inhibition of the import of AAC. These results suggest that MOM72 functions in an ATP-dependent manner with a possible role for ATP-hydrolysis in the release of the preprotein or the recycling of the receptor.

An interesting question is whether receptors can bind presequences directly or whether additional cytosolic factors are necessary for mediating this binding reaction. If so, this would resemble the situation in the endoplasmic reticulum (ER), where the signal recognition particle (SRP) targets the precursor proteins to the ER membrane (Walter, Gilmore & Blobel, 1984). In the ER, the precursors are not directly recognized by the import receptors, but instead, the SRP is recognized by its cognate receptor and transfers the bound protein to the translocation channel in the ER membrane (Ogg et al., 1992).

Several studies have suggested a requirement of cytosolic cofactors in mitochondrial protein import (*see* Becker et al., 1992 for review). Two possible major functions for cofactors were proposed: (i) a chaperone-like function for preventing misfolding and preserving a transport-competent conformation of preproteins (Ohta & Schatz, 1984; Pelham, 1986; Chen & Douglas, 1987*b*; Chirico, Waters & Blobel, 1988; Deshaies et al., 1988; H. Murakami et al., 1988; Rothman, 1989; Neupert et al., 1990; Murakami &

Mori, 1990; Sheffield, Shore & Randall, 1990) and (ii) a direct involvement in the targeting of precursors. The first function is apparently fulfilled by cytosolic heat-shock proteins (e.g., hsp70), which bind to preproteins and keep them in an import-competent conformation (H. Murakami et al., 1988; Hartl, Martin & Neupert, 1992).

A role of putative presequence-binding factors in targeting mitochondrial precursor proteins to mitochondria has been repeatedly suggested (K. Murakami et al., 1988; Ono & Tuboi, 1988; Murakami & Mori, 1990). These factors were identified by fractionating rabbit reticulocyte lysate and analyzing the fractions with regard to their ability to confer import competence to a precursor protein. A 50-kD protein called PBF (presequence binding factor) was found to be required for the import of precursors with a cleavable presequence into rat liver mitochondria. PBF was isolated due to its ability to form a complex with a purified mitochondrial precursor protein, namely pOTC (pre-ornithine transcarbamylase). This binding was inhibited by the corresponding prepeptide, suggesting that the factor indeed binds to the presequence (K. Murakami et al., 1988; Murakami & Mori, 1990; Murakami et al., 1992). A 28-kD protein which stimulated import into mitochondria of a peptide corresponding to the presequence of pOTC was purified from rabbit reticulocyte lysate (Ono & Tuboi, 1990*b*). This protein was also required for the binding of the presequence peptide to liposomes constituted of lecithin and the partially purified mitochondrial import receptor (Ono & Tuboi, 1988, 1990*a*). Another cytosolic factor, mitocondrial import stimulating factor (MSF), was reported to enhance the import of the precursor to the matrix-localized protein adrenodoxin. The protein is a heterodimer of 30 and 32 kD subunits. It may promote depolymerization of the oligomeric precursor and unfolding in an ATP-dependent manner (Hachiya et al., 1993).

To investigate whether cytosolic factors are essential for import or whether their function can be circumvented, a fusion protein was overexpressed in *E. coli* and purified to apparent homogeneity that consisted of the amino-terminal part of cytochrome b_2 fused to $F_1\beta$ (Becker et al., 1992). The purified protein was denatured in urea and therefore its import was independent of cytosolic hsp70 (Eilers & Schatz, 1986; Pfanner et al., 1990). This protein was added to mitochondria which had been extensively washed in buffer containing high salt to remove any attached cytosolic factors. Import of the purified protein into these mitochondria was independent of the addition of cytosolic factors and could not be further stimulated by the addition of reticulocyte lysate. The import of the fusion protein was inhibited by antibodies to MOM19 and showed all other characteristics of the authentic mitochondrial protein import. Therefore, it was concluded that protein targeting to mitochondria can occur independently of the addition of cytosolic recognition factors (Becker et al., 1992). This is in apparent contrast to the results mentioned above concerning the identification of presequence binding factors. Clearly, studies with intact cells are required to fully understand the role of cytosolic factors in mitochondrial targeting.

3. A Protein Complex in the Mitochondrial Outer Membrane Contains the Receptors and a General Insertion Pore

After binding to the mitochondrial outer membrane receptors, the precursor proteins become inserted into and translocated across the mitochondrial outer membrane. An important step towards a molecular understanding of mitochondrial protein uptake was the establishment of methods to arrest translocation at certain stages and thus generate translocation intermediates (Pfanner & Neupert, 1987; Hartl et al., 1989; Pfanner et al., 1990). In particular, analysis of the import of the ADP/ATP carrier provided useful information on the consecutive steps of translocation. In contrast to the receptor-bound intermediate of the AAC precursor the intermediate at the GIP-stage was found to be resistant to proteases added to intact mitochondria and to require ATP for its formation. AAC arrested at the GIP could be extracted by protein denaturants, but not by detergents suggesting the existence of a proteinaceous pore as had already been proposed earlier (Blobel, 1980).

What could be the molecular nature of these proteinaceous pores? In *N. crassa* (Kiebler et al., 1990) and in *S. cerevisiae* (Moczko et al., 1992), the two import receptors MOM19 and MOM72 are found in a high molecular weight complex in the mitochondrial outer membrane. In both organisms, the complex contains at least five additional proteins of 7, 8, 22, 30 and 38 kD (Table), termed MOM7, MOM8, MOM22, MOM30 and MOM38 (Kiebler et al., 1990; Moczko et al., 1992; Söllner et al., 1992). This mitochondrial receptor complex is thought to represent the translocation machinery of the outer membrane organized in a cooperating multi-subunit complex as found in other biological systems (Alberts & Miake-Lye, 1992). Interestingly, the AAC precursor only copurifies with the receptor complex if it is arrested at the GIP-site, but not if it is arrested at the receptor level or if it is fully imported and assembled, indicating that the GIP-site copurifies with the receptor complex (Kiebler et al., 1990).

Four of these proteins, MOM7, MOM8,

Table. Components of the mitochondrial receptor complex from *Neurospora crassa*

Component		Topology					Function			Equivalent in *S.c.*	
Name	Cloned	Alkaline extraction	No. of TMS	Proposed orientation	Degradation by proteases from:		Acts at the level of	Ab block import	Receptor needed for own import	Name	Essential
					Outside	Inside					
MOM72	Yes	No	1	C out, N in	Yes	ND	Receptor	Yes	MOM19	MOM72/Mas70	No
MOM38	Yes	No	>2	N out, C in	Yes	Yes	GIP	Yes	MOM19 and MOM72	ISP42	Yes
MOM30	No	No	ND	ND	ND	ND	GIP	ND	ND	MOM30	ND
MOM22	Yes	No	1	N out, C in	Yes	Yes	Transfer from receptor to GIP	Yes	MOM19 and MOM72	MOM22	ND
MOM19	Yes	No	1	C out, N in	Yes	No	Receptor	Yes	None	MOM19	No
MOM8	No	Yes	ND	ND	ND	ND	GIP	ND	ND	MOM8 ISP6?	ND
MOM7	No	Yes	ND	ND	ND	ND	GIP	ND	ND	MOM7 ISP6?	ND

ISP6, import site protein of 6 kD, suppressor of ISP42-mutation in a ts-mutant (M. Douglas, *personal communication*).
Abbreviations: TMS, transmembrane spanning segments; Ab, antibodies; ND, not determined; N, amino-terminus; C, carboxy-terminus; GIP, general insertion pore; *S.c.*, *Saccharomyces cerevisiae*

MOM30 and MOM38, are believed to be involved in the formation of the general insertion pore GIP that is responsible for the insertion of preproteins into the outer membrane and their translocation across this membrane (Pfaller et al., 1988). The ability of the mitochondria to form the GIP-intermediate of the AAC was lost upon protease treatment in a manner similar to the degradation of MOM19, MOM22 and MOM72; in contrast, the ability to transfer the AAC precursor from the GIP-stage into the inner membrane was resistant to much higher protease concentrations and correlated with the integrity of MOM38 (Table). Thus, MOM38 might be a candidate for a central component of the GIP. The cDNA-sequence of MOM38, a component of the receptor complex directly involved in translocation of precursor proteins, revealed at least one putative transmembrane-spanning domain (Kiebler et al., 1990).

The MOM38 homologue in yeast, ISP42 was identified by crosslinking to a precursor trapped in translocation contact sites (Vestweber et al., 1989). Antibodies directed against ISP42 inhibited import of precursor proteins after mitochondria had been pretreated with trypsin. This indicated that the antibody had only access to its antigen after removal of protease-sensitive components from the mitochondrial outer membrane (Ohba & Schatz, 1987; Vestweber et al., 1989). Depletion of the protein in yeast cells caused accumulation of uncleaved mitochondrial precursor proteins (Baker et al., 1990). Disruption of the ISP42 gene led to inviability of the yeast cells. Overexpression of ISP42 in yeast led to an incorrect (nonfunctional) assembly in the membrane suggesting that ISP42 needs other components for its assembly (Baker & Schatz, 1991).

Crosslinking of the AAC at the GIP-site revealed that besides MOM38, three other components of the receptor complex, MOM7, MOM8 and MOM30 seem to be in close contact to a precursor arrested at the GIP-site. Recently, it was shown that a precursor at the GIP-site is accessible to proteases once the intermembrane space is opened (Rassow and Pfanner, 1991). These data fit very well with the recently determined topologies of the receptor complex components. The receptors MOM19 and MOM72 each have one transmembrane-spanning domain in their amino-terminal sequences; thus, most of their domains are cytoplasmically exposed. In contrast, MOM38 has substantial parts of their domains located in the intermembrane space (K.-P. Künkele, W. Neupert and M. Kiebler, *manuscript in preparation*).

Taken together these data suggest that a preprotein arrested at the GIP-site is located partly in the outer membrane and partly in the intermembrane space between the outer and the inner boundary membrane. It remains to be investigated whether the various MOMs form a sealed stable channel or whether such a channel represents a dynamic structure which can easily associate and dissociate. It is conceivable that the preprotein might trigger the formation of a translocation channel in the outer membrane.

Preliminary data suggest that antibodies against a segment of MOM38 exposed to the intermembrane space inhibit import of precursor proteins after

swelling of mitochondria (K.-P. Künkele, W. Neupert and M. Kiebler, *manuscript in preparation*). Thus, parts of MOM38 in the intermembrane space may be essential for the GIP function. It is tempting to speculate that this part might be responsible for mediating the contact to a component of the translocation machinery of the inner membrane once the preprotein has been inserted into the outer membrane pore. In addition, these observations point to a direct involvement of MOM38 in the insertion process.

The function of MOM22, the seventh of the so far identified components of the receptor complex has recently been investigated in some detail. MOM22 exposes its amino-terminal half to the cytosol, this is followed by a transmembrane-spanning segment and by a carboxy-terminal portion in the intermembrane space. Remarkably, the amino-terminal domain of MOM22 contains a cluster of 18 negatively charged amino acid residues. MOM22 appears to play a central role in the import of precursor proteins (Kiebler et al., 1993). Antibodies directed against the amino-terminal domain inhibited the import of practically all precursors tested. The current hypothesis is that MOM22 functions after the initial binding of precursors to the receptors and that both receptors transfer the precursor proteins to MOM22. The negatively charged cytosolic domain of MOM22 may then facilitate the insertion of the positively charged signal sequences into the outer membrane.

Yeast mitochondria contain a receptor/import complex with a similar protein composition as found in *N. crassa* mitochondria (Moczko et al., 1992). So far, three of the components of this yeast complex have been identified. ISP42, the yeast homologue of MOM38 (Vestweber et al., 1989; Baker et al., 1990) and MAS70, the homologue of MOM72 (Steger et al., 1990; Hines et al., 1990) and MOM19/MAS22 (Moczko et al., 1993). It may be expected that the two complexes exhibit similar properties. In fact, *N. crassa* MOM19 coexpressed in yeast efficiently assembled into the yeast complex. The possibility to isolate chemical amounts of the yeast complex should allow the identification and characterization of the remaining components.

Interestingly, the receptor complex varies in its composition with different isolation procedures. Coimmunoprecipitation with the anti-MOM19 antibody identifies all known components of the original receptor complex. In contrast, the anti-MOM72 antibody only coimmunoprecipitates MOM7, MOM8 and part of MOM38 (M. Moczko and N. Pfanner *unpublished*). Yet different subcomplexes are detected with antibodies directed against MOM22 or MOM38 (M. Kiebler, *unpublished*). These data suggest the existence of distinct receptor subcomplexes. Future studies have to show which particular functions these subcomplexes fulfill during protein import.

In a different approach to understand the physical nature of the insertion pores in the mitochondrial outer membrane electrophysical methods have been applied. There is growing evidence that in the outer membrane in addition to the voltage-dependent anion channels (VDAC) or porin (Colombini, 1979; for review, *see* Manella, 1992) other channels might exist that function in protein import (Chich et al., 1991). These voltage-activated channels are blocked by synthetic peptides representing amino-terminal presequences of mitochondrial precursors. Interestingly, recent evidence also suggests that proteins are translocated across the ER (Simon & Blobel, 1991) and the *E. coli* membrane (Simon & Blobel, 1992) through aqueous translocation pores. It remains to be investigated whether the channels identified with electrophysiological techniques represent the translocation pores responsible for importing precursor proteins into isolated mitochondria.

4. Components of the Mitochondrial Receptor Complex Are Targeted to Mitochondria by Differing Pathways

As the receptors on the mitochondria determine which proteins are imported and thereby contribute to define the identity of the mitochondria one may ask how the receptors themselves are targeted to this organelle. The precursor of MOM72 has been shown to use MOM19 as receptor (Söllner et al., 1990). The precursor of MOM19, however, does not require any of the known receptors or protease-accessible surface components. This raises the interesting question of how mitochondria control the specific assembly of MOM19. A mistargeting of MOM19 to other organelles might allow the other components of the receptor complex to assemble with MOM19 and eventually lead to the import of mitochondrial precursor proteins into the wrong organelle. Since MOM19 is associated with MOM38 in the mitochondrial receptor complex, this interaction might provide the specificity of MOM19 import into mitochondria. In fact, when the precursor of *N. crassa* MOM19 was imported after prebinding of anti-MOM38/ISP42 antibodies to yeast mitochondria, the import of MOM19 was inhibited (H. Schneider et al., 1991). We speculate that the receptor-independent association of MOM19 with MOM38 represents an evolutionary remnant form of mitochondrial protein import allowing specific import without the requirement of surface receptors. The ability of MOM38 to target MOM19 into the mitochondrial outer membrane requires that mistargeting

of MOM38 is tightly controlled. Otherwise, the receptor complex could assemble and then import mitochondrial precursor proteins into other membranes. Recent studies have shown that import of MOM38 strictly depends on MOM19 and MOM72 (Keil et al., 1993) thus meeting the high specificity requirements. Interestingly, MOM22 (Kiebler et al., 1993) displays a similar dependence on both import receptors (Keil & Pfanner, 1993).

This complex process of targeting the components of the receptor complex to mitochondria by individual pathways appears to reflect the need for a highly specific and efficient control system that prevents mislocalization of the key components determining the identity of an organelle.

5. The Transport Machineries of the Inner and Outer Membrane are Functionally Independent, Yet Cooperate in Protein Import

Translocation intermediates have been described that span both mitochondrial membranes simultaneously, suggesting translocation machineries in both membranes to cooperate in the import of preproteins (Schleyer & Neupert, 1985; Vestweber & Schatz, 1988; *for review, see*: Hartl & Neupert, 1990; Baker & Schatz, 1991). Such two membrane-spanning intermediates could be accumulated under a variety of experimental conditions *in vivo* and *in vitro*, and with a large number of different precursors. Abundant experimental data thus suggest that normal import across the two membranes occurs by a coupled reaction. Furthermore, this process appears to take place at sites where the two membranes are in close contact. To investigate whether precursors are translocated in a linear fashion or whether folded domains have to cross the membranes, hybrid proteins between amino-terminal portions of cytochrome b_2 and the entire dihydrofolate reductase were accumulated in mitochondrial contact sites (Rassow et al., 1990). It turned out that about 50 amino acid residues were sufficient to span both mitochondrial membranes supporting the view that mitochondrial precursor proteins cross the membranes in a linear and extended state.

It is not entirely clear whether these sites of translocation are present all over the mitochondrial envelope (which is constituted by the outer membrane and the inner boundary membrane) or whether they are concentrated in distinct areas, the so-called morphological contact sites. These areas of adhesion have been described in electron microscopical studies for some time (Hackenbrock, 1968; Brdiczka, 1991). Translocation intermediates spanning both membranes have been observed by immunoelectron microscopy at contact sites (Schleyer & Neupert, 1985; Schwaiger, Herzog & Neupert, 1987; van der Klei et al., 1992) but this does not exclude that *in vivo* larger areas of the outer and the inner boundary membranes contain translocation sites.

Several new lines of evidence now support the view that the two mitochondrial membranes contain distinct protein translocation machineries which can operate independently of each other, but cooperate in terms of time and space during translocation of, e.g., matrix proteins.

Evidence for an independently acting translocation machinery in the outer membrane came from import studies with the cytochrome c heme lyase (CCHL), a peripheral protein of the inner membrane (Lill et al., 1992*b*) which catalyzes the attachment of the heme group to apocytochrome c in the intermembrane space or at the trans side of the outer membrane (Nicholson, Hergersberg & Neupert, 1988; C. Hergersberg and W. Neupert, *unpublished*). Import of CCHL is dependent on components of the receptor complex in the mitochondrial outer membrane, namely on MOM19 and on GIP, showing that the same machinery is used by CCHL as by matrix proteins. In contrast to matrix proteins, however, import of CCHL is independent of the membrane potential across the mitochondrial inner membrane indicating that it does not cross the inner membrane during its import. CCHL is also unusual in that it does not appear to require cytosolic hsp70 or the hydrolysis of free ATP to keep it competent for import. This might be due to a relatively extended conformation of the protein due to its unusually high content of proline residues (12%) which might enable the protein to maintain an import-competent conformation even without the aid of chaperones (Lill et al., 1992*a*).

In an approach to directly demonstrate the translocation activity of the outer membrane, it was found that highly purified outer membrane vesicles correctly inserted outer membrane proteins such as porin, MOM19, and MOM22. In addition, the vesicles were able to import the precursor of cytochrome c heme lyase into a protease-protected location (Mayer, Lill & Neupert, 1993). However, insertion of the AAC into these vesicles at the GIP-stage was not observed. An unknown component of either the intermembrane space or the inner membrane could be missing that would couple the translocation machinery of the outer membrane with that of the inner membrane. These results clearly indicate that the outer membrane translocation machinery can work independently of the inner membrane machinery but lacks the components needed for the translocation of inner membrane or matrix space proteins.

Evidence that the inner membrane can translo-

cate independently of the outer membrane came from import experiments into mitoplasts where the outer membrane of mitochondria had been disrupted by detergent or osmotic treatment and from import experiments into inner membrane vesicles with right-side-out orientation. In the first case, the translocation sites of the outer membrane were saturated with translocation intermediates spanning the two membranes. This treatment led to an import inhibition of other precursor proteins (Vestweber & Schatz, 1988; Rassow et al., 1989). However, import of some precursor proteins could be restored if the outer membrane of these mitochondria was opened (Hwang et al., 1989). In the second case, inner membrane vesicles virtually free of outer membrane components were shown to import several mitochondrial precursor proteins in an ATP- and membrane potential-dependent manner (Hwang et al., 1989). These data support the view that the inner membrane contains an import machinery which in principle can act independently of that in the outer membrane.

Further evidence that the translocation contact sites between the outer and the inner membrane do not represent stable sealed channels came from recent studies investigating the properties of translocation intermediates of preproteins accumulated at low levels of ATP in the matrix. These intermediates are protected against a proteolytic attack from outside. If, however, the outer membrane is disrupted and the intermembrane space is thereby opened, the intermediate becomes sensitive to added protease indicating that it is exposed to the intermembrane space (Hwang, Wachter & Schatz, 1991; Rassow & Pfanner, 1991). Similar results were obtained with a chimeric, artificial precursor protein containing pancreatic trypsin inhibitor at its carboxy-terminal domain which can be accumulated in the intermembrane space following depletion of matrix ATP (Jascur et al., 1992). Restoring the ATP level allows the translocation of this intermembrane space intermediate across the inner membrane. In conclusion, these data suggest that the import machineries of the two mitochondrial membranes are not connected by a single sealed channel. Still, none of the preproteins *en route* to the inner membrane was freely soluble in the intermembrane space. This emphasizes that transfer across the intermembrane space is usually coupled to translocation across both membranes.

The sequential and independent use of the two import machineries was directly demonstrated by dissecting the two translocation steps in time. A fusion protein between the presequence of the β-subunit of the F_1-ATPase ($F_1\beta$) and CCHL was constructed and used for import into intact mitochondria (Segui-Real et al., 1993). This fusion protein was able to use two import pathways: In the presence of a membrane potential, it was imported directly into the matrix using the receptor complex of the outer membrane. In the absence of a membrane potential, however, the protein was imported into the intermembrane space according to the normal import pathway for CCHL (*see above*). This intermembrane space localized species could be, however, further imported into the matrix, if a membrane potential was regenerated following the first import step. Taken together, these data show that the import pathway into the matrix can be experimentally resolved into two independent membrane translocation steps which usually occur simultaneously.

A current model allows the definition of the following steps occurring during translocation of a preprotein across mitochondrial membranes. First, binding takes place followed by insertion into and translocation across the outer mitochondrial membrane. The translocated signal sequence then interacts with a component of the inner membrane. This interaction allows the membrane potential-dependent translocation of the targeting signal across the inner membrane and leads to coupled translocation by both translocation machineries. Thus, the two translocation systems can act independently of each other, but during import of most precursor proteins cooperate in a dynamic manner in terms of space and time (Glick, Wachter & Schatz, 1991; Pfanner et al., 1992).

It has been suggested that intermembrane space components may aid in the process of cooperation between both membranes by facilitating the transfer of preproteins across the space between both membranes. Interestingly, an intermembrane space component has previously been claimed to be necessary for preprotein transport (Schwaiger et al., 1987). Such a component could have a chaperone-like function or could be involved in the coupling of the translocation machineries of the two membranes.

In contrast to the import pathway described so far, the transport pathway of cytochrome c is rather different. Its import is independent of the receptors MOM19 or MOM72 and of GIP. Cytochrome c is directly inserted into the mitochondrial outer membrane and imported into the intermembrane space (Stuart, Nicholson & Neupert, 1990). The specificity and efficiency of the import is ensured by a high affinity interaction of apocytochrome c with the enzyme CCHL (Nargang et al., 1988; Nicholson, Stuart & Neupert, 1989). CCHL resides in the intermembrane space close to the mitochondrial surface and may attach the heme group to apocytochrome c while the protein chain is still in transit (C. Hergersberg, *unpublished*). This might provide a driving force for the import of this exceptional protein (Lill

et al., 1992*a*). On the other hand, it has been proposed that apocytochrome c can freely traverse the outer membrane into the intermembrane space, where it binds with high affinity to CCHL. This binding thus serves to trap the apo precursor on the inside of the outer membrane (Dumont, Ernst & Sherman, 1988). Subsequent addition of the heme group causes a tight folding of the cytochrome c, which then is unable to traverse the outer membrane.

6. The Membrane Potential, hsp70 and ATP in the Matrix Are Essential for Translocation of Preproteins Across the Inner Membrane

What is the driving force of mitochondrial protein import? Import into mitochondria can occur in a post-translational manner *in vitro* (Korb & Neupert, 1978; Maccecchini, Rudin & Schatz, 1979) and *in vivo* (Hallermayer, Zimmerman & Neupert, 1977; Schatz, 1979; Wienhues et al., 1991), showing that transport is mechanistically independent of elongation. Therefore, polypeptide chain elongation cannot be the driving force for mitochondrial protein import.

Processing of translocated proteins to the mature species has been suggested to represent a possible driving force for protein transport (Simon, Peskin & Oster, 1992), but in most transport systems processing can occur independently of translocation. In mitochondrial import, proteins are cleaved by the matrix processing peptidase (α- and β-MPP) upon arrival in the matrix (Schneider et al., 1989; Arretz et al., 1991), but translocation can occur independently of processing (Schmidt et al., 1984). In bacterial protein export (Randall & Hardy, 1989), leader peptidase cleaves the presequence of proteins being translocated into the periplasm (Wickner, 1988), but export is independent of the cleavage reaction. In the endoplasmic reticulum signal peptidase cleaves the presequences of the proteins in transit to the lumen of the ER (Evans, Gilmore & Blobel, 1986).

As mentioned before, the translocation of preproteins requires the membrane potential across the mitochondrial inner membrane. Also, in bacterial protein export the proton motive force supports translocation of polypeptide chains. But the two systems differ in several aspects. First, the orientation of the potential with respect to the direction of protein transport is different. In mitochondria the direction of import is from outside (positive) to inside (negative). In bacteria protein, export is from inside (negative) to outside (positive). Secondly, in mitochondria the electrical component of the potential but not ΔpH is required, whereas in bacteria both components seem to be involved. Thirdly, protein translocation across the inner membrane of mitochondria absolutely requires $\Delta\Psi$ in all cases investigated. In bacteria, $\Delta\mu_{H^+}$ is essential only for some precursors, though in many cases stimulates the translocation process. In mitochondria, the membrane potential is required only for the insertion and presumably the translocation of the presequence. The mature part of the precursor can be translocated in the absence of a potential (Schleyer & Neupert, 1985). Therefore, it was proposed that the membrane potential is needed to translocate the (positively charged) presequence across the mitochondrial inner membrane in an electrophoretic manner (Martin, Mahlke & Pfanner, 1991). There are, however, several other possible roles for $\Delta\Psi$. The membrane potential might be involved in orienting the presequence with respect to the surface of the inner membrane or might influence the activity of a protein with a function in translocation (Nicholls & Ferguson, 1992).

In all protein transport systems studied, translocation requires the hydrolysis of nucleoside triphosphates (ATP or GTP). In mitochondria, it has been shown that protein import requires ATP in addition to a membrane potential (Pfanner & Neupert, 1986; Chen & Douglas, 1987*a*; Eilers, Oppliger & Schatz, 1987). Three different ATP-dependent steps in protein translocation have been identified: one needing ATP in the cytosol, another one at the level of the receptor MOM72 (discussed in section 2), and a third step located in the matrix.

At least part of the ATP requirement in the cytosol can be explained by the role of hsp70 proteins. Cytosolic hsp70 proteins are believed to help in keeping preproteins in an import-competent state by binding to segments that are not natively folded. Hydrolysis of ATP is needed for the release of the preproteins from the hsp70s during translocation (Hartl et al., 1992).

Hsp70 (Ssc1p) in the matrix is thought to facilitate the passage of precursor proteins into the matrix and to keep them in an unfolded conformation (Kang et al., 1990; Ostermann et al., 1990; Scherer et al., 1990; Manning-Krieg et al., 1991). In a yeast mutant with a partial defect in mitochondrial hsp70, protein translocation into the matrix was impaired. The presequences were driven into the matrix by the membrane potential but the precursor proteins were arrested during translocation in a membrane-spanning manner. By artificially unfolding the precursor protein in urea, the protein was translocated into a protease-protected location (Kang et al., 1990). In another hsp70-mutant allele described recently with a stronger phenotype, the protein in transit was not

even processed by MPP (D. Gambill, W. Voos, N. Pfanner, E. A. Craig, *unpublished*). These observations suggest that the mitochondrial hsp70 is essential for driving the translocation of preproteins into the mitochondrial matrix. The binding of hsp70 to the precursor protein in the mitochondrial matrix appears to confer unidirectionality (Neupert et al., 1990; Simon et al., 1992). The energetic input then would occur at the level of dissociation of hsp70 from the translocated preproteins which requires hydrolysis of ATP (Hartl et al., 1992). By binding to the incoming precursors in the matrix space, hsp70 may, in addition, drive the unfolding of precursors on the cytosolic side of the outer membrane (Neupert et al., 1990). According to this model, hsp70 binding would lead to a stepwise unfolding, and the unfolded segments could be sequestered in a vectorial fashion via a proteinaceous pore in the matrix space.

Mitochondrial hsp70 may stay associated with the preprotein until it is transferred to the hsp60 folding machinery (Cheng et al., 1989; Ostermann et al., 1989), a process which conceivably could require the cooperation of putative mitochondrial homologues of DnaJ and GrpE (Langer et al., 1992). Hsp60 seems to have a dual role in mitochondrial protein import: it functions in folding and assembly of imported proteins (Cheng et al., 1989).

7. Conclusions and Perspectives

As illustrated in Fig. 2, transport of proteins into mitochondria involves a series of steps. Recently, several components of the translocation machinery were identified and characterized: cytosolic hsp70, the receptor complex in the outer membrane with the two receptors MOM19 and MOM72, four components of the general insertion pore (GIP) MOM7, MOM8, MOM30 and MOM38, components of the translocation machinery in the inner membrane MPI1 and Mas6, the matrix processing peptidase MPP with its two components α- and β-MPP, the intermembrane space protease IMP1, the heat-shock proteins hsp70 and hsp60 in the matrix, as well as cytochrome c heme lyase which is specifically involved in import of cytochrome c into the intermembrane space.

Future work will have to focus on the identification and characterization of further components of the translocation machinery. A detailed understanding of the process of insertion of precursor proteins into the outer and inner membrane once they have bound to the receptors is necessary. A thorough analysis of the GIP-stage and the transport machinery in the inner membrane as well as the molecular characterization of the export channels remain to be performed. As in other transport systems, reconstitution of partial reactions will be required to obtain insight into all these steps.

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