MITOCHONDRIAL PREPROTEIN TRANSLOCASE

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ABSTRACT

Mitochondria import most of their proteins from the cytosol. Dynamic protein complexes in the mitochondrial outer and inner membranes are responsible for the specific recognition and membrane translocation of preproteins. The preprotein translocase of the outer mitochondrial membrane contains several import receptors and a general import pore. The preprotein translocase of the inner membrane consists of a channel interacting with preproteins in transit and an import motor that includes the matrix heat shock protein Hsp70. Acidic patches of import components are thought to guide the import of positively charged signal sequences (acid chain hypothesis). Energy input is derived from the inner membrane potential and ATP. Proteins in the mitochondrial matrix are required for proteolytic processing and folding of imported proteins. The dynamic nature of the membrane translocase permits sorting of preproteins at distinct stages of the import pathway.

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INTRODUCTION

Mitochondria are essential organelles of all eukaryotic cells. They contain two membranes, the outer membrane and the inner membrane, and their own genetic system and protein synthesis machine in the innermost compartment, the matrix. However, mitochondria synthesize only a small number of proteins in the matrix. More than 98% of mitochondrial proteins are encoded on nuclear genes and are synthesized as preproteins on cytosolic polysomes. Analysis of mitochondrial protein import focuses on three central issues (Kübrich et al 1995, Ryan & Jensen 1995, Lill & Neupert 1996, Schatz & Dobberstein 1996): specific targeting of preproteins to the correct organelle; translocation of entire proteins across biological membranes; and folding of imported proteins into their active conformations (Figure 1). Preproteins carry degenerate signal sequences, either as N-terminal extensions (presequences) or as internal targeting sequences, that direct their interaction with mitochondrial receptors and the translocation machinery of the mitochondrial membranes. Translocation across the membranes involves at least partial unfolding of the precursor polypeptides. Folding of imported proteins can be assisted by molecular chaperones. In the past, numerous components of the protein import machinery were identified, including cytosolic chaperones, import receptors, subunits of pores or import

Figure 1  Basic steps of mitochondrial protein import shown for a preprotein transported into the matrix. Nuclear-encoded mitochondrial preproteins are synthesized on cytosolic polysomes. Cytosolic chaperones prevent misfolding and aggregation of the preproteins in the usual post-translational import mechanism. Targeting sequences, typically present in presequences at the N terminus of preproteins (zig-zag line), are recognized by receptors (R) on the mitochondrial surface. Translocation across the outer membrane occurs through the general import pore (GIP). Transport through the translocase of the inner membrane (Tim) requires the membrane potential ($\Delta \psi$). A matrix-processing peptidase removes the presequences. During and after membrane translocation, the proteins interact with ATP-dependent heat shock proteins (Hsp) of the matrix and are folded to their active conformation.
Synthesis of preproteins on cytosolic polysomes

Cytosolic chaperones

Preprotein

Cytosol

Receptor

R

General import pore

GIP

Translocase of inner membrane

Tim

Heat shock proteins and folding helpers

Hsp

ATP

Processing peptidase

Active protein

Matrix

Intermembrane space

Inner membrane

Δψ

Outer membrane
channels, processing enzymes, matrix chaperones, and other folding helpers. Recent studies attempt to define the functions of the various import components with the aim of unraveling the mechanistic principles of translocation.

PREPROTEIN TARGETING TO MITOCHONDRIA

Post-Translational Translocation and Cytosolic Chaperones

There is ample evidence from in vitro import studies into isolated mitochondria that completely synthesized preproteins can be released from ribosomes and imported in a post-translational manner (summarized in Neupert et al 1990, Hönlinger et al 1995a, Schatz 1996). The majority of preprotein import in vivo probably also occurs by a post-translational mechanism (Wienhues et al 1991, Schatz 1996), although it cannot be ruled out that part of the import occurs cotranslationally, i.e., that the N-terminal portion of a preprotein is inserted into the mitochondrial membranes while other parts are still being synthesized on the ribosome (Kellems et al 1975, Verner 1993).

Cytosolic chaperones were shown to bind to preproteins in a post-translational import reaction in order to prevent misfolding or aggregation of the precursor polypeptides. The chaperones preserve the import competence of preproteins. Members of the heat shock protein 70 (Hsp70) family can interact with preproteins destined for mitochondria, other organelles, or the cytosol (Deshaies et al 1988, Murakami et al 1988, Craig et al 1995). The mitochondrial import stimulation factor (MSF) shows a preference for preproteins targeted to mitochondria (Hachiya et al 1993, Mihara & Omura 1996). MSF is a heterodimer of 32- and 30-kDa subunits and belongs to the family of 14-3-3 proteins that perform a diversity of functions in signal transduction, cell-cycle regulation, and exocytosis, probably by modulating interactions among components of signal transduction pathways (Aitken 1996). Both Hsp70s and MSF are ATPases. The functional importance of the chaperones Hsp70 and MSF for mitochondrial protein import in vivo remains to be established.

Mitochondrial Targeting Signals

Signal sequences of mitochondrial preproteins are typically 15–30 amino acid residues in length, enriched in positively charged residues, and able to form amphipathic α-helices (Hurt & van Loon 1986, Horwich 1990). Specific primary sequence motifs have not been found. In a majority of preproteins, signal sequences are present as N-terminal extensions, termed presequences, that are removed after import. Many preproteins, however, contain internal targeting sequences that have been poorly characterized. It is likely that internal targeting sequences also can form positively charged amphipathic signals (Pfanner et al 1987a, Fölsch et al 1996). The amphipathic signals function as matrix targeting
signals that can direct import of preproteins to receptors on the mitochondrial surface and subsequently across outer and inner membranes. In addition, some preproteins contain sorting signals that are typically more hydrophobic and direct their specific sorting to intramitochondrial compartments. Sorting signals can be located immediately adjacent to the matrix targeting signal, for example in a bipartite presequence or in the mature part of preproteins (Hurt & van Loon 1986, Hartl & Neupert 1990).

Import Receptors on the Mitochondrial Surface

The translocase of the outer mitochondrial membrane (Tom) consists of at least nine distinct proteins (Table 1). Four Tom proteins, Tom20, Tom22, Tom37, and Tom70, were identified as import receptors or subunits thereof [according to the new uniform nomenclature, the numerical part of the name indicates the approximate molecular mass in kDa (Pfanner et al 1996)]. They form two heterodimeric import receptors: Tom20-Tom22 and Tom70-Tom37. Tom20-Tom22 recognize typical mitochondrial preproteins that carry N-terminal presequences (Bolliger et al 1995, Höninger et al 1995b, Mayer et al 1995a). The membrane anchor of Tom20 is located at the immediate N terminus, and the remainder of the protein forms a domain on the cytosolic side of the membrane (Schneider et al 1991). Tom22, which contains a single internal membrane anchor, has an N-terminal domain exposed to the cytosol (Kiebler et al 1993). The purified cytosolic domain of each subunit of the Tom20-Tom22 receptor specifically binds presequences of cleavable mitochondrial preproteins, indicating that each receptor subunit can function as an independent presequence receptor. Presequences interact with Tom22 in an electrostatic manner, whereas the binding of presequences to Tom20 indicates properties of a hydrophobic interaction (Brix et al 1997). We suggest that the negatively charged Tom22 recognizes the positively charged face of an amphipathic presequence and that Tom20 binds to the hydrophobic (back) side of a presequence. It is unknown whether the two receptors bind a presequence simultaneously or in a sequential manner. In any case, after their interaction with Tom20-Tom22, the preproteins are inserted into the general import pore (Figure 2).

Tom70, which forms a heterodimer with Tom37 (Gratzer et al 1995), has a topology similar to that of Tom20, i.e. an N-terminal hydrophobic membrane anchor and a large cytosolic domain (Hase 1983, Sollner 1990). The purified C-terminal domain of Tom70 selectively binds mitochondrial preproteins with a preference for those with internal targeting information (Schlossmann et al 1994, Brix et al 1997). The numerous members of the family of inner membrane metabolite carriers, such as the ADP/ATP carrier, are among those preproteins with a preference for Tom70. After their interaction with Tom70,
### Table 1  Protein transport machinery of mitochondrial outer and inner membranes

<table>
<thead>
<tr>
<th>Component</th>
<th>Former/other names</th>
<th>Essential for cell viability</th>
<th>Membrane association</th>
<th>Proposed function</th>
<th>References</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tom72</td>
<td>Tom71</td>
<td>No</td>
<td>Integral</td>
<td>None (homologous to Tom70)</td>
<td>Bömer et al 1996 Schlossmann et al 1996</td>
</tr>
<tr>
<td>Tom70</td>
<td>Mas70, MOM72</td>
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<td>Integral</td>
<td>Receptor</td>
<td>Söllner et al 1990 Hines et al 1990</td>
</tr>
<tr>
<td>Tom22</td>
<td>Mas17, Mas22, MOM22</td>
<td>Yes</td>
<td>Integral</td>
<td>(Presequence) receptor and trans site</td>
<td>Bolliger et al 1995 Hönlinger et al 1995b</td>
</tr>
<tr>
<td>Tom7</td>
<td>MOM7</td>
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<td>Integral</td>
<td>Dissociation of Tom complex</td>
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</tr>
<tr>
<td>Tom6</td>
<td>Isp6, MOM8b</td>
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<td>Integral</td>
<td>Assembly of Tom complex</td>
<td>Kassenbrock et al 1993 Alconada et al 1995b Dietmeier et al 1997</td>
</tr>
<tr>
<td>Tom5</td>
<td>MOM8, MOM8a</td>
<td>No</td>
<td>Integral</td>
<td>Transfer from receptors to import pore</td>
<td>Hönlinger et al 1996</td>
</tr>
</tbody>
</table>


## Transport machinery of inner membrane

<table>
<thead>
<tr>
<th>Protein</th>
<th>MIM Number</th>
<th>Mpi</th>
<th>Location</th>
<th>Description</th>
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<tr>
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<td>Yes</td>
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<td>Import of inner membrane carriers</td>
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</tr>
<tr>
<td>Tim11</td>
<td>MIM17, Mpi2</td>
<td>No</td>
<td>Integral</td>
<td>Association with preprotein in transit</td>
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</table>

### Abbreviations and comments

- **Abbreviations and comments.** Essential for viability indicates a strict requirement for growth of *Saccharomyces cerevisiae* on both fermentable and non-fermentable carbon sources. Tom, translocon of outer mitochondrial membrane (~mol mass in kDa); Tim, translocon of inner mitochondrial membrane (~mol mass in kDa). mtHsp70 and Mge1 are matrix proteins; a fraction of the proteins transiently interacts with the Tim machinery. Further components involved in mitochondrial protein import (components of cytosol, intermembrane space and matrix, processing enzymes) are shown in Figures 2 and 5 and are listed in Kübrich et al (1995).
Figure 2  Targeting of preproteins to the mitochondrial surface. Cytosolic chaperones, mitochondrial import stimulation factor (MSF), and cytosolic heat shock proteins 70 (cHsp70), can bind preproteins and prevent aggregation. The main import receptor is a heterodimer of Tom20 and Tom22 (Tom, translocase of outer mitochondrial membrane) that recognizes the N-terminal positively charged targeting sequences (presequences). The second import receptor is a heterodimer of Tom70 and Tom37; it preferentially binds preproteins with internal targeting sequences and more hydrophobic preproteins. Preproteins recognized by Tom70-Tom37 are transferred to Tom20-Tom22 before insertion into the general import pore (GIP).

the preproteins are probably not directly inserted into the general import pore but are first transferred to the other import receptor Tom20-Tom22 (Figure 2) (Kiebler et al 1993, Haucke et al 1996). Tom22 functions as the central import receptor because it is required for cell viability under all growth conditions (Table 1) (Lithgow et al 1994, Hönlinger et al 1995b).

The exact function of Tom37 is not known; it is assumed to cooperate with Tom70 in recognition of preproteins with internal targeting information (Gratzer et al 1995). In addition, a homologue of Tom70, termed Tom72, which is expressed at low levels and is loosely associated with the import receptor, has been identified. However, a deletion of Tom72 does not significantly affect cell growth or mitochondrial function, suggesting that Tom72 does not play a major role in the Tom machinery (Bömer et al 1996a, Schlossmann et al 1996).

The cytosolic chaperones MSF and Hsp70 show a differential preference for the receptor subcomplexes (summarized in Mihara & Omura 1996). Preproteins interacting with MSF are mainly transferred to Tom70-Tom37, whereas preproteins bound to cytosolic Hsp70 are typically transferred directly to Tom20-Tom22 (Figure 2) (Hachiya et al 1995, Komiya et al 1996). However, cytosolic chaperones are not required for the specificity of targeting because a chemically pure cleavable preprotein was efficiently imported via Tom20 without the addition of chaperones or other cytosolic cofactors (Becker et al 1992).

A DYNAMIC TRANSLOCASE IN THE OUTERMEMBRANE

General Import Pore

After binding by the import receptors, the preproteins are transported across the outer membrane through the general import pore (GIP). Coimmunoprecipitation experiments showed a loose interaction of the import receptors with several other Tom proteins, Tom40, Tom7, Tom6, and Tom5 (Table 1) (Moczko et al 1992, Söllner et al 1992). Tom40 is embedded in the outer membrane and spans the membrane several times, most likely by $\beta$-strands. In fact, Tom40 reveals a structural similarity to porins present in bacterial and mitochondrial outer membranes that form pores for the transport of metabolites (Mannella et al 1996). Tom40 is essential for cell viability, and it likely forms the main constituent of the import pore of the outer membrane (Vestweber et al 1989, Baker et al 1990, Kiebler et al 1990). Tom5 possesses a single-membrane anchor at the C terminus; its N-terminal segment, which contains a negative net charge, is exposed to the cytosolic side (Figure 3). Tom5 is closely associated with Tom40 and represents the connecting link between import receptors and the general import pore. It is required for the transfer of preproteins to and insertion into the pore (Dietmeier et al 1997). The negatively charged cytosolic
Figure 3  The preprotein translocases of mitochondrial outer and inner membranes. The preprotein translocase of the outer mitochondrial membrane (Tom) is assembled from subcomplexes: the receptors Tom20-Tom22 and Tom70-Tom37, and Tom40, which is the main component of the general import pore. Tom5 is associated with Tom40 and promotes entry of preproteins into the import pore. Tom6 and Tom7 modulate assembly and dissociation of the translocase. The translocase of the inner membrane (Tim) contains the essential proteins Tim17, Tim23, and Tim44. Tim17 and Tim23 are major subunits of the import channel. Tim11 is associated with the import and sorting machinery. The matrix heat shock protein Hsp70 (mtHsp70) reversibly binds to the Tim machinery: Tim44 and Tim17 (or a closely associated protein). The membrane potential \( \Delta \psi \) is required for the initial transport of the positively charged presequences across the inner membrane. mtHsp70, in cooperation with the Tim machinery, and the co-chaperone Mge1 (mitochondrial GrpE) function as an ATP-driven import motor.
segment of Tom5 seems to position the preprotein directly above the import pore, therefore being crucial for efficient insertion.

**Tom6 and Tom7 Modulate the Dynamics of the Translocase**

Tom6 and Tom7 do not directly interact with a preprotein in transit but modulate the dynamics of the translocase. Tom6 promotes the association of both receptor subcomplexes, Tom20-Tom22 and Tom70-Tom37, with the import pore component Tom40 and thereby accelerates preprotein import by a factor of $\approx 3$ (Alconada et al 1995b). The effect of Tom6 on the two receptor subcomplexes is qualitatively, but not quantitatively, comparable. The efficiency of association of Tom40 with Tom20-Tom22 and Tom70-Tom37 is very different. In the presence of Tom6, the majority of Tom20-Tom22 can be found in association with Tom40, whereas more than 90% of Tom70-Tom37 readily dissociates from the import pore (Alconada et al 1995a,b). Tom7 preferentially affects the interactions between Tom20, Tom22, and Tom40. Surprisingly, Tom7 functions in an antagonistic manner to Tom6. A deletion of Tom7 stabilizes the interaction between these Tom proteins. In wild-type mitochondria, Tom7 favors the dissociation of Tom20-Tom22 from Tom40 and the dissociation of the Tom20-Tom22 subcomplex itself (Hönlinger et al 1996). The increased stability of the Tom machinery in mutant mitochondria lacking Tom7 only slightly impairs the import of preproteins that are targeted to the inner membrane or matrix; however, it severely inhibits the sorting of preproteins at the outer membrane. For example, transport of the precursor of porin into the outer membrane requires the presence of Tom7 (Hönlinger et al 1996). We conclude that Tom7 promotes a dynamic behavior of the outer membrane translocase. It supports a partial dissociation of the Tom machinery and thereby facilitates the lateral release of a preprotein into the outer membrane.

**Trans Binding Sites for Preproteins at the Outer Membrane**

During or after translocation through the general import pore, the preproteins are thought to interact with a \textit{trans} binding site on the intermembrane space side of the outer membrane (Pfanner et al 1987b, Bolliger et al 1995, Mayer et al 1995b). The molecular nature and functional importance of a \textit{trans} site was the subject of a controversial debate. In addition to its cytosolically exposed domain, Tom22 exposes a small C-terminal domain to the intermembrane space that carries a net negative charge (Figure 3). Bolliger et al (1995) showed binding of a chemically synthesized presequence peptide to the purified C terminus of Tom22 and suggested a function as a \textit{trans} binding site. In contrast, Nakai et al (1995) and Court et al (1996) reported that deletion of the intermembrane space domain of Tom22 revealed none or only a minor inhibitory effect on protein import and concluded that the domain did not function as a \textit{trans} site for preproteins.
Figure 4  Functions of Tom and Tim complexes in protein sorting to mitochondrial subcompartments. (a) Import pathway of a typical cleavable preprotein into the mitochondrial matrix via Tom20-Tom22, Tom5, Tom40, intermembrane space domain of Tom22, Tim23, Tim17, Tim44, and mtHsp70. When additional sorting signals are present in the preprotein, sorting to the inner membrane or intermembrane space can occur at the level of the Tim machinery. Some preproteins are completely imported into the matrix and are then exported across the inner membrane (conservative sorting; not shown in the figure) (Hartl et al. 1986). (b) Hydrophobic inner membrane proteins without a presequence such as the carrier proteins [e.g. ADP/ATP carrier (AAC)] are initially recognized by Tom70 and are then transferred to Tom22, Tom5, and Tom40; the trans site at the outer membrane is distinct from the intermembrane space domain of Tom22; insertion into the inner membrane is mediated by a complex containing Tim22. (c) Some intermembrane space proteins, such as the cytochrome c heme lyase (CCHL), use only the Tom machinery. (d) Outer membrane proteins, e.g. porin, use the Tom machinery. Tom7 promotes dissociation of the outer membrane translocase and thus facilitates a lateral release of porin into the membrane.
To resolve the controversy, a functional assay for the reversible accumulation of translocation intermediates of preproteins at the *trans* side of the outer membrane of intact mitochondria was used, thereby demonstrating the presence of two distinct *trans* sites: one for preproteins with N-terminal targeting sequences (presequences) (Figure 4a) and one for preproteins with internal targeting information (such as the ADP/ATP carrier) (Figure 4b) (M Moczko, A Hönliger & N Pfanner, unpublished data). The intermembrane space domain of Tom22 was indeed essential to form a *trans* site for presequence-containing preproteins, whereas the *trans* site for preproteins with internal targeting information has not been identified at a molecular level. The *trans* sites are of particular importance for productive accumulation of preproteins at the outer membrane, for example when further import into the inner membrane is blocked by a dissipation of the membrane potential $\Delta \psi$ across the inner membrane. In the presence of a membrane potential, preproteins traversing the outer membrane are rapidly transferred to the inner membrane. Outer membrane *trans* sites play only a limited role in this direct import; i.e. a deletion of the intermembrane space domain of Tom22 moderately inhibits, but does not block, the direct transfer of preproteins from the outer membrane to the inner membrane.

**THE PREPROTEIN TRANSLOCASE OF THE INNER MEMBRANE**

The preprotein translocase of the inner mitochondrial membrane (Tim) is not stably connected to the translocase of the outer membrane. Tim machinery was shown to function independently of the Tom machinery (Hwang et al 1989, Rassow & Pfanner 1991, Segui-Real et al 1993). The main building blocks of the Tim machinery are an inner membrane channel for preproteins and an import motor located on the matrix side. Five Tim proteins have been identified at a molecular level (Table 1). Tim17, Tim23, and Tim44 are essential elements of the general import pathway of a preprotein into the mitochondrial matrix (summarized in Pfanner et al 1994), whereas Tim22 and Tim11 perform more specialized roles (Sirrenberg et al 1996, Tokatlidis et al 1996).

**The Tim Channel**

Tim17 and Tim23 are thought to constitute the core of the import channel for preproteins (Figure 3) (Kübrich et al 1994, Berthold et al 1995, Blom et al 1995). Tim17 and Tim23 are hydrophobic proteins, homologous to each other in their membrane-spanning regions. Each protein is predicted to span the membrane four times (Emtage & Jensen 1993, Dekker et al 1993, Kübrich et al 1994, Maarse et al 1994, Ryan et al 1994). In addition, Tim23 contains a hydrophilic N-terminal domain on the intermembrane space side. This domain of Tim23 contains a net negative charge and has the ability to bind presequence...

Both Tim17 and Tim23 are in close contact with a preprotein in transit (Ryan & Jensen 1993, Kübrich et al 1994). However, they cannot substitute for each other even though they are similar in sequence because each protein is essential for cell viability (Table 1). Originally, it was suggested that the Tim channel is just a passive diffusion channel for preproteins (Ungermann et al 1994, Berthold et al 1995). In this model, it is difficult to explain how the channel can prevent the passage of small ions during translocation of the various side chains of preproteins, which must occur since the electrochemical proton gradient across the inner membrane is maintained during protein import. Recent work indicates that Tim17 and Tim23 actively interact with a preprotein in transit. The presence of a membrane-spanning precursor polypeptide stabilizes the interactions between the channel subunits Tim17 and Tim23 (PJT Dekker & N Pfanner, unpublished data). We suggest that the channel interior is dynamically changed during preprotein translocation.

Translocation of the N-terminal presequences strictly requires the membrane potential $\Delta \psi$ (negative on the inside). It is generally assumed that a major function of the $\Delta \psi$ in protein import is to exert an electrophoretic effect on the positively charged presequences (Martin et al 1991, Roise 1992). In support of the electrophoretic model, a presequence with a high number of positive charges was imported at a lower $\Delta \psi$ than a presequence with a lower positive charge (Martin et al 1991). Additionally, it is possible that the $\Delta \psi$ could modulate the conformation of Tim proteins and thereby help to open the channel. Interestingly, the N-terminal domain of Tim23 has the ability to dimerize, probably via a heptad leucine repeat motif (leucine zipper) (Bauer et al 1996). The dimerization is supported by the presence of a $\Delta \psi$. Once a preprotein is inserted into the channel, the Tim23 dimer dissociates again. Bauer et al (1996) suggested that $\Delta \psi$-driven dimerization of Tim23 represents an initial step for binding of preproteins to the inner membrane. The dimerization is not sufficient, however, to explain the essential function of the $\Delta \psi$ in protein translocation; in the absence of a $\Delta \psi$ a significant portion of Tim23 molecules dimerize, but protein transport into the inner membrane is blocked completely. Future studies will have to address the relevance of the dimerization of Tim23 for import and the function of the $\Delta \psi$.

Tim11 consists of a hydrophobic N-terminal portion and a hydrophilic C-terminal segment that may be located in the intermembrane space (Tokatlidis et al 1996). Tim11 has been cross-linked to a preprotein traversing the inner membrane, as well as a preprotein sorted to the intermembrane space. Although the exact role of Tim11 as part of the Tim machinery is not yet clear, it is likely...
that it participates in sorting of preproteins at the inner membrane (Haucke et al 1997).

**Import Motor: Cooperation of Matrix Hsp70 with the Tim Machinery**

Tim44, the first component of the Tim machinery identified (Maarse et al 1992, Scherer et al 1992), is stably associated with the inner membrane but is mainly exposed at the matrix side (Figure 3). Although Tim44 can be found in close contact with preproteins in transit (Blom et al 1993, Horst et al 1993), it is most likely not a constituent of the import channel itself. Tim44 dynamically interacts with a fraction of Tim23, forming a Tim44-Tim23 subcomplex in the absence of preproteins (Bömer et al 1997). A clue to the function of Tim44 in protein import was the observation that it functions as a reversible membrane anchor for matrix Hsp70 (mtHsp70) (Kronidou et al 1994, Rassow et al 1994, Schneider et al 1994).

mtHsp70 (termed Ssc1 in the yeast *Saccharomyces cerevisiae*) is an essential component of the mitochondrial import machinery (Kang et al 1990, Scherer et al 1990). After the initial $\Delta \psi$-driven translocation of the N-terminal pre-sequence, mtHsp70 is required for translocation of the remainder of the precursor polypeptide across the inner membrane in an ATP-dependent manner (Gambill et al 1993). mtHsp70 supports the unfolding of precursor polypeptides; i.e binding of mtHsp70 to an N-terminal portion of a preprotein in the matrix facilitates unfolding of the domains that are still located on the cytosolic side (Figure 3) (Voos et al 1993). Two models were proposed to explain the role of mtHsp70 in protein import (Pfanner & Meijer 1995): (a) Brownian ratchet (trapping); the Tom and Tim machineries form passive diffusion channels for preproteins. By Brownian motion, precursor polypeptides can slide back and forth in the channels. mtHsp70 binds to the segments of a preprotein emerging on the matrix side, thereby preventing back-sliding and favoring its import (Ungermann et al 1994). (b) Import motor (pulling); mtHsp70 bound to Tim44 interacts with the precursor polypeptide (Horst et al 1996). mtHsp70 is an ATPase and changes its conformation upon binding of ATP (von Ahsen et al 1995). The conformational change of membrane-bound mtHsp70 generates mechanical force that pulls the preprotein into the matrix (Glick 1995). In particular, folded domains that are still located on the cytosolic side are pulled against the outer membrane import pore and unfolding is facilitated (Figure 3).

Recent evidence indicates that both mechanisms—pulling and trapping—cooperate in protein import (Voos et al 1996). Binding of mtHsp70 to the membrane anchor is crucial for the import of preproteins, which contain folded domains (pulling). A mutant of mtHsp70 defective in binding to the membrane...
anchor is still able to efficiently promote import of unfolded preproteins. Because a soluble mtHsp70 cannot generate a directional mechanical force, this mutant mtHsp70 most likely supports import by a trapping mechanism. In any case, the reaction cycle of mtHsp70 depends on binding and hydrolysis of ATP and thus represents a major site where metabolic energy is converted into driving force for import.

Tim44 is not the only partner protein of mtHsp70 engaged in preprotein import. The co-chaperone Mge1 (mitochondrial GrpE) is a matrix protein homologous to the nucleotide exchange factor GrpE of bacteria (Bolliger et al 1994, Laloraya et al 1994). Mge1 interacts with mtHsp70 bound to a precursor polypeptide (Figure 3) and promotes the reaction cycle of mtHsp70, thereby allowing nucleotide release (Voos et al 1994, Nakai et al 1994, Laloraya et al 1995, Westermann et al 1995, Schneider et al 1996, Dekker & Pfanner 1997, Miao et al 1997). Surprisingly, mtHsp70 can also transiently bind to the Tim17-Tim23 channel in a Tim44-independent reaction. Most likely the Tim machinery contains two membrane anchor sites for mtHsp70, Tim44 and Tim17, or a protein closely associated with it (Bömer et al 1997). It is not yet understood why two distinct membrane anchors for mtHsp70 are needed. One may speculate that the interaction of mtHsp70 with Tim44 is required in cases where a strong pulling force has to be generated, whereas binding to the Tim17-Tim23 channel serves to bring mtHsp70 as close as possible to the exit of the import channel. Because mtHsp70 only transiently interacts with each membrane anchor, the presence of two anchor sites could facilitate a continuous action of mtHsp70 molecules on a precursor polypeptide emerging on the matrix side.

The Tim22 Machinery for Insertion of Inner Membrane Carrier Proteins

The presence of a separate Tim machinery for carrier proteins, with the ADP/ATP carrier as a prominent member (Figure 4b), was originally suggested by the observation that a mutation in TIM17 affects import of cleavable preproteins, but not that of the ADP/ATP carrier (Pfanner et al 1994). Similarly, a mutant form of Tim23 inhibits the matrix targeting pathway of cleavable preproteins, but does not influence the insertion of carrier proteins. Sirrenberg et al (1996) identified Tim22, a hydrophobic mitochondrial inner membrane protein with homology to Tim17 and Tim23. Depletion of Tim22 does not affect the general import pathway of presequence-carrying preproteins into the matrix, but does inhibit the insertion of hydrophobic proteins into the inner membrane, in particular members of the family of inner membrane carriers. Tim22 is present in a protein complex of the inner membrane distinct from the Tim17-Tim23 complex (Sirrenberg et al 1996).
The pathway for the import of noncleavable inner membrane proteins (Figure 4b) is thus distinct from that of presequence-containing preproteins (Figure 4a). At the outer membrane, the ADP/ATP carrier uses Tom70 as the initial receptor and a trans site distinct from the intermembrane space domain of Tom22. At the inner membrane, the Tim22 machinery is essential for its import. Matrix ATP and mtHsp70 are not essential for import of the noncleavable inner membrane proteins (Pfanner et al 1987b, Wachter et al 1992, Bömer et al 1996b, 1997, Sirrenberg et al 1996), although a role of mtHsp70 for the import of noncleavable preproteins cannot be fully excluded (Ostermann et al 1990). Common elements for the import of cleavable preproteins and carrier proteins are located in the core of the Tom machinery: Tom22 (cytosolic domain), Tom5, and Tom40 are required for import of both types of preproteins. The membrane potential $\Delta \psi$ is needed for import of any preprotein into the inner membrane.

FOLDING OF PROTEINS IMPORTED INTO THE MATRIX

The presequences of preproteins entering the matrix are proteolytically removed by the dimeric mitochondrial processing peptidase (MPP) (for a further description of mitochondrial peptidases for preproteins, see Kalousek et al 1993). During or after proteolytic processing, the imported proteins fold into their active conformations. A number of folding helpers in the matrix can assist refolding of imported proteins (Figure 5). Depending on the type and conformation of imported proteins, the dependence on the various folding helpers can be quite different.

We first describe the basic functions of the mitochondrial folding helpers and secondly discuss the network of functional interaction.

1. The molecular chaperone mtHsp70 binds to most, if not every preprotein during import into the matrix, with Mge1 acting as the nucleotide exchange factor to facilitate cycles of binding and release. In addition, mtHsp70 interacts with completely imported preproteins and is required for folding in the matrix (Kang et al 1990, Voos et al 1994). Besides Mge1, a further co-chaperone, Mdj1, the homologue of bacterial DnaJ, cooperates with mtHsp70. Mdj1, which is also a chaperone in its own right, can bind preproteins directly (Westermann et al 1996). In analogy to the detailed studies with bacterial chaperones (summarized in Hartl 1996), it is assumed that mtHsp70 and Mdj1 keep preproteins in an unfolded state and prevent misfolding and aggregation.

2. The chaperonin Hsp60, which forms a 14-mer, functions together with the chaperonin 10 (Cpn10), a heptamer. Hsp60 promotes productive folding of

3. Peptidyl-prolyl cis/trans isomerases (PPIases) catalyze the cis/trans isomerization of peptide bonds preceding a prolyl residue (Schmid 1993). In the absence of PPIases, the isomerization is very slow and represents one of the rate-limiting steps of folding of several proteins. In mitochondria, a PPIase of 20 kDa was identified as component of the folding apparatus (Rassow et al 1995, Matouschek et al 1995). This PPIase can bind the immunosuppressive drug cyclosporin A and is termed cyclophilin 20.

The classical folding pathway in the mitochondrial matrix includes the following steps (Figure 5). A preprotein entering the matrix is bound by mtHsp70. ATP and Mge1 help to release mtHsp70 from the membrane anchor. Mdj1 together with mtHsp70 prevents misfolding of the imported protein. It is not known whether several different mtHsp70s sequentially bind and release from the preprotein or whether the same mtHsp70 interacts throughout the import process. In either case, release of mtHsp70 from the polypeptide is stimulated by the action of the nucleotide release factor Mge1 and the subsequent binding of ATP. The released protein interacts with Hsp60/Cpn10. The folding cage provided by the chaperonin promotes folding of the protein, typically requiring several ATP-dependent reaction cycles. When prolyl isomerization is a critical step, a PPIase such as cyclophilin can act on the protein released from mtHsp70 and/or Hsp60 and accelerate folding. Finally, the active conformation of the imported protein is obtained.

As shown by Rospert et al (1996), all these steps do not have to be followed by all preproteins, but productive folding can occur without interaction with at least some of these components (Figure 5). In the simplest case, a matrix chaperone (mtHsp70) is required only for membrane translocation, and the folding itself occurs spontaneously. Other preproteins use mtHsp70 together with Mdj1 to prevent misfolding but do not need to interact with Hsp60/Cpn10. Therefore, only some proteins are transferred on to the chaperonin. Hsp60 may then act alone, i.e. without Cpn10 (Höhfeld & Hartl 1994), or the full chaperonin cycle may be required. Thus a number of folding pathways exist in the mitochondrial matrix that probably do not form strictly linear pathways but build up a network of interactions. An interesting example was provided by Rassow et al (1995) with an imported protein that mainly required the mtHsp70 system and the PPIase cyclophilin 20. Upon inactivation of cyclophilin 20, folding was delayed, but the protein did not aggregate or misfold. It accumulated at mtHsp70 and, in addition, at Hsp60 (in a non-native form) and was finally folded to the active form after release from the chaperones. This example indicates that a network
Figure 5  Folding of proteins imported into the mitochondrial matrix. Matrix Hsp70 together with the co-chaperone Mge1 is required for membrane translocation of preproteins. In addition, mHsp70 can participate in folding of imported proteins in cooperation with mitochondrial DnaJ (Mdj1) and mitochondrial GrpE (Mge1). The chaperonin heat shock protein 60 (Hsp60) forms a 14-mer and, in cooperation with chaperonin 10 (Cpn10), provides a folding cage for some preproteins. Peptidyl prolyl cis/trans isomerases (PPIase) can accelerate folding. The mitochondrial processing peptidase (MPP) removes the N-terminal presequences of preproteins.
of interactions of folding helpers bears the advantage that a functional impairment of one component may be compensated to a certain extent by other folding helpers.

MECHANISTIC PRINCIPLES OF PROTEIN TRANSLOCATION

The molecular characterization of mitochondrial protein import in the past years has provided us with a first impression of the mechanisms that may govern the specific translocation of preproteins across the two mitochondrial membranes. We conclude this review by discussing hypotheses that explain mechanistic principles of translocation.

Acid Chain Hypothesis

Many import components contain patches of negatively charged residues. These acidic patches could direct the import of the positively charged targeting sequences, typically the N-terminal presequences but also internal targeting sequences (acid chain hypothesis) (Hönlinger et al 1995b). Acidic patches are found in the cytosolic chaperone MSF; in the cytosolic domains of the import receptors Tom70, Tom20, and Tom22; in the cytosolic segment of Tom5; in the intermembrane space tails of Tom22 and Tom40; in the intermembrane space domain of Tim23; and in the matrix-processing peptidase. In addition, the membrane potential across the inner membrane is negative on the matrix side and is critical in directing import of the presequences. A stepwise interaction of targeting sequences with the acidic patches could serve as (part of) a guiding system for import of preproteins (Bolliger et al 1995, Hönlinger et al 1995b).

Dynamic Translocases

Characterization of the Tom machinery yielded the first evidence for a dynamic nature of the membrane transport complexes. The currently available results suggest that a comparable principle governs the action of the Tim machinery. We propose that subcomplexes continuously assemble and dissociate and thereby facilitate transfer of preproteins and sorting of proteins at distinct stages of the import pathway.

At practically every import step, a lateral release of a preprotein is possible due to the partial dissociation of the Tom or Tim machinery. Sorting of the most abundant outer membrane protein, porin, into the membrane requires the action of Tom7 to dissociate the Tom complex and allows lateral release of the porin precursor (Figure 4d). Preproteins spanning the Tim machinery can be laterally released to allow sorting into the inner membrane or to the...
intermembrane space (Figure 4a) (Glick et al 1992, Gärtner et al 1995, Fölsch et al 1996). Coverage of the controversial discussion about intramitochondrial protein sorting can be found in Glick & von Heijne (1996), Schatz (1996), and Stuart & Neupert (1996).

The import machinery for noncleavable inner membrane proteins, the Tim22 complex, is separate from the main Tim machinery Tim17-Tim23-Tim44. Based on our mechanistic understanding of the Tom machinery, we propose that the two inner membrane Tim machineries are not completely separated but that they cooperate in preprotein import, particularly, for hydrophobic inner membrane proteins containing both internal targeting information and a presequence.

It is still not known how the Tom and Tim complexes cooperate. They can be physically connected by a membrane-spanning preprotein (Figure 3) (Horst et al 1995). However, in the absence of a preprotein, no evidence for a stable interaction between Tom and Tim complexes has been found. Tom complexes can function by themselves, for example for transport of the precursor of cytochrome c heme lyase into the intermembrane space (Figure 4c) (Lill et al 1992). In contrast to previous assumptions, the number of Tom complexes is substantially larger than that of the Tim17-Tim23 complexes. Only $\approx 20\%$ of the Tom complexes are needed to transfer preproteins to the Tim17-Tim23 complexes (Figure 4a) (PJT Dekker & N Pfanner, unpublished data). The other Tom complexes are engaged in different tasks, such as preprotein transfer to Tim22 and sorting of preproteins to the intermembrane space or to the outer membrane (Figure 4b–d). Tom complexes can thus be recruited for different functions. The lack of a stable interaction between the Tom complexes and the Tim17-Tim23 channel facilitates a multifunctional mode of action of the Tom machinery.

Energetics of Import and Generation of Driving Force
The electrical potential gradient $\Delta \psi$ and the ATP-dependent function of mtHsp70 are the two main driving forces of import. Both operate at the level of the Tim machinery. (a) $\Delta \psi$ is essential for initial entry of a preprotein into the inner membrane, both at the Tim17-Tim23 channel for presequence-carrying preproteins and at the Tim22 machinery for import of carrier proteins. The likely model for both transport systems is an electrophoretic effect on the positively charged targeting sequences. In addition, $\Delta \psi$ may induce conformational changes and assembly reactions of Tim proteins, such as the promotion of dimerization of Tim23. (b) The reaction cycle of mtHsp70 converts the energy stored in ATP into mechanical force that pulls a preprotein across the membranes (import motor). An ATP-induced conformational change of mtHsp70 can generate a directional force as long as mtHsp70 is bound to a membrane anchor. In the Brownian ratchet model (trapping model), binding of soluble mtHsp70 to the preprotein in transit does not generate a pulling force, but can
trap preprotein segments in the matrix and prevent back-sliding via the import channel. ATP is needed to release the preproteins from mtHsp70. In the trapping model, the energy of ATP is used to shift the equilibrium of preproteins sliding back and forth in the import channel toward the inward-directed forms.

When a preprotein is spanning both membranes, the driving force of mtHsp70 extends across the inner membrane to the outer membrane. Preprotein domains can thus be unfolded during entry into the Tom machinery. However, not all proteins are able to interact with mtHsp70 because they never enter the matrix. It is unknown what import driving force is provided by the Tom machinery itself for preproteins such as porin or cytochrome c heme lyase that do not require the Tim machinery, or for translocation intermediates at the outer membrane that are accumulated when entry into the inner membrane is blocked. It is likely that the import driving force at the outer membrane is considerably lower than that provided by the Tim/Hsp70 machinery, because in the absence of mtHsp70 function, stably folded domains cannot be unfolded and thus are unable to traverse the outer membrane (Gartner et al 1995).

The cytosolic chaperones MSF and cytosolic Hsp70 are ATPases. No evidence thus far indicates that the cytosolic chaperones can provide a driving force for membrane translocation of preproteins even though transfer of preproteins from MSF to the surface receptors requires ATP (Figure 2). Surprisingly, the transfer of preproteins from cytosolic Hsp70 to surface receptors seems to be ATP-independent (Komiya et al 1996).

In addition to the forces generated by the Tim/mtHsp70 translocation motor, free energy released by folding of imported proteins into their active conformation may be used to generate a (weak) import driving force. Such folding energy can be derived from proteins inserted into the membranes, e.g. porin or ADP/ATP carrier, or proteins refolded in a soluble subcompartment, e.g. cytochrome c heme lyase. Future studies will be required to determine whether the Tom machinery generates any driving force for translocation and if additional energy sources, e.g. nucleoside triphosphatases, are used in this process.

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