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Each eukaryotic cell contains several membrane-bound organelles in which distinct metabolic processes take place. Most of the proteins that perform these processes are encoded by the nuclear genome, synthesized in the cytosol and translocated co- or post-translationally to the organelle in which they function. Translocation of proteins into and across organelle membranes is an essential step in the biogenesis of a number of cell organelles, such as the endoplasmic reticulum, mitochondria, chloroplasts and peroxisomes. Since the majority of these proteins is synthesized in the cytosol, a well-organized intracellular protein trafficking system is required.

As a model system to study the process of protein translocation across membranes we have chosen the import of proteins into mitochondria of the bakers' yeast *Saccharomyces cerevisiae*. Yeast is an ideal model organism to study mitochondrial biosynthesis since it can be easily grown in large quantities, which is favourable for the isolation of mitochondria. Mitochondria can be easily isolated from yeast and used in an *in vitro* system to study mitochondrial protein import. In addition, a wide variety of both classical and modern genetic techniques can be applied to yeast, which include gene deletion, mutant generation and introduction of foreign DNA. Recently, the sequence information of the entire genome of *S. cerevisiae* has become available. This has facilitated the identification of genes encoding proteins involved in mitochondrial protein import.

Mitochondria are important organelles that contain hundreds of proteins that function in a variety of metabolic routes, the Krebs cycle and the respiratory chain. Electron transport through the respiratory chain is a source of the energy production of the cell. Mitochondria consist of four compartments formed by the outer and inner membranes, the intermembrane space and the matrix space (Figure 1). The inner membrane contains the protein complexes of the respiratory chain and the matrix space harbours many water-soluble enzymes including those of the Krebs cycle.
In fungal and animal cells, mitochondria are the only cytoplasmic organelles with their own genome. In yeast, only eight polypeptides are encoded by mitochondrial DNA and synthesized within the mitochondrion. All other mitochondrial proteins are encoded by chromosomes in the nucleus and synthesized on cytosolic ribosomes. These mitochondrial preproteins are synthesized with a mitochondrial targeting sequence or signal sequence, which is recognized by cytosolic chaperones that keep the preproteins in an import-competent conformation. The targeting sequence is then recognized by receptors on the mitochondrial surface which enables the mitochondria to selectively import the proper preproteins into the organelle. The subsequent translocation of the preproteins across both mitochondrial membranes to the matrix space is assisted by two distinct multisubunit protein complexes located in the mitochondrial outer and inner membrane. Finally, upon arrival of the preproteins in the mitochondrial matrix space, the targeting sequence is cleaved off by specific processing peptidases and mature proteins are folded into their functional conformation. A simplified overview of mitochondrial protein import is schematically depicted in Figure 2.

It is only a few years ago that initial progress was made in unraveling the mitochondrial protein import system with the identification of the first components of a multisubunit protein complex located in the mitochondrial outer membrane, now known as the Tom complex (translocase of the outer mitochondrial membrane). More recently, three subunits of a protein translocation system in the inner membrane were discovered, which was named Tim for translocase of the inner mitochondrial membrane. Even more recently, seven new Tim components were identified and all appeared to be subunits of another distinct Tim complex which is involved in the translocation of a specific subset of inner membrane proteins.

In this chapter an overview will be presented of our current knowledge of the system for protein translocation into mitochondria.

**Figure 2.** Simplified overview of the basic steps of protein import into mitochondria. Nuclear encoded mitochondrial proteins are synthesized with a mitochondrial targeting signal on ribosomes in the cytosol. Preproteins are recognized by cytosolic chaperones which prevent them from aggregation and keep them in a loosely folded import-competent conformation. Preproteins are then targeted to receptors (R) on the mitochondrial surface, followed by translocation through the general insertion pore (GIP) in the outer membrane. Preproteins destined for the mitochondrial matrix are subsequently translocated through the inner membrane translocation channel (Tim); this step requires a membrane potential (Δψ) across the inner membrane. Upon arrival of preproteins in the matrix space, N-terminal targeting sequences are cleaved off by specific processing peptidases. Matrix heat shock proteins (Hsp) assist in the complete translocation of the proteins into the matrix space and in folding of the processed proteins into their functional conformation.
Synthesis of mitochondrial preprotein on cytosolic ribosomes

Targeting by cytosolic chaperones

Recognition by receptors

Translocation through general import pore

Translocation through Tim complex

Folding mediated by heat shock proteins

Processing peptidase

Mature protein
Preproteins synthesized in the cytosol

Mitochondrial targeting signals

To allow the selective uptake of only mitochondrial preproteins into mitochondria, preproteins contain a mitochondrial targeting signal. This signal is usually present as an N-terminal extension of the mature protein and is cleaved off upon import of the preprotein into the mitochondrial matrix space, or is part of the mature protein. Although the location of the latter signal is in general assumed to be at the N-terminus, several examples are now known of mitochondrial proteins containing a targeting signal in the middle part or even at the C-terminus of the protein [33, 58, 97, 119]. The mitochondrial inner membrane carrier family is an example of a group of at least 34 proteins that is synthesized without cleavable targeting signal.

Although no consensus sequence has been established, N-terminal mitochondrial targeting signals have some specific structural properties. The length of the signal sequence can vary from 10 to 80 amino acids and is characterized by the presence of predominantly hydrophobic amino acids, a net positive charge and a lack of acidic residues. The sequence has the ability to form an amphipathic \( \alpha \)-helix with the hydrophobic residues all clustered at one face and the positively charged residues all grouped at the opposite face of the helix.

Completion of the yeast genome sequencing project has potentially revealed the sequence of all genes of \textit{S. cerevisiae} [51]. Newly identified genes often encode proteins with unknown functions and unknown cellular localization, amongst them in principle also new mitochondrial proteins. To predict the cellular localization of a protein, programs such as PSORT have been designed [141]. The algorithm of PSORT is based on the analysis of various sequence features of protein sorting signals. However, the results presented in Chapter 6 indicate that this and other currently available computational programs are not satisfactory for predicting the presence and location of a mitochondrial targeting signal in a protein sequence and illustrate the complexity of these targeting sequences. Definition of a consensus sequence or better definition of the features of a mitochondrial targeting signal can be helpful in the improvement of predictive programs that localize such a signal in a protein sequence, which will benefit the specificity of programs as PSORT that predict the cellular localization of proteins.

Recently, a new type of mitochondrial targeting signal was identified in the precursor protein of DNA helicase Hmi1 of \textit{S. cerevisiae} [119]. This helicase is involved in mitochondrial DNA inheritance and is synthesized with a C-terminal mitochondrial targeting signal which is cleaved off after import. This targeting signal is very similar to the known N-terminally located targeting signals with respect to the presence of a stretch of positively charged amino acids and the ability to form an amphipathic \( \alpha \)-helix. As expected, this C-terminal targeting signal directs import of helicase in a C- to N-terminal direction rather than in the N-to C-terminal direction as is the case for preproteins with the targeting signal at the N-terminus. This helicase may thus be a
representative of a new class of mitochondrial proteins using a novel targeting signal and possibly an exceptional import pathway. Cellular protein localization prediction programs such as PSORT only search for N-terminal mitochondrial targeting signals to predict a mitochondrial localization for a protein and mitochondrial proteins bearing a C-terminal targeting signal have therefore been overlooked so far.

**Intramitochondrial sorting signals**
Mitochondrial targeting signals at the extreme N-terminus of preproteins are generally used to direct precursor proteins to the matrix space, whereas internal mitochondrial signal sequences are often found in inner membrane proteins such as the carrier family proteins and most Tim proteins. More complex signal sequences are required for intramitochondrial protein sorting of preproteins such as cytochrome \( b_2 \) and cytochrome \( c_1 \) to the intermembrane space. These proteins contain a bipartite targeting sequence that consists of two targeting signals juxtaposed at the N-terminus and which direct the preproteins into the intermembrane space in two discrete steps. The signal at the very N-terminus directs the precursor to the matrix, where it is cleaved off by the mitochondrial processing peptidase. A second signal sequence which consists of basic residues and a stretch of hydrophobic amino acids is then exposed and acts as a sorting signal for targeting to the intermembrane space. This sorting signal is then finally cleaved off by one of two processing peptidases that are located at the intermembrane space side of the inner membrane (see section 'Proteolytic processing of mitochondrial targeting signals').

**Cytosolic chaperones involved in mitochondrial protein import**
For translocation into mitochondria, newly synthesized precursor proteins must maintain a loosely folded conformational state to allow their translocation across the mitochondrial membranes. Aggregation and misfolding is prevented by binding of cytosolic chaperones. The first cytosolic chaperone that was shown to function in mitochondrial protein import was Hsp70, a 70 kDa heat shock protein [49]. Hsp70 does not only function in mitochondrial protein import, but also in protein translocation across the membrane of the endoplasmic reticulum. Another cytosolic protein with a chaperone function, but specific for mitochondrial preproteins, is the mitochondrial import stimulation factor (MSF) from rat (the yeast homologue of MSF has not yet been identified). MSF is a heterodimer of 30 and 32 kDa subunits and specifically binds to N-terminal mitochondrial targeting sequences [135]. The recently discovered cytosolic chaperone Mft52 is also required for import of preproteins with N-terminal targeting sequences [34]. Mft52 contains a C-terminal located domain with a net negative charge, which shares sequence homology with a domain in the outer membrane translocase proteins Tom20 and Tom22. This domain is proposed to specifically bind to basic amphipathic N-terminal targeting sequences of mitochondrial preproteins.
MSF and Hsp70 are very similar with respect to their chaperone functions. Both recognize aggregated proteins, unfold them in an ATP-dependent manner and associate with unfolded proteins to stabilize their unfolded conformation. After binding to a mitochondrial preprotein, both Hsp70 and MSF deliver the bound preprotein to receptor complexes on the surface of the mitochondrial outer membrane.

**Translocation of preproteins across the outer membrane**

**Import receptors on the mitochondrial outer membrane**

After binding to cytosolic chaperones, preproteins are directed to receptor complexes on the mitochondrial surface. These receptor complexes are components of a dynamic multisubunit protein complex that is involved in the translocation of preproteins across the outer membrane, the Tom complex. This complex consists of at least nine proteins, that can roughly be divided into two classes: i) receptors on the cytosolic face of the outer membrane that are involved in the recognition of preproteins and ii) membrane embedded components that constitute and regulate the translocation channel or the general insertion pore (GIP) of the outer membrane (Table 1).

Tom20, Tom22, Tom37, and Tom70 were defined as receptor proteins on the mitochondrial surface and form the heterodimeric complexes Tom22-Tom20 and Tom70-Tom37 (numbers indicate the apparent molecular mass in kDa; new nomenclature according to Pfanner et al. [150]). Each protein contains a domain that protrudes into the cytosol and which functions as a docking site for incoming preproteins.

Tom20 is an integral membrane protein with its carboxy-terminus protruding into the cytosol and binds preproteins with N-terminal presequences and preproteins with internal targeting signals [30]. Tom20 binds to preproteins probably via hydrophobic interactions with the hydrophobic face of the amphipathic presequence [30].

Tom22 is one of two Tom proteins regarded to be essential for the viability of yeast [83, 122, 142]. It is attached to the outer membrane as an integral membrane protein, containing a cytosolically exposed amino-terminus and a small carboxy-terminal domain protruding into the intermembrane space. Both the N- and C-terminus of Tom22 contain several negatively charged amino acids, which are proposed to interact with the positively charged presequence of translocating preproteins via electrostatic interactions (see also section 'Acid chain hypothesis').

It is speculated that preproteins move from the cis binding site at the cytosolic N-terminus of Tom22 to the trans binding site at its IMS located C-terminus during translocation of a preprotein across the outer membrane. Tom 22 and Tom20 together form the most important receptor complex of the mitochondrial outer membrane. The purified cytosolic domains of Tom20 and Tom22 bind presequences without a requirement for other Tom proteins and display
an opposite binding affinity to presequences in the presence of salt, suggesting that they recognize different surfaces of a presequence [30]. Tom70 is the largest receptor unit and is also integrally attached to the outer membrane with its N-terminus and exposes a large C-terminal domain into the cytosol [72, 81, 188]. Tom70 preferentially binds mitochondrial preproteins bearing internal targeting signals such as the members of the family of carrier proteins [30, 174]. Tom72, which is highly homologous to Tom70, is loosely associated with the Tom complex and just like Tom70 it contains a large domain protruding into the cytosol. However, the protein is expressed at a very low level and deletion of the gene encoding Tom72 has no effect on the growth of *S. cerevisiae* on either fermentable or non-fermentable carbon sources [19]. Therefore, the protein does not seem to play an important role in mitochondrial protein import. The fourth receptor protein is Tom37. Although the exact function of Tom37 is not clear, it is thought to form a heterodimer with Tom70 to cooperate in the binding of preproteins with internal targeting information [67].

**Preprotein targeting to receptor complexes by cytosolic chaperones**

Newly synthesized preproteins containing an N-terminal mitochondrial targeting signal are bound by the cytosolic chaperone Hsp70. This complex of Hsp70 and preprotein is targeted to the surface of the outer membrane, where the precursor is handed over from Hsp70 to the Tom22-Tom20 receptor complex. Transfer of the preprotein does not require binding of Hsp70 to the receptor [135, 136]. At this stage of import extramitochondrial ATP is not consumed, either because the preprotein no longer requires Hsp70 to maintain a loosely folded state, or because Hsp70 can dissociate spontaneously from the precursor upon interaction of the Hsp70-precursor complex with the Tom22-Tom20 receptor complex. Precursor proteins carrying an internal mitochondrial targeting signal are bound by the cytosolic chaperone MSF. The MSF-preprotein complex is then targeted to the surface of the outer membrane, followed by the docking of the MSF-preprotein complex onto the Tom70-Tom37 receptor complex, forming an MSF-preprotein-receptor complex. MSF is released from this transient complex in an ATP-dependent manner [135, 136]. The precursor is then transferred from the Tom70-Tom37 receptor to the Tom22-Tom20 complex before it is handed over to the translocation channel for further passage across the outer membrane. Therefore, independent of the presence or absence of an amino-terminal targeting signal, all preproteins enter the general insertion pore via the Tom22-Tom20 complex. Both the Hsp70- and the MSF-dependent pathways function in parallel and their relative importance may be determined by the affinity of the precursor protein for Hsp70 or MSF.
The translocation channel of the outer membrane: the general insertion pore

After the recognition and binding of preproteins by the Tom70-Tom37 or the Tom22-Tom20 receptor complexes, all translocating preproteins enter the outer membrane translocation channel via the Tom22-Tom20 receptor complex. The translocation channel or general insertion pore is composed of Tom40 and the three small proteins Tom7, Tom6 and Tom5. Tom40 was the first component of the outer membrane translocation channel and was identified by photo-crosslinking to a precursor protein that was arrested during import into isolated yeast mitochondria. Tom40 appeared to be essential for the viability of yeast [5, 103, 196]. Although the protein lacks an obvious transmembrane sequence, it behaves as a typical integral membrane protein and is resistant to digestion by protease added to mitochondria [5]. As Tom40 is in close contact to preproteins in transit and several regions of its protein sequence are amphipathic, it was proposed that Tom40 contributes to the formation of a protein translocation channel with a hydrophilic interior. Later experimental evidence has confirmed that Tom40 forms the hydrophilic pore for transport of preproteins across the outer membrane [80].

**Figure 3.** Schematic representation of the initial steps of protein import into mitochondria: recognition and binding of mitochondrial preproteins by cytosolic chaperones MSF and Hsp70 followed by docking of the preprotein-chaperone complexes to the receptors Tom22-Tom20 and Tom70-Tom37 at the surface of the mitochondrial outer membrane. MSF, mitochondrial import stimulation factor; Hsp70, heat shock protein of 70 kDa; Tom, translocase of the outer mitochondrial membrane (numbers indicate the apparent molecular mass in kDa); GIP, general insertion pore, translocation channel of the outer membrane; OM, outer membrane.
The primary sequence of the integral membrane protein Tom7 does not show significant homology to any known protein. Loss of Tom7 produces yeast cells that are viable on both fermentable and non-fermentable carbon sources [82]. Co-immunoprecipitation experiments demonstrate that in mitochondria lacking Tom7, the interactions between Tom20, Tom22 and Tom40 are stabilized and that the import of matrix and inner membrane proteins is not affected. Absence of Tom7 appears to have an opposite effect on the interactions between Tom proteins compared to the effect caused by loss of Tom6, which results in a destabilization of the interactions between Tom22/Tom20, Tom40 and Tom70.

<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>
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<td>Tom72</td>
<td>Tom71</td>
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<td>Tom40</td>
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<td>Yes</td>
<td>Integral</td>
<td>Main component of GIP</td>
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<td>Mas37</td>
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<td>Integral</td>
<td>Receptor</td>
<td>[67]</td>
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<tr>
<td>Tom22</td>
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<td>Integral</td>
<td>Receptor and part of GIP</td>
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<td>Mas20, Mom19</td>
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<td>Receptor</td>
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<td>[82]</td>
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<tr>
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<td>Integral</td>
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<td>[3, 100]</td>
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<tr>
<td>Tom5</td>
<td>Mom8, Mom8a</td>
<td>No</td>
<td>Integral</td>
<td>Transfer from receptors to GIP</td>
<td>[50]</td>
</tr>
</tbody>
</table>

Table 1. Components of the transport machinery of the mitochondrial outer membrane. Tom, translocase of the outer mitochondrial membrane; essential for cell viability, requirement for growth on both fermentable and non-fermentable carbon sources.

The gene encoding Tom6 was isolated as a high copy suppressor of a tom40 mutant (isp42-3; [100]). Tom6 is a membrane spanning protein, not essential for cell viability and lacking significant homology with other proteins [100]. Tom6 contains its targeting signal at the C-terminus and is imported via an exceptional import route, since import takes place even in the absence of the known protein receptors on the mitochondrial surface [32]. Tom6 promotes cooperation between receptors and components of the GIP and thereby supports the efficient
transfer of preproteins [3]. Since Tom6 and Tom7 perform complementary functions it is proposed that they modulate the dynamics of the outer membrane translocase.

Tom5 was identified by co-immunoprecipitation with antibodies directed against Tom40 and is an integral membrane protein with a cytosolic N-terminal domain which has a net negative charge [50]. Preproteins interacting with the Tom22-Tom20 receptor complex are transferred to Tom5 and this is probably facilitated by the negative patches in the amino-terminal domain of Tom5. The close association of Tom5 and Tom40 may represent the functional link between the receptor complex and the GIP. Tom5 might thereby function as part of an 'acid chain' of increasing negative charges that drives translocation of the positively charged presequences of imported preproteins (see section 'Acid chain hypothesis') [50].

**Figure 4.** Schematic representation of the protein translocation complexes in the mitochondrial outer and inner membrane. OM, outer membrane; IMS, intermembrane space; IM, inner membrane; Tom, translocase of the outer mitochondrial membrane; Tim, translocase of the inner mitochondrial membrane; mtHsp70, mitochondrial heat shock protein of 70 kDa; Mge1, mitochondrial GrpE (nucleotide exchange factor).
Translocation of preproteins across the inner membrane

After translocation of the precursor proteins across the outer membrane with the help of the Tom complex, preproteins are handed over to Tim proteins that are involved in the translocation of the preproteins into and across the inner membrane. Two types of Tim complexes have now been discovered: the first one is the Tim23-Tim17 complex, the other one the recently identified Tim54-Tim22 complex. The Tim23-Tim17 complex is involved in translocation of precursor proteins with matrix space targeting presequences, whereas Tim54 and Tim22 are specifically involved in the translocation and membrane insertion of multipass inner membrane proteins such as the members of the carrier family proteins. Properties of the components of both complexes will be discussed in more detail in the next sections.

The Tim23-Tim17 complex: translocase for preproteins with a matrix targeting signal

Tim17 and Tim23 were identified with a genetic screen which was based on the mislocalization of a chimeric protein consisting of the presequence of the mitochondrial matrix protein SOD (manganese superoxide dismutase) and the URA3 gene product (a cytosolic enzyme with OMP decarboxylase activity). In a wild type strain carrying a deletion of the chromosomal URA3 locus, the chimeric protein is efficiently imported into mitochondria. Transformant cells can therefore not grow in the absence of added uracil. After mutagenesis of the strain harbouring this test plasmid, cells with mutations in proteins involved in the translocation of the chimeric protein into the mitochondria were selected by means of their ura+ phenotype [45, 127, 128]. TIM17 and TIM23 were then isolated by complementation-transformation of these mutants with a yeast genomic DNA library. The TIM23 gene was simultaneously identified using a different genetic screen [54]. TIM17 was also isolated as a multicopy suppressor of a tim23 mutation [161].

Both Tim17 and Tim23 are encoded by essential genes (initially termed MPI2 and MPI3, respectively [150]) and synthesized without cleavable presequences. The proteins have significant homology with regard to their protein sequence and structural features. The hydrophilic N-terminal domain of Tim23 of about 100 amino acids is however lacking in Tim17. The hydrophilicity plots of the entire Tim17 sequence and the corresponding C-terminal domain of Tim23 predict the presence of four membrane spanning domains [45]. Localization studies and sodium carbonate extraction experiments have shown that Tim17 and Tim23 are associated with the mitochondrial inner membrane and that they behave as integral membrane proteins. Chemical crosslinking indicated that Tim23 and Tim17 are in close proximity to preproteins in transit across the mitochondrial membranes, suggesting their involvement in
protein import, probably by constituting the protein translocation channel of the inner membrane [45, 128].

The region encompassing amino acids 50 to 100 of the intermembrane space localized N-terminus of Tim23 contains a leucine-zipper motif which may be involved in Tim23 dimerization [7]. After dimerization in an anti-parallel fashion in response to the membrane potential \( \Delta \psi \), a cluster of negatively charged amino acid residues will become exposed at the surface of the associated N-termini, and this may provide a binding site for positively charged N-terminal targeting sequences of translocating preproteins. Dimerization of Tim23 is a reversible process, since the dimer dissociates again upon interaction with an incoming preprotein, which probably triggers opening of the translocation channel and allows further translocation of the preprotein [7].

<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>
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<td>Tim44</td>
<td>Isp45, Mim44,</td>
<td>Yes</td>
<td>Peripheral</td>
<td>Membrane anchor for mtHsp70</td>
<td>[111, 127, 155, 171, 180]</td>
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<td></td>
<td>Mpi1</td>
<td></td>
<td></td>
<td></td>
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<td>Mas6, Mim23,</td>
<td>Yes</td>
<td>Integral</td>
<td>Import channel</td>
<td>[45, 54]</td>
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<td></td>
<td>Mpi3</td>
<td></td>
<td></td>
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<tr>
<td>Tim17</td>
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<td>Integral</td>
<td>Import channel</td>
<td>[112, 128, 161]</td>
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<td></td>
<td>Sma1</td>
<td></td>
<td></td>
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<td>mtHsp70</td>
<td>Ssc1</td>
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<td>Transient</td>
<td>ATP-dependent chaperone involved in protein import and folding</td>
<td>[40, 98, 199]</td>
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<td>Mge1</td>
<td></td>
<td>Yes</td>
<td>Transient</td>
<td>Nucleotide exchange factor, co-chaperone of mtHsp70</td>
<td>[16, 115, 181]</td>
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Table 2. Components of the mitochondrial inner membrane protein import machinery for matrix proteins. Tim, translocase of the inner mitochondrial membrane (numbers indicate the apparent molecular mass in kDa); essential for cell viability, requirement for growth on both fermentable and non-fermentable carbon sources; mtHsp70 and Mge1 are soluble matrix proteins that transiently associate with the Tim complex during protein import.

The Tim44-mtHsp70 protein translocation motor

The first protein that appeared to be involved in translocation of preproteins across the inner membrane was Tim44, initially termed Mpi1 [127]. This protein was identified using the same genetic screen as used for the isolation of \( \text{TIM23} \) and \( \text{TIM17} \) [45, 128]. Tim44 was also identified with a biochemical approach (thereby named Isp45) [171]. Antibodies raised against
Isp45 inhibited protein import into inner membrane vesicles and cross-linking and immunoprecipitation experiments indicated that Isp45 directly interacted with a precursor protein in transit across the inner membrane [171].

Tim44 is encoded by an essential gene and is synthesized as a precursor protein of 48.8 kDa. Upon import into mitochondria the presequence is cleaved off, resulting in a mature protein of 44 kDa. The hydrophilicity plot of Tim44 indicates that it is a hydrophilic protein and that the sequence lacks a significant hydrophobic region. Localization studies showed that Tim44 is a peripheral membrane protein that is localized at the matrix side of the inner membrane [13]. A c-myc epitope tag at the C-terminus of the protein is exposed to the intermembrane space, while antibodies directed against Tim44 inhibited protein import into inner membrane vesicles. Both observations suggest that Tim44 spans the inner membrane at least once [127, 171]. Nevertheless, the membrane topology of Tim44 is still the subject of debate. In contrast to our data, it was recently reported that a c-myc epitope tag at the C-terminus of Tim44 may not be exposed to the IMS [139].

Tim44 is associated with a small fraction of mitochondrial Hsp70 (mtHsp70) and this complex forms a matrix localized ATP-dependent motor for translocation of preproteins across the inner membrane. The SSC1 gene, which encodes mtHsp70, is essential for the viability of yeast [98, 170]. The co-chaperone Mge1 cooperates with mtHsp70 in the translocation of preproteins into mitochondria. Mge1 is also encoded by an essential gene and is a homologue of the prokaryotic nucleotide exchange factor GrpE. Mge1 promotes the release of nucleotides from mtHsp70 in the ATP-dependent reaction cycle of this chaperone system [16, 93].

The mechanism of the Tim44-mtHsp70 protein translocation motor

Two different mechanisms have been postulated for the role of the Tim44-mtHsp70 complex in the translocation of preproteins. They are referred to as the ‘Brownian ratchet’ model and the ‘pulling motor’ model [152]. In the first model, the Tom and Tim translocases form passive diffusion channels through which the preprotein can slide back and forth by Brownian motion. As soon as the preprotein emerges in the matrix space, it is caught by mtHsp70 which traps the preprotein and prevents it from sliding back into the cytosol. By Brownian motion, the preprotein will move a little further into the matrix space and will be bound by another mtHsp70 molecule. By the consecutive action of several bound mtHsp70 molecules, the preprotein will be completely translocated across the inner membrane. According to the ‘pulling motor’ model, a preprotein emerging in the matrix space will be bound by mtHsp70, which itself is attached to the inner membrane via Tim44. Binding of ATP to this mtHsp70 molecule will then induce a conformational change of the membrane-bound mtHsp70 and this generates a mechanical force which actively pulls the preprotein towards the matrix space [64, 85, 197]. Several cycles of ATP binding, ATPase activity and release of ADP enables mtHsp70 to translocate the complete
preprotein across the inner membrane. The 'pulling motor' model can also explain the unfolding of folded domains of translocating preproteins that are still located in the cytosol, the so-called unfoldase activity of mtHsp70. Recent evidence indicates that the 'pulling and trapping' mechanisms cooperate in the translocation of preproteins across the mitochondrial inner membrane [199].

**Figure 5.** Schematic representation of the molecular organization of the Tim complex in the mitochondrial inner membrane. A leucine zipper motif in the N-terminus of Tim23 is involved in dimerization of Tim23 molecules at the intermembrane space side of the inner membrane. Putative coiled-coil structures in the N-terminus of Tim44 may contribute to the dimerization of Tim44 at the matrix side of the inner membrane. Tim complex, translocase of the inner mitochondrial membrane; IMS, intermembrane space; IM, inner membrane; matrix, matrix space; 17, Tim17; 23, Tim23; 44, Tim44; mtHsp70, mitochondrial Hsp70.

The N-terminal domain of Tim44 contains a region predicted to form coiled-coils, which is conserved in Tim44 of different species. Coiled-coil structures are involved in protein-protein interactions and may mediate dimerization of Tim44 molecules. In crosslinking experiments it was shown that Tim44 can indeed form dimers that can recruit two mtHsp70 molecules to the site of protein import [139]. The cooperation of two Tim44-bound mtHsp70 molecules which sequentially bind incoming segments of a precursor protein in transit in a hand-over-hand mechanism may increase the efficiency of protein import in comparison with the presence of only a single mtHsp70 molecule at the protein import site [139] (Figure 5).
The Tim54-Tim22 complex: translocase for polytopic inner membrane proteins

The recent identification of additional Tim proteins indicated the existence of a distinct Tim complex, with a function different from the Tim23-Tim17 complex. This novel Tim complex is specifically involved in the translocation and membrane insertion of members of the mitochondrial carrier family proteins and other polytopic membrane proteins into the inner membrane. Members of the carrier family, for example AAC and the phosphate carrier PiC, have six membrane spanning domains, are very hydrophobic and are synthesized without a cleavable N-terminal targeting sequence. They are not imported via the Tim23-Tim17 machinery.

The first member of this alternative inner membrane translocation complex is Tim22, which was identified during the yeast genome sequencing project due to its significant homology to Tim17 and Tim23 (>50% similarity) [185]. The TIM22 gene encodes a protein of 21.8 kDa that is essential for the viability of yeast and whose C-terminal region is very similar to the hydrophobic domains of Tim23 and Tim17. Tim22 is an integral inner membrane protein and is present in a 300 kDa complex [185].

Another protein required for the import and membrane insertion of polytopic carrier proteins into the inner membrane is Tim54. It was identified in a two-hybrid screen as a partner protein of Mmm1, a protein involved in the maintenance of mitochondrial morphology [101]. The essential TIM54 gene encodes a 54.2 kDa integral membrane protein with one or two membrane spanning domains and a C-terminus which faces the intermembrane space. It was shown that Tim54 both genetically and physically interacts with Tim22 but not with Tim23 and Tim17, providing additional evidence for the existence of two distinct Tim complexes [101].

The recently discovered Tim complex consists of the two membrane proteins Tim22 and Tim54 and in addition the smaller subunits Tim8, Tim9, Tim10, Tim12 and Tim13. The intermembrane space localized peripheral inner membrane protein Tim12 and the soluble intermembrane space protein Tim10 were identified as multicopy suppressors of the respiratory-deficient mrs2-1 mutation and were previously termed Mrs5 and Mrs11, respectively [95, 96]. The MRS5 and MRS11 genes encode proteins of 13 and 12 kDa, respectively, and are the first known intermembrane space proteins that are essential for the viability of yeast. Depletion of either protein results in the accumulation of Hsp60 in the cytosol and other severe defects in mitochondrial function, implying a role for them in mitochondrial protein import. Mrs5 and Mrs11 finally appeared to specifically mediate import of carrier proteins into mitochondria as components of the Tim54-Tim22 system and were therefore from then on called Tim12 and Tim10 [186].
Tim9 was identified in a search for extragenic suppressors of the temperature-sensitive *tim10-1* mutation [108] and also by immunoprecipitation with anti-Tim10 antibodies [1]. The *TIM9* gene is essential and encodes for a protein that is 25% identical to Tim10 and Tim12.

<table>
<thead>
<tr>
<th>Component</th>
<th>Essential for cell viability</th>
<th>Membrane association</th>
<th>Proposed function</th>
<th>References</th>
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</thead>
<tbody>
<tr>
<td>Tim54</td>
<td>Yes</td>
<td>Integral</td>
<td>Insertion of carrier proteins into inner membrane</td>
<td>[101]</td>
</tr>
<tr>
<td>Tim22</td>
<td>Yes</td>
<td>Integral</td>
<td>Insertion of carrier proteins into inner membrane</td>
<td>[185]</td>
</tr>
<tr>
<td>Tim13</td>
<td>No</td>
<td>Soluble</td>
<td>Unknown</td>
<td>[107]</td>
</tr>
<tr>
<td>Tim12</td>
<td>Yes</td>
<td>Peripheral</td>
<td>Insertion of carrier proteins into inner membrane</td>
<td>[96, 186]</td>
</tr>
<tr>
<td>Tim11</td>
<td>No</td>
<td>Integral</td>
<td>Sorting of intermembrane space proteins at the inner membrane</td>
<td>[4, 192]</td>
</tr>
<tr>
<td>Tim10</td>
<td>Yes</td>
<td>Transient</td>
<td>Delivery of carrier proteins from Tom to Tim complex</td>
<td>[95, 186]</td>
</tr>
<tr>
<td>Tim9</td>
<td>Yes</td>
<td>Soluble, fraction membrane-associated</td>
<td>Delivery of carrier proteins from Tom to Tim complex</td>
<td>[1, 108]</td>
</tr>
<tr>
<td>Tim8</td>
<td>No</td>
<td>Soluble</td>
<td>Unknown, DDP1 homologue</td>
<td>[107]</td>
</tr>
</tbody>
</table>

Table 3. Components of the mitochondrial inner membrane protein import machinery for carrier proteins. Tim, translocase of the inner mitochondrial membrane; essential for cell viability, requirement for growth on both fermentable and non-fermentable carbon sources.

All three small Tim proteins contain the four conserved cysteine residues of a so-called 'twin CX3C' motif, which could function as a zinc-finger in protein-protein interactions. The majority of Tim9 is associated with Tim10 as a soluble intermembrane space localized 70 kDa complex; a small fraction of Tim9 is part of a 300 kDa membrane-bound complex which also contains Tim10, Tim12, Tim22 and Tim54 [1, 108]. Tim9 is probably also present in another, less abundant 70 kDa complex consisting of Tim9/Tim10/Tim12, which is tightly associated with the inner membrane [1].
General introduction

Figure 6. Schematic representation of the protein translocases of the mitochondrial inner membrane. Mitochondrial preproteins destined for the matrix- and intermembrane space are translocated by the Tim23-Tim17/Tim44-mtHsp70 complex, while mitochondrial inner membrane proteins carrying internal targeting sequences are directed to the inner membrane by the Tim54-Tim22 system. The latter system also consists of soluble components in the intermembrane space, which probably function as chaperones for transport of hydrophobic polypeptides through the aqueous phase of the intermembrane space to bring preproteins emerging at the trans site of the outer membrane to the Tim54-Tim22 complex. OM, outer membrane; IMS intermembrane space, IM, inner membrane; matrix, matrix space; 9, Tim9, 10, Tim10; 12, Tim12.

The most recently identified translocase components are Tim8 and Tim13. Both were identified by screening the yeast genome database for additional homologues of Tim9, Tim10 and Tim12 [107]. The genes encoding Tim8 and Tim13 are not essential and deletion of their chromosomal loci has no significant effect on the growth of yeast cells. Tim8 and Tim13 both display the 'twin CX3C' motif of Tim9, Tim10 and Tim12. Both proteins are localized in the intermembrane space and associated with a 70 kDa complex which also contains a small fraction of Tim9. They are not present in the 300 kDa membrane bound complex. Tim8 is the yeast homologue of the human DDP1 protein, a protein of the intermembrane space with an unknown function. Mutations in DDP1 result in human deafness dystonia, a mitochondrial disease. The homology
between Tim8 and DDP1 suggests that the deafness dystonia syndrome is a disease that is probably caused by a defective mitochondrial protein import machinery.

**Intramitochondrial protein sorting of intermembrane space proteins**

Almost all preproteins destined for the mitochondrial matrix space are synthesized with a cleavable N-terminal targeting sequence. These preproteins are imported into mitochondria via the general import pathway, which is specifically mediated by the Tim23-Tim17 complex. The identification and characterization of several components of another Tim complex in the inner membrane led to the understanding that a specialized complex is involved in import and assembly of a number of polytopic inner membrane proteins with internal targeting sequences. Proteins that are destined for the intermembrane space, such as cytochrome \( \text{b}_2 \) and cytochrome \( \text{c}_1 \), carry a bipartite targeting sequence which directs these proteins to their proper location via a complex intramitochondrial sorting route. The first part of this presequence is located at the extreme N-terminus and resembles a typical matrix targeting signal, which is proteolytically removed by mitochondrial processing peptidase (MPP) in the matrix space to generate an intermediate-sized protein. The second part of the bipartite sequence consists of a stretch of hydrophobic residues which directs the protein to the IMS. This sorting signal is then cleaved off by the IMS localized catalytic site of inner membrane protease 1 (IMP1).

Two conflicting models have been proposed to explain the mechanism of intramitochondrial protein sorting. The first one suggests that the entire precursor polypeptide is translocated into the matrix space, where the N-terminal matrix targeting signal is cleaved off by MPP. Reexport across the inner membrane delivers the intermediate-sized protein into the IMS \([70, 71]\). Since mitochondrial sorting signals are similar to targeting sequences of secretory bacterial proteins, and the reexport of proteins with bipartite presequences resembles the mechanism of protein export in bacteria, this model is called the 'conservative sorting' model \([68, 70, 109]\).

According to the 'stop-transfer' model, the sorting signal causes an arrest of preprotein translocation in the inner membrane, followed by lateral movement of the precursor into the inner membrane without further translocation into the matrix space \([11, 87, 99]\). The 'stop-transfer' model suggests that sorting of IMS proteins can be performed by components of the inner membrane translocase, whereas the 'conservative sorting' model may require the presence of a separate reexport system for sorting of these proteins.

The first indication for the existence of proteins specifically involved in the sorting of IMS proteins came with the identification of Tim11. This 11 kDa inner membrane protein was identified by chemical cross-linking to the sorting sequence of cytochrome \( \text{b}_2 \). Tim11 is not essential for the viability of yeast but required for optimal mitochondrial function \([192]\). However, it was then found that Tim11 is not associated with Tim complexes but that it is
identical to subunit e of the yeast F$_1$F$_0$-ATPase, a subunit so far only known in mammalian ATPases [4]. Whether Tim11 has a dual function, both as a subunit of the ATPase and in mitochondrial protein translocation, remains to be elucidated.

Molecular chaperones and isomerases of the matrix space

Mitochondrial Hsp70, Hsp60, Hsp10 and PPIases

As discussed in previous sections, the chaperone mtHsp70 plays a crucial role in the import of preproteins into mitochondria. In addition, mtHsp70 is required for the folding of many completely imported proteins in the matrix space [98]. The activity of the bacterial homologue of Hsp70, DnaK, is regulated by the co-chaperones GrpE and DnaJ [62, 183]. GrpE functions as a nucleotide exchange factor for Hsp70 by enabling the chaperone to shift from an ADP-bound state to an ATP-bound state [148]. Mdj1, the mitochondrial DnaJ homologue, is a membrane protein that can function as a chaperone independently of mtHsp70 [203] and only functions in protein folding steps after completion of preprotein translocation [86, 158]. The presence of a small domain in Tim44 with limited sequence similarity to DnaJ suggested that the interaction of Tim44 and mtHsp70 may resemble that of DnaK and DnaJ. This DnaJ resembling fragment is needed for efficient interaction of Tim44 and mtHsp70 and deletion of this domain is lethal in yeast [134]. Mge1, the mitochondrial GrpE homologue of S. cerevisiae, is an essential protein and required for mitochondrial protein import. Mge1 functions as a nucleotide exchange factor to promote the release of ADP from mtHsp70 during the reaction cycle of this chaperone [16, 89, 117].

Following the interaction with mtHsp70, imported proteins can be transferred to the heat shock protein Hsp60 which further mediates folding of the polypeptide. Hsp60, the functional homologue of bacterial GroEL, is constituted of 14 identical subunits of approximately 60 kDa that form two donut-like shaped heptameric rings stacked on top of each other, forming a cavity in which protein folding presumably takes place [36, 157]. Matrix ATP and the bacterial GroES homologue Hsp10 (Cpn10) are required for activity of Hps60. Hsp10 consists of a single heptameric ring structure of 10 kDa subunits, which can bind onto the Hsp60 structure and functions as a lid to cover the Hsp60-cavity [118].

Another class of proteins that constitute the mitochondrial protein folding apparatus are the cyclophilins. Cyclophilins were first identified by their ability to bind the immunosuppressive agent cyclosporin A, but were later found to have peptidyl-prolyl cis/trans isomerase (PPIase) activity. PPIases catalyze the cis/trans isomerization of peptide bonds preceding a prolyl residue and thereby accelerate the slow refolding processes of proteins that require peptidyl-prolyl isomerization. In mitochondria, a 20 kDa PPIase called cyclophilin 20 was identified which was shown to be a component of the mitochondrial protein folding machinery and to cooperate with
Hsp70 and Hsp60 [132, 156]. In the absence of cyclophilin 20, preproteins are still imported into mitochondria and folded into their active conformation, although the folding process is very slow [132, 156].

**Proteolytic processing of mitochondrial targeting signals**

**Mitochondrial processing peptidase and mitochondrial intermediate peptidase**

Translocation of proteins across membranes is often accompanied by proteolytic removal of targeting signals from the transported precursor proteins. This process is catalyzed by a group of proteases that includes soluble as well as membrane-bound enzymes. Most mitochondrial preproteins destined for the matrix space contain a cleavable N-terminal mitochondrial targeting signal which is proteolytically removed upon import of the preprotein into the matrix. Most of these proteins are processed by a single proteolytic cut of the targeting sequence by the mitochondrial processing peptidase (MPP). In fungi and mammals MPP is a soluble enzyme of the mitochondrial matrix space, whereas in plants it is localized in the inner membrane as part of the cytochrome \( bc_1 \) complex of the respiratory chain [29]. The processing activity of yeast MPP requires the presence of divalent metal ions [15]. In yeast, MPP is a soluble heterodimer composed of the two structurally related subunits \( \alpha \)-MPP and \( \beta \)-MPP. \( \alpha \)-MPP is thought to be involved in the recognition and binding of substrates, while \( \beta \)-MPP functions as the catalytic subunit. Both MPP subunits are required for processing activity [15, 126]. MPP recognizes a large number of N-terminal presequences. These vary in length from 8 to 69 amino acids and lack notable primary sequence similarity, although conserved arginine residues are located at positions -2 and -3 with respect to the MPP cleavage site. Most preproteins with N-terminal targeting sequences are processed in one step by MPP, but others, such as the precursors of cytochrome oxidase subunit IV and the Rieske Fe/S protein of the cytochrome \( bc_1 \) complex bear bipartite targeting sequences, which are cleaved off in two steps. The first maturation step of these preproteins is performed by MPP, while further processing of an N-terminal octapeptide results in the mature protein. This second cleavage step is accomplished by another matrix peptidase, the mitochondrial intermediate peptidase (MIP) [90, 91]. Why maturation of these precursor proteins occurs in two steps is not known.

**Inner membrane protease**

Import of precursor proteins of a number of intermembrane space proteins such as cytochrome \( b_2 \) involves another bipartite targeting sequence which is also removed in two proteolytic steps. In the first step the matrix targeting signal is cleaved off by MPP, generating an intermediate-sized form of cytochrome \( b_2 \). In the second step, a sequence for sorting cytochrome \( b_2 \) to the
intermembrane space is proteolytically removed in the intermembrane space by the inner membrane protease (IMP), thereby releasing the mature enzyme. IMP is a complex of two structurally similar subunits, Imp1 and Imp2, which have different substrate specificities. Imp1 mediates the processing of cytochrome $b_2$, the mitochondrially encoded cytochrome oxidase subunit 2 (Cox2) and NADH-cytochrome $b_5$ reductase. It is a 21.4 kDa integral inner membrane protein with a C-terminal part exposed on the outer face of the membrane [176, 177]. Imp1 requires divalent cations and acidic phospholipids for activity and is expressed at a low level [178]. Imp2 shares 25% sequence identity with Imp1 and is also an inner membrane protein with a large domain protruding into the intermembrane space. Imp2 catalyzes the second processing step of the cytochrome $c_1$ precursor and is required for the stability of Imp1 [147].

A third protein involved in the maturation of preproteins in the intermembrane space was named Soml [55]. It was identified as a high copy suppressor of an imp1 mutant. Soml is a small, weakly expressed protein of 8.4 kDa and localization studies suggest that it is membrane associated, although a membrane spanning domain cannot be predicted from its amino acid sequence. Soml is essential for the proteolytic maturation of Cox2 and for the stability of cytochrome $b_2$. Soml represents a novel factor that is essential for the correct function of IMP and/or the protein sorting machinery [55].

**Mechanistic principles of protein translocation**

**Energy requirements for mitochondrial protein import**

An early step in mitochondrial protein import is the binding of the cytosolic chaperones MSF and Hsp70 to preproteins and the subsequent targeting of bound preproteins to the mitochondrial surface. Both MSF and Hsp70 are ATPases and MSF hydrolyses ATP upon release of the bound preprotein to the Tom70-Tom37 receptor complex at the outer membrane surface. The presence of ATP in the cytosol is therefore necessary for the function of MSF and Hsp70 and is the first energy requirement for mitochondrial protein import.

Another energy requirement for the import of preproteins is a membrane potential Δψ across the inner membrane, which generates a negative charge on the matrix space side and positive charge on the intermembrane space side. Transport of preproteins into or across the inner membrane is absolutely dependent on the presence of Δψ [59, 173]. Δψ is only essential during the initial steps of preprotein translocation across the Tim channel, when the positively charged presequence traverses the inner membrane, and not during translocation of the mature part of a preprotein [131, 193]. It has been suggested that Δψ exerts an electrophoretic effect on the targeting sequences of precursor proteins. The negative charge of Δψ at the matrix space side of the inner membrane may exert an electrophoretic force on the positively charged amino-terminal targeting signal to drive its translocation across the inner membrane.
A further energy requirement for import of preproteins is the presence of ATP in the matrix space. Translocation of the targeting signal is dependent on a membrane potential $\Delta \psi$, but translocation of the complete protein requires ATP in the matrix space. After translocation of the targeting signal across the inner membrane, the preprotein emerges in the matrix space where it is bound by mtHsp70. Tim44-bound mtHsp70 mediates translocation of the entire preprotein across the inner membrane. Since the function of mtHsp70 is driven by several consecutive rounds of binding and hydrolysis of ATP, this final stage of protein import explains at least part of the importance of matrix ATP.

'Acid chain' hypothesis
Protein translocation across the outer membrane is driven in the absence of a membrane potential or an ATP-dependent system which may provide the energy for protein transport. It is thus not clear which force drives unidirectional protein transport across the outer membrane. Most of the Tom proteins contain clusters of negatively charged amino acids in their protein sequence, which may interact with the positively charged targeting signals of preproteins. In fact, not only Tom proteins contain patches with a net negative charge, but several other proteins mediating mitochondrial protein import harbour clusters with negatively charged amino acids, such as the cytosolic chaperone Mft52 and the inner membrane protein Tim23. The presence of these negatively charged clusters in these proteins along the import pathway led to the 'acid chain' hypothesis, which proposes that a preprotein is translocated across the mitochondrial membranes via the interaction of its positively charged presequence with the net negative clusters of the import components [17, 83, 167]. This suggests that each successive import component must harbour a patch with an increasing net negative charge to allow the effective transfer of preproteins from one patch to the next patch. The hypothesis is based on an analogy with the 'methionine bristles' that are present on the 54 kDa subunit of the signal recognition particle (SRP), which were suggested to bind the hydrophobic targeting sequences of proteins destined for the endoplasmic reticulum [9].
In the cytosol, the chaperone protein Mft52 contains two negatively charged domains which are homologous to those domains of Tom20 and Tom22. These domains in Mft52 specifically interact with preproteins bearing a basic, amphipathic N-terminal targeting sequence, allowing Mft52 to enhance preprotein delivery to the mitochondrial surface [34]. At the cis site of the mitochondrial outer membrane, the cytosolic domains of the receptors Tom20 and Tom22 display patches of negatively charged amino acids that are thought to interact with the positively charged presequences of preproteins. Tom5 may be the functional link between the surface receptors and Tom40 and contains an N-terminal cytosolic domain with a net negative charge. The pore-forming protein Tom40 also contains clusters of negatively charged amino acids. After translocation through the GIP, preproteins are thought to interact with the trans site of the outer
membrane, probably via the negatively charged amino acids of the intermembrane space domain of Tom22. Finally, preproteins containing an N-terminal targeting signal are probably transferred to the intermembrane space localized N-terminus of Tim23, which harbours another cluster of negatively charged amino acid residues and which may thus provide a cis binding site for preproteins on the inner membrane. It is not clear whether the predicted increase in negative charge is actually present in successive patches of the acid chain.

**Molecular organization and dynamics of the Tim and Tom translocases**

Tom and Tim complexes cooperate transiently during translocation of precursor proteins, but can also function independently from each other in the import process [6, 88, 120]. Preproteins cross the mitochondrial membranes at so-called translocation contact sites, where the two membranes are closely juxtaposed. The Tom and the Tim complexes are physically connected by a translocating preprotein [84]. In the absence of a translocating precursor protein, a salt-resistant complex was detected between the C-terminal domain of Tom22 which protrudes into the IMS and the N-terminus of Tim23, which also faces the IMS [110]. This suggests that the intermembrane space domains of Tom22 and Tim23 mediate a transient association of the outer and inner membrane protein transport channels.

Tom40 and Tom22 are stably associated in a 400 kDa complex together with the small Tom proteins Tom7, Tom6 and Tom5 [46-48]. The majority of Tom20 and Tom70 is not part of this 400 kDa complex. In mutant mitochondria lacking Tom6, the interaction between Tom40 and Tom22 is destabilized, leading to a dissociation of Tom22 from the 400 kDa GIP complex to a 100 kDa complex containing Tom40, Tom7 and Tom5. A lack of Tom6 thus causes a destabilization of the 400 kDa complex. The interaction between Tom40 and Tom22 could be restored by the import of Tom6 into isolated tom6Δ mutant mitochondria, indicating that Tom6 is required to promote the association between Tom40 and Tom22 [48].

Tim17 and Tim23 are present in a 90 kDa complex, which is required for the import of preproteins carrying N-terminal targeting signals. Preproteins in transit spanning both mitochondrial membranes connect most of the 90 kDa Tim complexes with ± 25% of the 400 kDa Tom complexes, forming a 600 kDa supercomplex [46]. This indicates that about 1 in 4 Tom complexes is connected to a Tim complex in translocation contact sites and suggests that the other Tom complexes may have different functions, such as preprotein sorting to the outer membrane and the intermembrane space and preprotein transfer to the Tim54-Tim22 complex.

Bömer et al. showed that Tim23 is present at the mitochondrial inner membrane in two pools, a Tim44-Tim23-containing subcomplex and a Tim23-Tim17-containing subcomplex [18]. On the other hand, recent results suggest that Tim17, Tim23, Tim44 and mtHsp70 are all present in a unique complex, with a ratio of Tim17:Tim23:Tim44:mtHsp70 being approximately 1:1:1:30 [139]. These authors were unable to detect two different subcomplexes both containing Tim23.
It could be shown that Tim44 is associated with the Tim23-Tim17 complex by ionic interactions and that Tim44 is in close contact to a membrane protein of 14 kDa. This protein might be similar to a previously identified 14 kDa protein, which was proposed to be a subunit of the Tim23-Tim17 complex and which was named Tim14 [10]. Previous immunoprecipitation experiments have suggested the existence of several new putative components of the Tim complex, which were proteins with molecular masses of 14, 20, 33 and 44 kDa [10, 12]. However, none of these proteins has been identified yet.