Translocation of Proteins into Mitochondria

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Abstract

About 10% to 15% of the nuclear genes of eukaryotic organisms encode mitochondrial proteins. These proteins are synthesized in the cytosol and recognized by receptors on the surface of mitochondria. Translocases in the outer and inner membrane of mitochondria mediate the import and intramitochondrial sorting of these proteins; ATP and the membrane potential are used as energy sources. Chaperones and auxilliary factors assist in the folding and assembly of mitochondrial proteins into their native, three-dimensional structures. This review summarizes the present knowledge on the import and sorting of mitochondrial precursor proteins, with a special emphasis on unresolved questions and topics of current research.

Contents

INTRODUCTION

Translocase: a membrane-embedded protein complex that mediates translocation of polypeptides from one side of the membrane to the other side

Almost the whole complement of proteins that constitute the mitochondria are encoded in the nucleus. These proteins are translated on ribosomes in the cytosol as precursors, which differ from the functional forms in the mitochondria in a number of ways. Most of them, if not all, are in an unfolded conformation and associated with chaperones, which maintain them in a translocation-competent conformation. They are endowed with signals for targeting to the surface of the mitochondria and for transport and sorting to the various mitochondrial subcompartments. In order to become functional proteins, these

precursors have to be recognized by receptors, threaded through pores in the membranes of the mitochondria, proteolytically processed, folded and often inserted into membranes, and assembled with cofactors and other proteins to macromolecular complexes.

During the past 10 years, a large body of information has been collected on the pathways, and the translocases and protein components that mediate these processes. Both the number of pathways and the number of components discovered have grown beyond what was generally expected. We have now a much more detailed picture of how the plethora of nuclear-encoded proteins are targeted to the mitochondria and sorted to the various mitochondrial subcompartments. The present review is based on an overview, which was published 10 years ago in the *Annual Review of Biochemistry* (1). It is focused on the progress made since then and can be read as a continuation of the previous review.

MITOCHONDRIAL PRECURSOR PROTEINS

A large body of evidence suggests that mitochondrial proteins are synthesized on free ribosomes in the cytosol, released as completed precursor polypeptide chains, and then imported into mitochondria in a posttranslational fashion. Observations to support this view have been obtained by experiments in vivo and in vitro. This, however, does not mean that proteins cannot be imported cotranslationally (1). Because most preproteins have N-terminal targeting signals, translocation could well start before the polypeptide chain is completed. In fact, in some cases, such a mechanism has been proposed to explain the particularities of the biogenesis of certain proteins such as fumarase (2, 3). It was proposed that mechanisms exist that direct messenger RNAs to the surface of the mitochondria where they could be translated on ribosomes bound to the outer membrane (4, 5). Definite proof for the existence of such targeting mechanisms, however, has not been provided.

Cytosolic Precursors

Precursor proteins in the cytosol are present as complexes with factors that are thought to stabilize them, as they are not in their final conformation and therefore are prone to degradation and aggregation. Several such factors have been described; the only ones for which convincing evidence has been provided are cytosolic chaperones Hsp70 and Hsp90 (6, 7). It is still unclear what the signals are that lead to the binding of these chaperones, when they are bound and released, and whether there is a specific role for the targeting signals present on the precursors.

Mitochondrial Targeting Signals

Cytosolic precursors of mitochondrial proteins contain information that is necessary and sufficient to direct them to the mitochondria. In most cases, this information is present as a cleavable sequence at the N terminus, also called presequence or prepeptide. However, many precursors lack such sequences and instead contain internal targeting signals.

Matrix targeting signals. The N-terminal targeting sequences are also called matrix-targeting sequences (MTSs) because they also bring the N terminus across the inner membrane into the matrix. In the absence of further sorting information, they direct proteins into the matrix. They have been studied in considerable detail, and their main characteristics have been known for more than 10 years. They consist of about 10–80 amino acid residues that have the potential to form amphipathic helices with one hydrophobic and one positively charged face. There is no consensus in the primary structure, which often differs considerably even between closely related orthologs. However, the general properties of these amphipathic helices are widely conserved among fungi and animals. In plants, the MTSs are still similar, although usually longer and richer in serine residues (8). The conserved properties of MTS allow searches

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PROGRAMS USED TO IDENTIFY MATRIX-TARGETING SEQUENCES

There are several prediction programs, based on different algorithms, that can identify mitochondrial proteins and distinguish them from proteins of other cellular subcompartments. Some of these programs and their Internet addresses are listed here.

Name	Internet address	Reference
TargetP	http://www.cbs.dtu.dk/services/TargetP/	(185)
PSORT II	http://psort.ims.u-tokyo.ac.jp/	(186)
MITOPRED	http://bioinformatics.albany.edu/~mitopred/	(187)
MitoProt II	http://ihg.gsf.de/ihg/mitoprot.html	(188)
Predotar	http://urgi.infobiogen.fr/predotar/predotar.html	(189)

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for it with prediction programs. Several of these programs are publicly available and listed in the sidebar Programs Used to Identify Matrix-Targeting Sequences.

N-terminal targeting signals are in most cases cleaved from precursors by the mitochondrial-processing peptidase (MPP) as soon as the cleavage sites reach the matrix (for reviews see References 9 and 10).

The N-terminal positioning of the MTS is important for its function. However, artificial transplantation of an MTS to the C terminus of a protein still led to its translocation into mitochondria, but in the C to N direction (11). Remarkably, there exists a protein in yeast, the DNA helicase Hmi1, in which the MTS is naturally present at the C terminus (12). In contrast, the insertion of an MTS in the central regions of proteins did not lead to mitochondrial targeting. There is, however, the interesting situation that some inner membrane proteins contain internal MTS-like sequences, which are C terminal to hydrophobic stretches. These structures might form hairpin loops that mimic the amphipathic structures of a typical MTS. Examples are Bcs1, Tim14, and Mdj2.

Preprotein: the precursor form of a mitochondrial polypeptide, usually unfolded or loosely folded, which often contains targeting signals (presequences)

Presequence (or prepeptide): a mitochondrial preprotein's N-terminal extension, often removed by the mitochondrial processing peptidase, following translocation into the matrix

Mitochondrial-processing peptidase (MPP): a matrix metalloprotease, consisting of two subunits, αMPP and βMPP, that removes presequences from preproteins

Internal targeting signals. Many mitochondrial precursors do not contain N-terminal presequences but instead have internal signals. These typically lack consistent patterns, and the nature of these signals remains largely elusive. Internal signals are present in proteins that are destined to all mitochondrial subcompartments. Precursors of outer membrane proteins all have internal signals. Although the targeting signals in β-barrel proteins remain unidentified, mitochondrial targeting of tail-anchored outer membrane proteins, such as bcl-2, Tom5, or Fis1, is dependent on the signal-anchor domain. A moderate hydrophobicity of this region and positive charges at the very N terminus were found to be indicative for these proteins and allow their distinction from signal-anchor proteins of the endoplasmic reticulum (13–16).

The internal signals that target proteins to the intermembrane space (IMS) or the inner membrane are described in detail below.

TRANSLOCATION OF PROTEINS INTO THE MATRIX

The majority of mitochondrial proteins reside in the matrix of the mitochondria. They are imported into this innermost subcompartment by the cooperation of the main two mitochondrial preprotein translocases, the TOM complex in the outer membrane and the TIM23 complex in the inner membrane. These complexes interact with each other to transfer the preproteins across both membranes in a concerted manner.
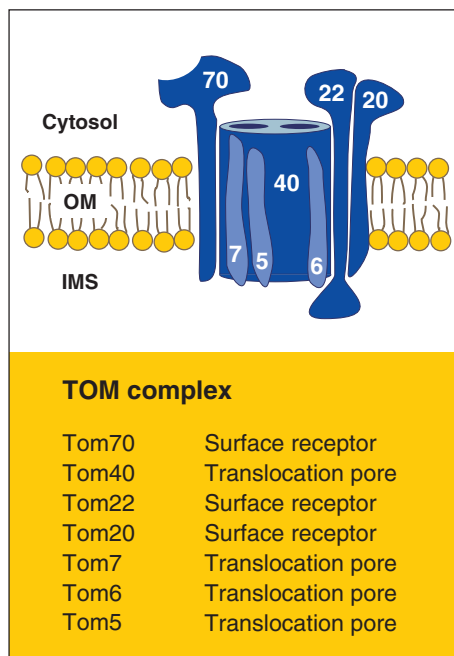
The TOM Complex of the Outer Membrane

The TOM complex is the translocase in the outer membrane that mediates the import of virtually all proteins of the mitochondria. It recognizes the precursor proteins in the cytosol, facilitates release of cytosolic-binding factors, contributes to the unfolding of cytosolic protein domains, transfers the polypeptides through pores across the outer membrane, and mediates insertion of some resident outer membrane proteins.

Architecture of the TOM complex. The composition and structure of the TOM complex of various organisms have been investigated in some detail. Most of what is known about the TOM complex comes from studies of the fungi *Neurospora crassa* and baker's yeast where only very minor differences have been observed. Even in more distantly related organisms, such as animals and plants, the structure and function of the TOM complex appears highly comparable to that in fungi (17–19).

Seven components were found to constitute the TOM complex (**Figure 1**). They can be grouped according to their functions into

TOM complex

Tom70	Surface receptor
Tom40	Translocation pore
Tom22	Surface receptor
Tom20	Surface receptor
Tom7	Translocation pore
Tom6	Translocation pore
Tom5	Translocation pore

Figure 1

Composition of the TOM complex. The TOM (translocase of the outer membrane) complex consists of the receptor subunits Tom70, Tom22, and Tom20 and the membrane-embedded subunits Tom40, Tom7, Tom6, and Tom5, which form the translocation pore. Abbreviations: IMS, intermembrane space; OM, outer membrane.

receptor and pore components. Tom20 and Tom70 are the major receptors that recognize preproteins. Both proteins are anchored with N-terminal transmembrane segments in the outer membrane and expose hydrophilic domains to the cytosol. Tom70 and Tom20 differ in their substrate specificity, but both receptors overlap in their function and can partly substitute for each other. The cytosolic domain of Tom70 contains 11 tetratricopeptide repeat motifs (20, 21), which show a substrate preference for hydrophobic precursors that contain internal targeting signals. These motifs not only recognize the precursors, but also interact specifically with cytosolic chaperones, namely with Hsp70 and, in animals, with Hsp90. Binding of ATP then triggers the release of the precursors from the chaperones and their insertion into and passage through the TOM pore (7).

Tom20 is the main receptor for N-terminal presequences. Structural analysis by NMR of a part of the cytosolic domain of Tom20 in complex with a prepeptide demonstrated a binding groove for the hydrophobic face of the MTS (22). In animals, Tom20 interacts with the cytosolic factor AIP. This protein contains tetratricopeptide repeats and may interact with preprotein-binding chaperones in a way similar to Tom70 (23).

Tom22 spans the outer membrane in an N_{out}-C_{in} orientation. It exposes a highly negatively charged N-terminal domain to the cytosol and a short C-terminal domain to the IMS. Tom22 connects Tom20 to the central translocation pore and may cooperate with Tom20 in the binding and unfolding of precursor proteins. In addition, Tom22 plays a critical role for the general integrity of the TOM complex (24, 25).

The general import pore of the TOM complex consists of the central component Tom40 and three small associated subunits, Tom5, Tom6, and Tom7. Tom40 is a membrane-embedded protein that presumably forms a β-barrel structure. Even in the absence of other TOM subunits, purified Tom40 forms pores in artificial membranes

that show characteristics similar to that of the entire TOM complex (26–28). It is, however, not clear whether in the TOM complex the pores are formed by single or multiple Tom40 molecules. The small TOM proteins consist of about 50 to 70 amino acid residues. They all form tail-anchored, probably α-helical structures with only a few residues exposed to the IMS. The loss of individual small TOM proteins leads only to minor effects, but the simultaneous deletion of all three proteins is lethal (29–31). They appear to stabilize the TOM complex, yet their individual functions are still unclear.

The TOM complex, when purified using the mild detergent digitonin, has a molecular mass of roughly 490–600 kDa (32–34). Single-particle imaging of a negatively stained TOM complex showed particles with two or three pore-like structures (**Figure 2a**). Purification procedures, using less gentle detergents like dodecylmaltoside, lead to the loss of the peripheral receptors Tom70 and Tom22 and yield the TOM core complex of 350–450 kDa, which contains two pores of about 20 Å in
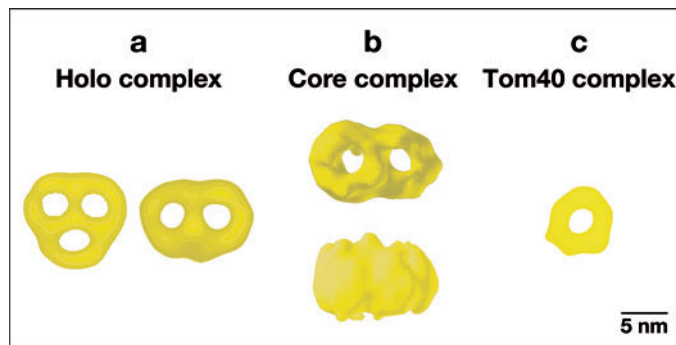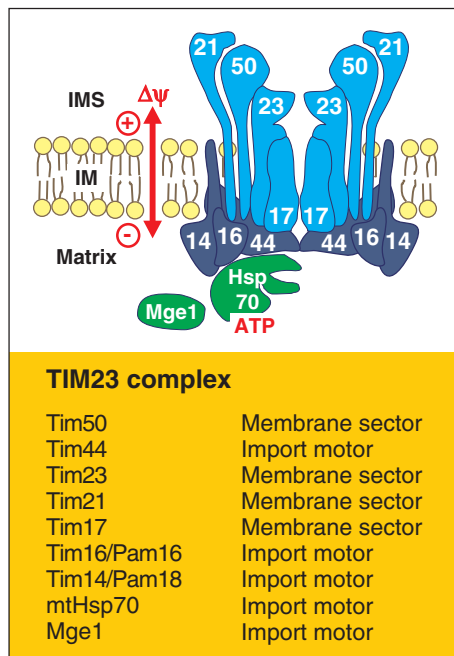

Figure 2

Electron micrograph of the TOM complex. Single-particle image reconstitution after electron microscopic analysis and correlation averaging of negatively stained isolated TOM complexes represent: (*a*) the entire TOM complex (32), (*b*) the TOM core complex without Tom70 and Tom20 (35), and (*c*) the homooligomeric Tom40 complex (34). Note that the holo complex shows two or three pores, whereas the core complex contains two, and the Tom40 complex, only one pore. It was suggested that two of the pores in the holo complex represent aqueous translocation channels. The third cavity might represent peripheral receptor subunits associated to the core complex. The bar corresponds to 5 nm. Reprinted with permission of *The Journal of Cell Biology* (34, 35) and of Elsevier for *Cell* (32).

diameter (35) (**Figure 2b**). Purified Tom40 on its own forms homooligomeric structures, which consistently show one cavity of dimensions similar to the TOM pores (**Figure 2c**).

Function of the TOM complex. The TOM complex contains several binding sites for precursor proteins. These sites are present on the cytosolic domains of Tom70, Tom20, and Tom22, which are often referred to as *cis*-binding sites. In addition, binding sites are present on the IMS-exposed surface of the TOM complex. The nature of the *trans*-binding site is not entirely clear, but Tom22, Tom7, and Tom40 appear to contribute to precursor binding in the IMS (36–38). An increase in the binding affinity presumably drives the vectorial translocation of the presequence across the outer membrane (39) and can facilitate even the unfolding of the precursor protein (38, 40). Notably, the TOM complex can act in this way as a molecular chaperone. Precursors bound to the *trans* site are protected against aggregation apparently by removal from the pool of unfolded species in the cytosol (38, 41, 42).

The TIM23 Translocase

The TIM23 complex is the major preprotein translocase in the inner membrane of mitochondria. In cells that are highly active in oxidative metabolism, such as fungal cells or metazoan muscle cells, its client proteins may make up some 20% of total cellular protein. The TIM23 complex translocates all precursors of matrix proteins, most inner membrane proteins, and many proteins of the IMS. Translocation by the TIM23 complex is energetically driven by the electrical membrane potential across the inner membrane ($\Delta\psi$) and the hydrolysis of ATP. The components of the TIM23 complex can be subdivided into two groups, which likely operate in a sequential and cooperative manner: (*a*) those that form the protein-conducting channel (the membrane sector) and (*b*) those

Figure 3

Composition of the TIM23 complex. Schematic representation of the subunits of the TIM23 translocase. Tim 21, Tim50, and Tim23 expose domains to the intermembrane space (IMS), which interact with incoming polypeptides. Tim17 and Tim23 are constituents of the translocation channel. Tim44, Tim16, Tim14, Hsp70, and Mge1 are associated at the matrix side. They make up the import motor. Other abbreviations: $\Delta\psi$, electrical membrane potential; IM, inner membrane.

that drive the translocation into the matrix space (the import motor) (**Figure 3**).

The membrane sector of the TIM23 complex. The membrane sector is composed of the three essential subunits, Tim50, Tim23, and Tim17, and a recently identified fourth protein, Tim21, which is dispensable for TIM23 function (43). Tim23 and Tim17 form the membrane-embedded core of the TIM23 complex. Both proteins share a phylogenetically related membrane domain consisting of four transmembrane segments. Additionally, Tim23 exposes an N-terminal hydrophilic region to the IMS. In yeast, this region

consists of two parts. The N-terminal part was found to span the outer membrane, at least under certain conditions, and might play a role in the positioning of the TIM23 translocase in proximity to the outer membrane (44). The second part (residues 50–100) contains an essential coiled-coil domain, which is critical for dimerization of Tim23 and for substrate binding (45–47). Recombinant Tim23 showed conductances of various sizes between 0.14 and 1.4 picosiemens (pS) after reconstitution into lipid membranes (48) and, hence, is able to form a channel; whether this channel indeed represents the protein-conducting channel of the TIM23 complex is not known.

The function of Tim17 is not clear. In contrast to Tim23, Tim17 exposes only a very short N-terminal tail of about 11 to 14 residues to the IMS. Nevertheless, this tail contains conserved negative charges, which are critical for preprotein import. Tim17 was proposed to play a critical role in gating of the TIM23 pore (49).

Tim50 is anchored into the inner membrane by an N-terminal transmembrane domain and exposes a large domain to the IMS (46, 47, 50). Tim50 interacts with incoming polypeptide chains as they reach the *trans* site of the TOM complex and presumably passes them on to Tim23. Tim50 not only functions as a passive import receptor but also seems to play a critical role in the regulation of import channel's permeability and, thus, might coordinate the translocation process of preproteins by the TIM23 complex (51).

Even less is known about the structure and function of Tim21, which was only recently identified (43). Tim21 was observed to interact with the IMS domain of Tom22, suggesting a role in the interaction of the TIM23 complex with the TOM complex (43, 52). Another role of Tim21 was proposed, regulating the association of the import motor with the membrane sector of the TIM23 complex. The existence of two forms of the TIM23 complex was suggested, one with both the membrane sector and the import motor and another one without the import motor (43). The

first form, free of Tim21, may be specialized on translocation into the matrix, whereas the second one may occur on translocation of precursors, which are subject to stop transfer. It is important to further analyze the structure and composition of the TIM23 complex to help verify this interesting proposal.

The import motor. The membrane sector of the TIM23 complex can transfer only the MTS of precursor proteins, a reaction driven by the $\Delta\psi$. Then the import motor has to take over. The following proteins play a role in mediating the further vectorial movement of the unfolded polypeptide chain: Tim44, Tim14 (Pam18), Tim16 (Pam16), mtHsp70, and Mge1.

Tim44 is a hydrophilic matrix protein that is completely (in fungi) or partially (in animals) attached to the inner membrane. In yeast, a fraction of Tim44 can be coisolated with the membrane sector of the TIM23 complex (53, 54). The crystal structure of a C-terminal fragment of Tim44 showed a large hydrophobic pocket, which was proposed to represent the membrane-binding site (55). Tim44 is in close contact with import intermediates and might function as a binding platform for motor subunits, bringing them in close proximity to the incoming chains.

One interaction partner of Tim44 is the Hsp70 chaperone of the matrix, mtHsp70. As with all Hsp70 chaperones, mtHsp70 consists of an N-terminal ATPase domain and a C-terminal substrate or peptide-binding domain (56, 57). Hsp70s switch between ATP-bound, ADP-bound, and empty states. The ATP form has an open binding pocket for substrates with high on and off rates. In the ADP form, the binding pocket is closed, and the on and off rates are low. The nucleotide-free form is an intermediate in the cycle. The exchange of ADP by ATP occurs through the nucleotide-free state. This step requires the action of the nucleotide exchange protein Mge1, a homolog of the bacterial GrpE.

The ability of Tim44 to recruit mtHsp70 is a key element in the import motor

because it brings mtHsp70 to the very site at which the precursor polypeptides appear in the matrix. Tim44 seems to recruit ATP-bound mtHsp70, which then can immediately grasp the incoming, unfolded polypeptide as its substrate-binding site is open. The ADP form appears to have a lower affinity for Tim44. This suggests that upon hydrolysis of ATP mtHsp70 is released from the import site (58–60).

Substrate binding to Hsp70 chaperones is typically regulated by DnaJ-like proteins. The import motor contains two subunits with DnaJ-like structures, Tim14/Pam18 (60–62) and Tim16/Pam16 (63–65). Tim14 contains an N-terminal membrane anchor that tethers the DnaJ-like matrix domain to the inner membrane. Cross-linking experiments indicate that Tim14 interacts with Tim44 and mtHsp70 in a nucleotide-dependent fashion: When ATP levels are low, Tim14 can be cross-linked to Tim44; when they are high, Tim14 can be cross-linked to mtHsp70. Presumably, Tim14 stimulates ATP hydrolysis in the mtHsp70 upon substrate binding and thereby triggers the release of the mtHsp70 precursor complex from Tim44.

Tim14 forms a complex with the second DnaJ homolog, Tim16. However, in contrast to Tim14, Tim16 is not a functional DnaJ-like protein because it lacks the HPD motif that is critical for interaction with Hsp70. Consistently, Tim16 does not influence the ATPase activity of mtHsp70 in vitro and even counteracts the ATPase-stimulating activity of Tim14 (66, 67). The recently solved crystal structure of the Tim14-Tim16 complex (68) revealed a tight interaction between both subunits, in particular by a unique N-terminal arm on Tim14 that embraces Tim16. Deletion of this arm leads to a destabilization of the complex and is lethal in yeast. In the crystal structure, the loop in Tim14, containing the HPD motif, is in direct contact with Tim16, fixed by several hydrogen bonds, and, thus, presumably unable to interact with Hsp70. This would confirm a function of Tim16 as a negative regulator of Tim14, which exhibits

its function by physically blocking the contact of Tim14 with mtHsp70.

Two additional components, Pam17 (69) and Mmp37/Tam41 (70, 71), were recently identified, and their involvement in the assembly or function of the import motor was suggested. The precise molecular role of these proteins is unknown.

The mechanism of TIM23-mediated translocation. A working model for TIM23-mediated preprotein import is presented in **Figure 4**. Preproteins are directed from the TOM complex to the TIM23 complex by binding the IMS domains of Tim50 and Tim23 (**Figure 4**, step 1). The translocation of the MTS across the TIM23 translocase requires $\Delta\psi$ either for providing energy to transfer the positively charged presequence or for gating the TIM23 channel, or for both processes (**Figure 4**, step 2). In the matrix, Tim44 binds the incoming preprotein and passes it on to mtHsp70 in the ATP-bound state (**Figure 4**, step 3). Tim14 stimulates ATP hydrolysis, leading to a tight binding of mtHsp70 to the preprotein and to the dissociation of mtHsp70 from Tim44 (**Figure 4**, step 4). At this stage, the preprotein can only slide into the matrix because the bound mtHsp70 prevents backsliding. Repetition of these trapping reactions leads to stepwise vectorial translocation of the whole preprotein into the matrix (**Figure 4**, step 5). Facilitated by the nucleotide exchange factor Mge1, mtHsp70 is released eventually from the preproteins, which then undergo folding and assembly. The TIM23 complex forms an oligomeric structure so that more than one mtHsp70 molecule is positioned at the outlet of the TIM23 channel. This is instrumental for rapid binding of mtHsp70 to incoming preproteins ("hand-over-hand model") (54).

A matter of debate has been the mechanism by which mtHsp70 generates the vectorial movement of precursor proteins (72, 73). Two different mechanisms have been proposed. The Brownian ratchet mechanism suggests that the precursors are subject to spontaneous
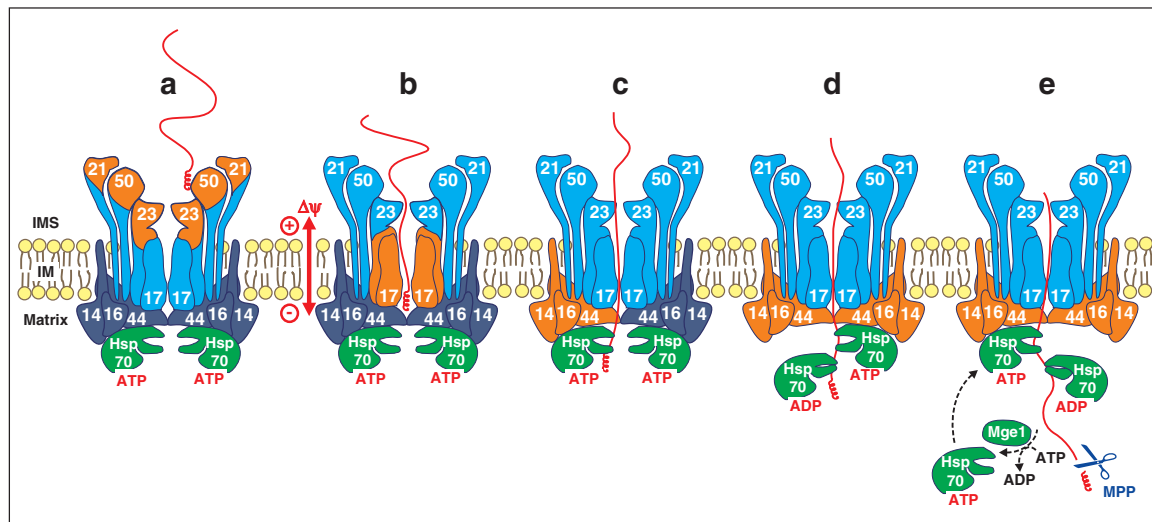
Figure 4

Stages of TIM23-mediated protein translocation. (*a*) Preproteins are directed by the intermembrane space (IMS)-exposed domains of Tim21, Tim50, and Tim23 (21, 50, and 23, respectively) to the protein-conducting channel of the TIM23 translocase (step 1). (*b*) Upon gating of the TIM23 channel, the presequences reach the matrix in an electrical membrane potential ($\Delta\psi$)-dependent step (step 2), (*c*) where they interact with the ATP-bound state of mtHsp70 (step 3). (*d*) Upon ATP hydrolysis, mtHsp70 tightly binds the incoming chain and is released from the TIM23 complex (step 4). (*e*) Repeated mtHsp70-binding cycles drive complete translocation of the precursor protein. mtHsp70 is finally released upon exchange of the bound ADP for ATP, a reaction stimulated by Mge1 (step 5). In most cases, the presequence is proteolytically removed by the mitochondrial-processing peptidase (MPP). Abbreviations: 14, Tim14; 16, Tim16; and 44, Tim44.

sliding reactions in the import machinery, consisting of TOM and TIM23 complexes (74). Binding of mtHsp70 would trap translocated segments because retrograde movement is blocked (75, 76). This ratchet-type mechanism then leads to a stepwise inward movement. The molecular motor in this mechanism is energetically powered by the ATP hydrolysis that is needed in the reaction cycle for binding of the polypeptide chain to mtHsp70 and for release of mtHsp70 from Tim44.

The power stroke mechanism, by contrast, proposes that mtHsp70 acts as a lever arm, which mechanically pulls on the precursor chain by a conformational change that occurs upon ATP hydrolysis (72, 77, 78).

In the past years, a large body of evidence has provided support for the first mechanism, and there is little doubt that unfolded pre-cursors can oscillate in the import machinery and be vectorially moved by trapping on the matrix side by mtHsp70 (59, 73, 79). Moreover, the capacity of a domain to be imported does not depend on its global unfolding, but instead, it is correlated with the ability to undergo limited unfolding of N-terminal segments following the MTS (73, 80, 81). Forces required to unfold domains, as measured by atomic force microscopy, were not related to their capacity to be imported (82–84). These observations are completely compatible with a ratcheting mechanism of the motor, and evidence for an active pulling of mtHsp70 is still lacking. The possibility that mtHsp70 in the import motor can exert a relatively minor force, which is determined by the energy of ATP hydrolysis and the length of a possible segment moved in the inward direction, cannot be excluded. Such a force most likely

would not be sufficient to mechanically pull
in a folded polypeptide chain, such as a titin
domain.

IMPORT OF OUTER MEMBRANE PROTEINS

The TOM complex does not only mediate
transfer of precursors across, but also mediates
the insertion of proteins into the outer membrane. There are different classes of outer
membrane proteins that, as far as we know,
use varied pathways for the insertion process.

One class comprises the proteins of Tom70
and Tom20, which are anchored in the outer
membrane with a transmembrane helix close
to their N terminus. These precursors do
not use the receptors, and competition experiments suggested they also do not use
the protein-conducting channel of the TOM
complex (85). Still, these proteins need the
TOM translocase, possibly because the complex can facilitate protein insertion at its
protein-lipid interface (86).

The outer membrane in addition contains
a number of proteins of different topologies,
such as proteins with C-terminal anchors (i.e.,
the small subunits of the TOM complex) or
a pair of internal transmembrane domains
(i.e., Fzo1). Whether the TOM complex is
involved in the binding or insertion of these
proteins is not known.

A particularly interesting class is the
β-barrel membrane proteins. Their insertion into the membrane requires the
function of both the TOM complex and
the translocase of outer membrane β-barrel proteins (TOB)/sorting and assembly machinery (SAM) complex, a translocase
specialized in this process.

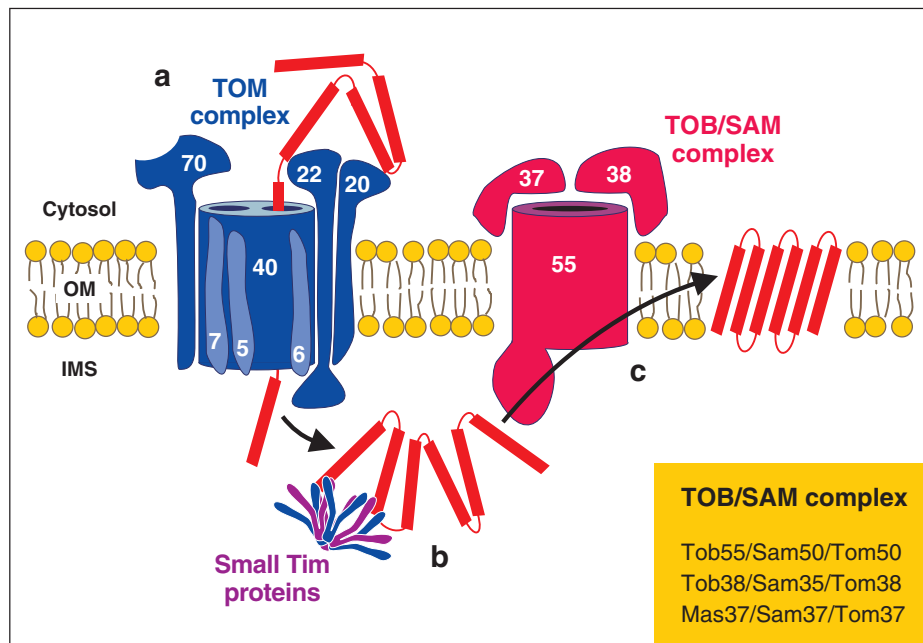### Topogenesis of Mitochondrial Outer Membrane β-Barrel Proteins

The outer membranes of mitochondria and
chloroplasts are the only membranes in eukaryotic cells that contain β-barrel proteins.
This is likely to reflect the evolutionary origin of these organelles from bacterial ancestors. So far identified β-barrel proteins in
mitochondria are porin (or VDAC), Tom40,
Tob55, Mdm10 and Mmm2. β-barrel proteins contain multiple membrane-spanning
β-strands each formed by 9–11 amino acid
residues (87). Precursors of β-barrel proteins
are imported via the TOM complex but require a second outer membrane complex. This
recently identified translocase is named TOB
(topogenesis of mitochondrial outer membrane β-barrel) (88) or SAM (sorting and assembly machinery) (89) complex.

Composition of the TOB complex. The
main component of the TOB complex is
Tob55, which was also named Sam50 or
Tom50 (**Figure 5**). It was identified by
three independent approaches: first, by applying proteomic and biochemical analysis of
N. crassa mitochondrial outer membranes
(88); second, by copurification with Mas37
(90), previously assigned to the TOM complex of yeast and found to be involved in the
biogenesis of Tom40 (89); and third, by sequence similarity to the bacterial Omp85 protein (91). Tob55 consists of two domains: The
N-terminal hydrophilic region is exposed to
the IMS and forms a characteristic structure,
called the POTRA (polypeptide translocation
associated) domain (92). The C-terminal domain presumably forms a β-barrel domain
with 14 - 16 transmembrane β sheets. Homologs of Tob55 are ubiquitously found in
eukaryotes where they are present in the outer
membranes of mitochondria and chloroplasts.
They are also found in the outer membrane of
gram-negative bacteria, and they are referred
to as Omp85 or YaeT (93, 94). Both the N-terminal POTRA domain and the C-terminal
β-barrel region are conserved among bacterial and eukaryotic homologs, which have
been implicated consistently in the biogenesis of β-barrel proteins.

In addition to Tob55, the TOB complex contains two hydrophilic subunits, which
are located at its cytosolic surface. These
proteins are Mas37 (Sam37/Tom37) and

Figure 5

The topogenesis of mitochondrial outer membrane β-barrel proteins (TOB) complex mediates membrane insertion of β-barrel proteins (88, 89). (*a*) Precursors of β-barrel proteins are translocated across the outer membrane (OM) by the TOM complex (step 1), (*b*) bound by small Tim proteins in the intermembrane space (IMS) (step 2) and (*c*) inserted and assembled by the TOB complex of the outer membrane (step 3). Synonyms of TOB components are shown in the inset. Abbreviation: SAM, sorting and assembly machinery.

Tob38 (Sam35/Tom38) (89, 95–97). Both proteins have clear homologs only in fungi, but it was suggested that the mammalian protein metaxin-1 represents a distant relative of Mas37 (98). However, evidence for a Mas37-like function of metaxin-1 is still lacking.

Tob55 and Tob38 are essential for viability of yeast cells. Depletion of these proteins selectively blocks the insertion and assembly of newly imported β-barrel proteins (88, 90, 91). In contrast, Mas37 is largely dispensable, but mutants show defects in the biogenesis of β-barrel proteins at elevated temperatures. The specific functions of the three TOB subunits are not clear, but the high conservation of Tob55 suggests that it is the central functional unit of the complex and that the other subunits have rather an accessory function.

Topogenesis of mitochondrial β-barrel proteins. Precursors of β-barrel proteins interact with the receptors of the TOM complex, mainly Tom20, and presumably move through the TOM pore to insert into the outer membrane from the IMS site (**Figure 5**, step 1). This is indicated by the observation that clogging the TOM channel by arresting matrix-targeted precursors blocked the import of β-barrel proteins (88, 89). In the IMS, complexes of small Tim proteins (see below) may guide these precursors from the TOM to the TOB complex (**Figure 5**, step 2). Import across the TOM channel and insertion by the TOB machinery are functionally coupled because when precursors were imported into mitochondria lacking Tob55, they did not reach the IMS but accumulated in the TOM complex. The POTRA domain

of Tob55 may be involved in the recognition of β-barrel precursors and might pass them on to the membrane-embedded C-terminal region of Tob55, which then facilitates membrane insertion and assembly of the β-barrel proteins (**Figure 5**, step 3). The direction of insertion from the IMS into the outer membrane presumably reflects the evolutionary origin of mitochondria from bacteria and the assembly process of β-barrel proteins is still exhibited by a phylogenetically old machine in principally the same manner as in prokaryotes.

IMPORT OF INNER MEMBRANE PROTEINS

The inner membrane of mitochondria harbors a large variety of different membrane proteins. Although some of these proteins are synthesized by mitochondrial ribosomes, most inner membrane proteins are imported from the cytosol. Three different import routes to the inner membrane were identified. They are depicted in **Figure 6**. First, inner membrane proteins with internal "hydrophobic loop" signals use a specialized translocation complex in the inner membrane, the
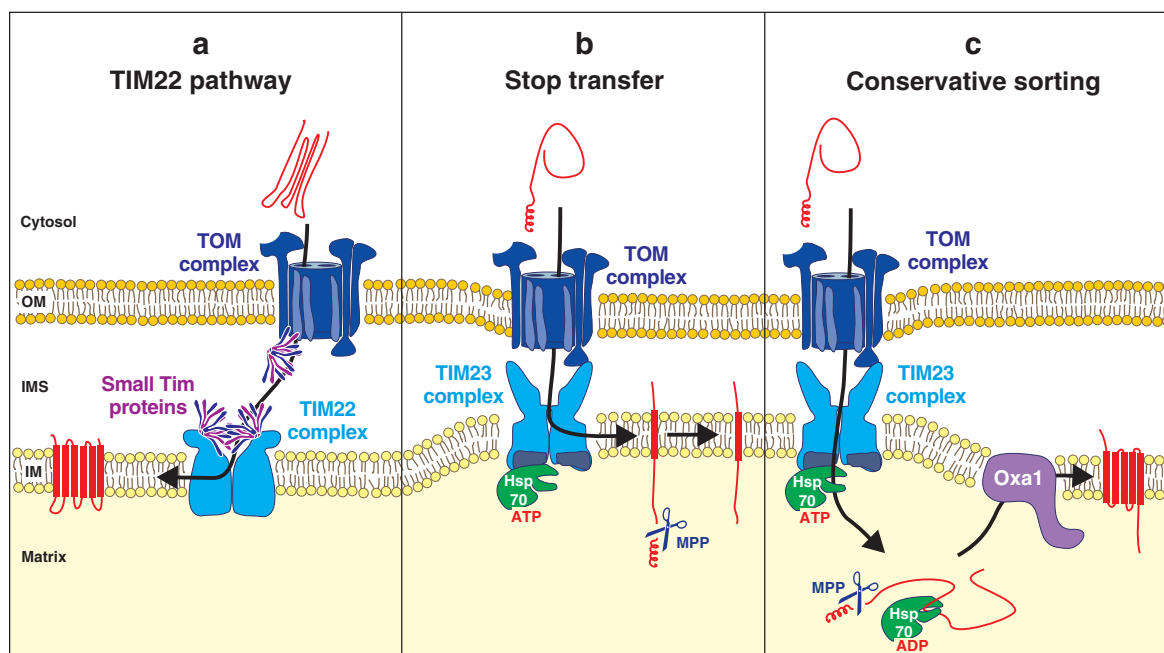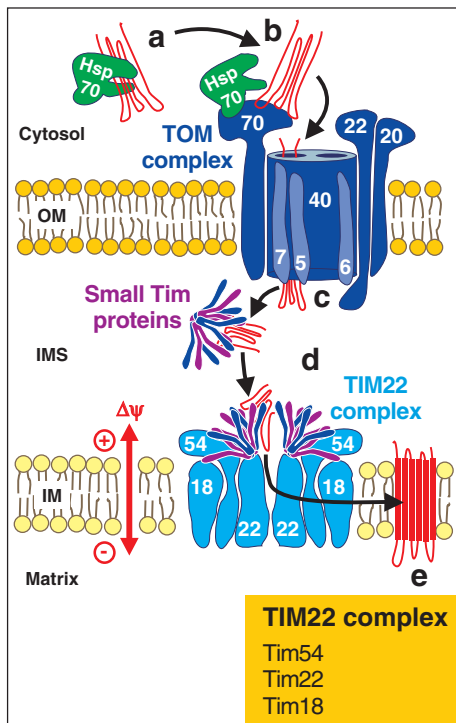

Figure 6

Sorting pathways of inner membrane proteins. Proteins are sorted to the inner membrane by several pathways, which differ with regard to the components involved and the direction of insertion. (*a*) Solute carriers and hydrophobic TIM (translocase of the inner mitochondrial membrane) subunits are inserted into the inner membrane by a dedicated inner membrane complex, the TIM22 translocase. Small Tim proteins in the intermembrane space (IMS) presumably function as chaperones, which guide the hydrophobic precursors across the IMS. (*b*) Inner membrane proteins, which contain only one transmembrane span, are arrested at the level of the TIM23 complex. They are integrated into the inner membrane by lateral exit. (*c*) A class of inner membrane proteins are sorted via soluble translocation intermediates in the matrix. Membrane insertion occurs here from the matrix side and, at least in certain cases, is facilitated by the Oxa1 (oxidase assembly) complex of the inner membrane.

TIM22 complex. Second, preproteins can be arrested at the level of the TIM23 complex and laterally integrated into the inner membrane; this transport route is referred to as the stop-transfer pathway (99, 100). Third, some inner membrane proteins are initially translocated into the matrix, and from there they are inserted in an export-like step into the inner membrane. Because the direction of the insertion reflects that of the prokaryotic ancestors of mitochondria, this pathway was called the conservative sorting pathway (101, 102).

The TIM22 Pathway

The Tim22 or carrier pathway employs three mitochondrial protein complexes that mediate in a successive order of steps the import and membrane insertion of the solute carrier family members and of hydrophobic TIM subunits (**Figure 7**). These complexes are the TOM complex in the outer membrane (see above), complexes of small Tim proteins in the IMS, and the TIM22 translocase in the inner membrane.

Small Tim proteins. In the IMS, the small Tim proteins comprise polypeptides of 8 to 12 kDa, which are characterized by a central "twin Cx_3C motif," i.e., two short motifs each comprising a pair of cysteine residues that are separated by three residues. The regions flanking this characteristic signature form α-helices, leading to a helix-loop-helix organization of these proteins. Small Tim proteins form hexameric structures of about 70 kDa in size. Structural analysis of the Tim9•10 complex revealed a ring-like organization in which alternating Tim9 and Tim10 subunits are arranged like the blades of a propeller (103–105). The core of this structure is formed by the central loop domains of the subunits and is surrounded by 12 arms formed by the α-helical termini of the six subunits. Thus, the Tim9•10 complex resembles a flattened jellyfish-like structure with 12 rather flexible tentacles. It was speculated that these arms can accommodate the hydrophobic regions of carrier proteins in the IMS, thereby

Figure 7

Protein insertion by the TIM22 pathway.
(*a*) Substrates of the TIM22 pathway are bound in the cytosol by chaperones (stage1) and
(*b*) recognized by receptors of the mitochondrial surface (stage 2). (*c*) The preproteins then traverse the outer membrane through the protein-conducting channel of the TOM complex (stage 3). (*d*) In the intermembrane space (IMS), small Tim proteins guide the precursors to the inner membrane (stage 4). (*e*) There they are inserted by the TIM22 translocase in an electrical membrane potential ($\Delta\psi$)-dependent manner (stage 5). The membrane-embedded Tim22 subunit of the TIM22 complex presumably serves as the central component of this translocase and is assisted by the accessory subunits Tim54 and Tim18. Abbreviation: IM, inner membrane.

shielding them from unproductive interactions (104). The structure to the Tim9•10 complex bears some resemblance to the chaperones prefoldin and Skp (106–108). Skp is a component of the bacterial periplasm that guides β-barrel proteins from the inner to the outer membrane. This function is analogous to the function of small Tim proteins, which

TIM22 complex: an inner membrane protein complex that mediates the insertion of carrier proteins and subunits of the TIM complexes

TIM: translocase of the inner mitochondrial membrane

also interact with β-barrel proteins (109–111). Whether this similarity in function and structure originates from a common phylogenetic ancestry of both components is unknown. Although the idea of the small Tim proteins being jellyfish-like chaperones that shield hydrophobic regions of their substrates is intriguing and attractive, it should be stressed that at present this is still a hypothetical. The molecular mechanisms by which small Tim proteins fulfill their function in protein translocation are still largely unclear.

The twin Cx_3C motifs are of special relevance for the formation of the structure of small Tim complexes, and the mutation of individual cysteine residues is sufficient to block complex assembly (112, 113). The cysteine residues can form pairs of intramolecular disulfide bridges in which the two central and the two distal cysteines are bonded (114–116). By contrast, in their reduced state, small Tim proteins can bind zinc ions, presumably by forming a zinc finger-like structure (112, 117, 118). Indeed, trapping experiments under anaerobic conditions revealed a reduced state of the cysteine residues (118). The observations on the oxidized or reduced states of the cysteine residues are apparently contradicting, but it is conceivable that the state of the cysteine residues is not permanently fixed. For example, during the biogenesis of small Tim proteins, the cysteine residues may initially bind zinc ions before hexamerization of the monomers induces formation of disulfide bridges (119). Indeed, in the absence of zinc ions, Tim10 rapidly oxidizes under the cytosolic redox conditions and thereby loses its import competence. Binding of zinc prevents this oxidation and thereby facilitates import of Tim10 (119). A change in the redox state of small Tim proteins might, however, not be limited to their biogenesis, but rather the complexes might permanently cycle between reduced and oxidized states (120, 121). The IMS protein Hot13 was implicated as a critical factor in the redox regulation of small Tim proteins (121). However, knowledge about the specific function of Hot13 for the redox dynamics of small Tim proteins is still scarce, especially because in vitro systems to analyze their activity with natural substrates have not yet been developed.

The TIM22 complex. The TIM22 translocase is a 300-kDa complex that consists of the three membrane proteins Tim22, Tim54, and Tim18 with which the three small Tim proteins, Tim9, Tim10, and Tim12, are associated. Tim22 is related in amino acid sequence to Tim17 and Tim23 (122). It represents the essential core of the complex that can mediate the insertion of carrier proteins even in the absence of Tim54 and Tim18, although at strongly reduced levels (123). Tim54 appears to be an important, but not essential, accessory component of the TIM22 complex (123, 124). Tim18 is a distant homologue of subunit 3 of the succinate dehydrogenase, Sdh3, and, to date, has been found only in fungal mitochondria (125, 126). The precise functions of Tim54 and Tim18 are not known.

A complex consisting of the small Tim subunits Tim9, Tim10, and Tim12 is permanently tethered to the IMS side of the TIM22 complex. Tim54 might contribute to the binding of this Tim9•10•12 complex because its association with Tim22 was destabilized in *tim54* deletion mutants (123). Tim12 is an essential protein, and the Tim9•10•12 complex may play a vital role for substrate recognition by the TIM22 complex.

Precursors using the TIM22 pathway. The TIM22 pathway is a sorting route selectively used by members of the solute carrier family and by the membrane-embedded TIM subunits Tim17, Tim22, and Tim23. All known substrates of the TIM22 complex represent membrane proteins with even-numbered transmembrane segments that expose their N and C termini into the IMS.

Members of the carrier protein family consist of three pairs of transmembrane segments, forming sequence-related internal repeats or modules (127–129). The targeting information is deciphered at three levels of the

import reaction: at the surface of the mitochondria to mediate binding to the Tom70 receptor, in the IMS to bind to the Tim9•10 complex, and at the level of the inner membrane for insertion by the TIM22 translocase. Peptide-binding scans suggested that the regions around the hydrophobic transmembrane domains are preferentially recognized by Tom70 (130, 131) and the Tim9•10 complex (115, 132). Each of the modules appears to contribute to these two initial recognition events. Import experiments with truncated carrier proteins suggested that each individual module contains targeting information for translocation into the IMS (127, 133). In contrast, only certain modules appear to mediate productive insertion into the inner membrane. In the ATP/ADP carrier and the dicarboxylate carrier only the most C-terminal module contains the information necessary and sufficient for membrane integration (127, 133). The molecular nature of the insertion signal in the third module remains still elusive.

Protein import by the TIM22 pathway.
The TIM22 pathway can be divided into several steps (134) (**Figure 7**). Following their synthesis, carrier proteins are bound by the cytosolic chaperones, Hsp70 and Hsp90 (stage 1). These complexes bind to the receptors of the TOM complex (stage 2) (7, 135). In mammals, Hsp90 and Hsp70 dock onto the tetratricopeptide repeat domain of Tom70. Hsp90 thereby appears to play an active role in the translocation of carrier proteins across the TOM channel because the binding of Hsp90 to Tom70 is vital for the import of carrier proteins into mitochondria. In yeast, Hsp70 rather than Hsp90 is used in import, and Hsp70 docking is required for the formation of a productive complex of preproteins with Tom70 (7).

Carrier proteins are then delivered to the TOM channel (stage 3) where they can acquire a topology in which the N and C termini remain exposed to the cytosol (128, 136). These translocation intermediates interact in

the IMS with Tim9•10 complexes, which presumably shield the hydrophobic domains of carrier proteins and accompany them across the IMS from the TOM to the TIM22 complex (115, 117, 137–139). Finally, carrier proteins are taken over by the TIM22 complex and inserted into the lipid bilayer of the inner membrane in a membrane potential-dependent reaction (stage 4). Following their release from the TIM22 complex, the carrier proteins assemble into their dimeric native state (stage 5). This dimerization occurs very rapidly and is stimulated by the hydrophilic head groups of phosphatidylcholine, which induce a structural rearrangement in the carriers (140, 141).

The import process of the TIM subunits Tim17, Tim22, and Tim23 is less well characterized than that of carrier proteins but seems to adhere to the same principles (142–146). For its biogenesis, Tim23 makes use of an alternative small Tim complex in the IMS, consisting of Tim8 and Tim13 subunits. In contrast to Tim9 and Tim10, Tim8 and Tim13 are not essential components in yeast, presumably because the Tim9•10 complex can functionally replace the Tim8•13 complex to some degree.

In humans, loss-of-function mutations of the Tim8 homologue DDP1 (deafness dystonia polypeptide 1) cause the Mohr-Tranebjaerg syndrome (OMIM 304700), a recessive X-linked neurodegenerative disorder, characterized by hearing loss, cortical blindness, dystonia, mental retardation, and paranoia (147, 148). How mutations in DDP1 interfere with mitochondrial function is not entirely clear. DDP1 appears to play a role in the import of human Tim23 (113, 146, 149) and also in the import of human carrier proteins citrin and aralar1 (150). Both proteins contain extended hydrophilic regions at their N termini. Tim23 also contains a long N-terminal hydrophilic region, and it was proposed that the Tim8•13 complex (or DDP1•Tim13 complex in humans) interacts specifically with substrates that contain hydrophilic extensions. Consistent with

this idea, the Tim8•13 complex appears to specifically interact with the N-terminal hydrophilic region of the Tim23 precursor, whereas the Tim9•10 complex interacts with the hydrophobic loops of the membrane-embedded region of Tim23 (145).

The Stop-Transfer Pathway

Many proteins of the inner membrane are synthesized with typical MTSs. Most of them contain only one transmembrane domain and adopt an N_{in}-C_{out} topology in the inner membrane. Subunit 5a of the cytochrome oxidase of budding yeast (Cox5a) was one of the first representatives of this class whose biogenesis was studied in detail (99, 151, 152). Other proteins that appear to be imported by the same principle are Cbp4, Cox11, Dld1, Hem14, MWFE, Mia40, Oms1, Pet117, Qcr7, Sco1, Sco2, She9/Mdm33, Tim50, Yme1, and Yme2.

Import experiments with purified mitochondria suggested that Cox5a is inserted into the inner membrane by a stop-transfer mechanism, i.e., the transmembrane domain functions as a critical sorting signal that causes the arrest of the precursor during the import reaction at the level of the inner membrane and inserts it laterally into the lipid bilayer. The import reaction is mediated by the TOM and TIM23 translocases. Upon deletion of its transmembrane domain, Cox5a is mistargeted to the matrix (99, 153). The import of Cox5a, similar to that of matrix proteins, is driven by the membrane potential and the mtHsp70-mediated hydrolysis of ATP. However, in contrast to matrix proteins, Cox5a can be imported into mitochondria of mutants in which the function of mtHsp70 is impaired (152). When the transmembrane segment of Cox5a, which normally is placed in the C-terminal third of the protein, was moved to the N terminus right after the presequence, ATP and mtHsp70 became completely dispensable for import (151). In this case, the insertion into the lipid bilayer apparently prevented backsliding of the polypeptide.

Topogenic signals in stop-transfer proteins. The transmembrane segments of precursors that use the stop-transfer pathway have to be recognized by the TIM23 translocase. The mere presence of a hydrophobic segment, however, is not sufficient for translocation arrest. Precursors of conservatively sorted proteins, despite the presence of transmembrane segments in their sequences, do not become arrested but rather pass though the TIM23 complex into the matrix. Studies with mutant preproteins revealed at least three characteristic properties of arrested proteins that play a role as topogenic signals that are deciphered by the TIM23 translocase:

1. Arrested transmembrane segments are typically followed at their C termini by clusters of charged amino acid residues (154). The relevance of these charges was studied in some detail with the D-lactate dehydrogenase of yeast, Dld1. A sequence of six residues with two positively and three negatively charged residues (Arg-Glu-Thr-Lys-Glu-Asp) is critical for translocation arrest and deletion of this stretch, which caused mistargeting of Dld1 to the matrix. Similar clusters of charged residues are often found in proteins that are inserted on a stop-transfer route (153). Conceivably, the TIM23 complex interacts with these charges or with the increased diameter of the polypeptide chain because increased hydration of the charged regions slows down the import process and facilitates lateral insertion of the transmembrane domain.

2. The transmembrane spans of arrested preproteins, on average, are more hydrophobic than those of conservatively sorted proteins. Especially tyrosine and phenylalanine residues are relatively enriched in arrested sequences, whereas serine, threonine, asparagine, and glutamine residues are more abundant in transferred transmembrane domains (153).

3. Proline residues within the transmembrane domains are important determinants for intramitochondrial sorting (153). Proline residues are typically absent in arrested transmembrane domains but often are present in transferred domains. The insertion of a proline residue into the transmembrane domain of Cox5a caused the mistargeting of the protein to the matrix. By contrast, the removal of proline residues from a transmembrane domain of the conservatively sorted protein Oxa1 led to arrest of the Oxa1 precursor upon import and mediated its insertion into the inner membrane.

Stop-transferred proteins with N_{out}-C_{in} topology. Although most precursors undergoing stop-transfer are arrested with the N terminus facing the matrix (N_{in}-C_{out}), some proteins reach the reverse orientation (N_{out}-C_{in}). These proteins typically contain an internal transmembrane span C-terminally followed by an amphipathic helix with a hydrophobic and a positively charged face. It was proposed that, during the import process, this sequence forms a hairpin-like structure, which resembles the structural features of a presequence, to promote protein import on a TIM23-dependent import route (155). Examples are the AAA protein, Bcs1, and the J-proteins, Tim14 and Mdj2.

The Conservative Sorting Pathway

Among the proteins that are synthesized with presequences are several inner membrane proteins with more than one transmembrane domain. Examples of this group are subunit 9 of the F_oF_1-ATPase of *N. crassa*, Oxa1, Cox18/Oxa2, Mrs2, and Yta10 (156–159). These proteins are initially translocated to the matrix where they are bound by mtHsp70. From the matrix, they integrate into the inner membrane in an export-like reaction, which is still ill defined. At least in vivo, import into the matrix and insertion into the inner membrane

can be separated into two subsequent reactions, suggesting that they form functionally independent processes (156, 157, 160). The protein segments that are transferred across the inner membrane in most cases show a bias for negatively charged residues. Membrane insertion strongly depends on the membrane potential, presumably because the transfer of the negatively charged regions to the positively charged IMS drives the insertion reaction (156, 157, 160). The Oxa1 complex of the inner membrane facilitates the insertion of at least some of these inner membrane proteins (161, 162). Oxa1 belongs to a large family of proteins with members in mitochondria, chloroplasts, and bacteria (for a review, see References 163 and 164). In all these systems, the members of the Oxa1 family mediate the membrane insertion of proteins.

The term "conservative sorting" was initially coined for the sorting pathway of the Rieske iron sulfur protein (101). This protein consists of a large C-terminal IMS domain that contains the iron sulfur cluster. In vitro import experiments indicated that the entire preprotein is initially imported into the matrix (165). There, the iron sulfur cluster of the protein appears to be incorporated before the catalytic domain, presumably in a folded conformation, is exported into the IMS, and is assembled into the bc_1 complex. Novel evidence for a conservative sorting of the Rieske protein recently came from genetic experiments in which transfer of the gene to the mitochondrial genome was shown to result in a functional protein that is properly placed into the bc_1 complex (166). The mechanism by which the Rieske protein is exported across the inner membrane is still completely unknown.
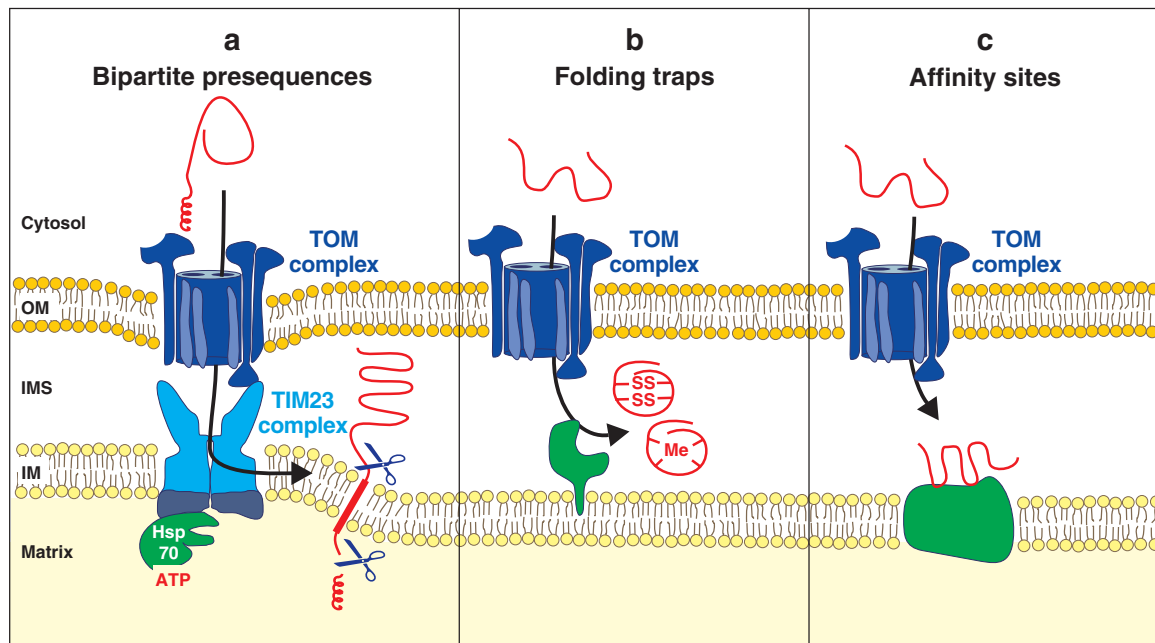
PROTEIN IMPORT INTO THE INTERMEMBRANE SPACE

Proteins of the IMS have essential functions in a variety of different processes, such as in metabolic and bioenergetic reactions, in the transfer of metabolites and proteins between the outer and the inner membrane,

Oxa1 complex: a protein complex that functions in the insertion of membrane proteins from the matrix into the inner membrane

Figure 8

Protein translocation into the intermembrane space (IMS). Three classes of IMS proteins can be distinguished on the basis of their import mechanisms. (*a*) Proteins with bipartite presequences embark on a stop-transfer route to the inner membrane; the mature parts of these proteins are then released into the IMS by proteolytic cleavage. (*b*) Translocation of a class of small proteins is achieved by their folding, mediated by IMS-located factors after transfer across the TOM pore; trapping in the IMS makes translocation vectorial. Formation of disulfide bridges (SS) or binding of metal cofactors (Me) occurs in many cases. (*c*) A class of proteins requires binding to affinity sites in the IMS for net translocation. Abbreviations: IM, inner membrane; OM, outer membrane.

and in the control of regulated cell death. Although the function of many of these proteins was studied in detail, knowledge of their biogenesis is still scarce. On the basis of their import characteristics, most IMS proteins can be sorted into one of three categories (**Figure 8**):

1. Some IMS proteins contain a canonical N-terminal MTS, followed by a hydrophobic sorting sequence. Their import is mediated by the TOM and TIM23 complexes and depends on the membrane potential across the inner membrane and, in most cases, on ATP. Such bipartite presequences are removed by proteolytic processing releasing the mature proteins into the IMS (**Figure 8*a***).

2. Many, if not most, IMS proteins are of low molecular weight, and their net translocation across the TOM complex requires their folding in the IMS. The folding is triggered by the acquisition of cofactors or by intramolecular disulfide bridges. According to the folding trap hypothesis, the folded state prevents back-translocation out of the mitochondria and thereby confers unidirectional net import of these proteins (**Figure 8*b***).

3. Some IMS proteins are permanently associated with components in the IMS, and the affinity to these factors appears to drive the import reaction (**Figure 8*c***). Examples are the import of cytochrome *c* heme lyase or creatine kinase.

In the following section, specific representatives of the first two of these classes will be described in more detail.

Proteins with Bipartite Presequences

Proteins with bipartite presequences are released into the IMS after proteolytic cleavage. Although the targeting of these proteins appears to adhere to a consistent principle, a surprising variety of processing peptidases is employed to mediate proteolytic release of these IMS proteins. Some proteins and their processing sites are sketched in **Figure 9**. Among these proteins are many of the apoptotic components that are contained in the IMS [similar to the apoptosis-inducing factor (AIF)], i.e., HtrA2, Endonuclease G, and Smac/Diablo (**Figure 9a**). The import process of Smac/Diablo was characterized recently (167). Smac/Diablo contains a classical mitochondrial presequence, followed by a hydrophobic sequence. Following import by the TOM and TIM23 translocases, the hydrophobic sequence is arrested at the level of the inner membrane and laterally integrated into the lipid bilayer. This sorting

intermediate, which resembles an inner membrane protein in N_{in}-C_{out} topology, is recognized by the inner membrane peptidase (IMP) complex, which separates the transmembrane anchor from the C-terminal mature part of
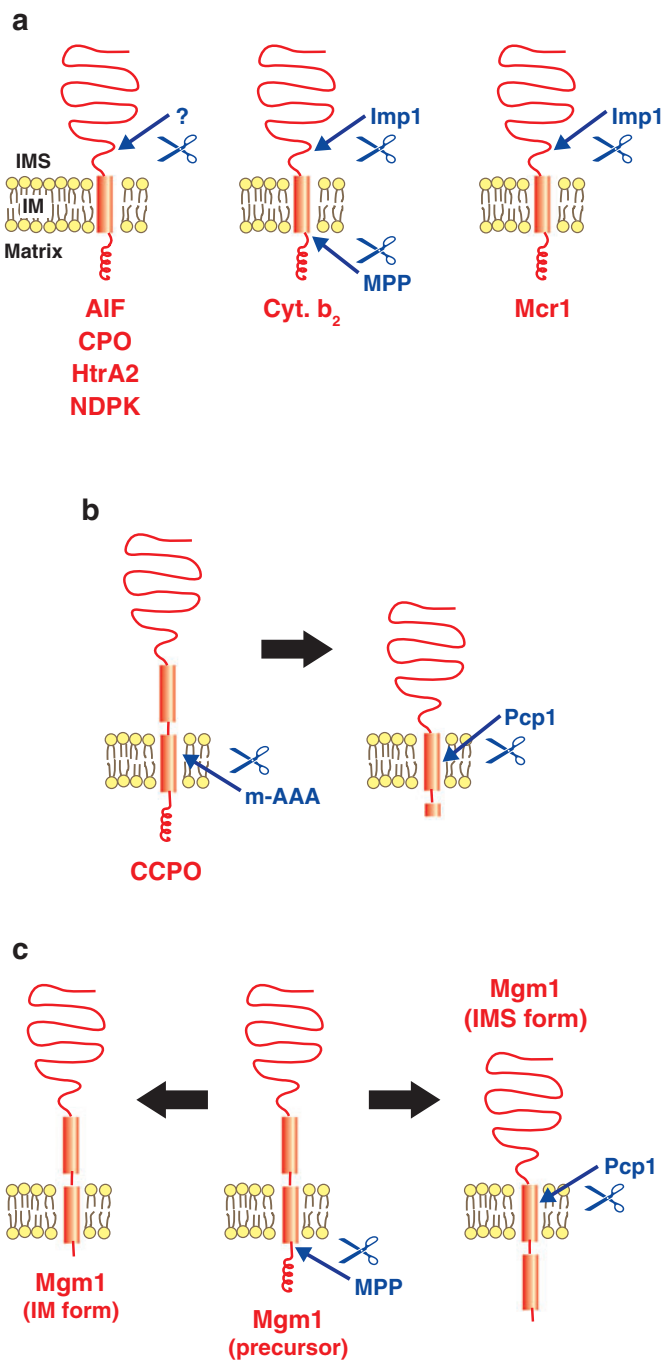
Figure 9

Preproteins with bipartite presequences. Schematic representation of different precursor forms. Shown are the membrane-inserted sorting intermediates. Cleavage sites are indicated by arrowheads. (*a*) For most proteins, the bipartite presequence is removed by cleavage at the intermembrane space (IMS) close to the inner membrane. (*b*) In the case of cytochrome *c* peroxidase (CCPO), the m-AAA protease removes an N-terminal part of the protein and contributes to the further translocation of the intermediate, allowing its cleavage by Pcp1. (*c*) Two alternative forms of Mgm1 are produced by alternative processing of different insertion intermediates. Abbreviations: AIF, human apoptosis-inducing factor; CPO, coproporphyrinogen oxidase; Cyt. b_2, cytochrome b_2; HtrA2, human proapoptotic serine protease Omi/HtrA2; IM, inner membrane; m-AAA, m-AAA protease; Mcr1, cytochrome b_5 reductase; Mgm1, mitochondrial dynamin-like protein; NDPK, nucleotide diphosphokinase.

Smac/Diablo. The IMP complex contains the two proteolytically active subunits, Imp1 and Imp2, which are related to bacterial leader peptidases (168).

Some proteins employ more than one processing peptidase. In the case of cytochrome b_2, the mitochondrial targeting is cleaved by MPP in the matrix before the mature region of the protein is released into the IMS by cleavage of the Imp1 protease. The cleavage by MPP therefore is not a prerequisite for import of cytochrome b_2 because mutants in which the MPP processing site was deleted were still properly sorted to the IMS. In contrast, both processing peptidases appear to play a crucial role for the import of yeast cytochrome c peroxidase (CCPO) (169–171). CCPO contains an N-terminal mitochondrial targeting sequence, followed by two hydrophobic sequences (**Figure 9b**). After its integration into the inner membrane, the N-terminal hydrophobic sequence is cleaved by the m-AAA protease, an ATP-hydrolyzing metalloprotease (172). The CCPO intermediate is then further imported and matured by a second cleavage event, catalyzed by the rhomboid protease Pcp1. Pcp1, in a way similar to the m-AAA protease, cleaves its substrates at hydrophobic transmembrane regions. In the case of CCPO, this cleavage occurs at the second hydrophobic region and thereby releases the mature CCPO protein into the IMS. The presence of the m-AAA protease is essential for the import of CCPO, potentially because this oligomeric ATP-consuming enzyme is critical for pulling the tightly folded IMS domain of CCPO across the outer membrane.

Even more complex is the topogenesis of Mgm1, a dynamin-related component of the mitochondrial fission machinery (173). Mgm1 is present in mitochondria in two differentially processed isoforms (see **Figure 9c**): The short variant of Mgm1 is an IMS protein comprising residues 161–902 of the Mgm1 precursor; the long variant is anchored to the inner membrane and comprises residues 81–902 of the precursor. Both variants are produced in comparable amounts

by a process called "alternative topogenesis." The N terminus of Mgm1 contains two hydrophobic sorting sequences. For generation of the long isoform, the N-terminal hydrophobic sequence is inserted into the inner membrane from which the mitochondrial targeting sequence is removed by MPP. In about half of the precursor proteins, the N-terminal hydrophobic sequence fails to integrate into the membrane and is transferred farther into the matrix until the second hydrophobic region arrests translocation. This region is then cleaved by Pcp1, and the short isoform is released to the IMS. The ratio between both variants depends on the energetic state of the mitochondria. Increased levels of ATP thereby promote the transfer of the N-terminal sorting sequence in the matrix and, thus, the generation of the short isoform. By contrast, depletion of ATP leads to increased levels of the long isoform. It was proposed that in vivo this is used to measure the ATP levels in mitochondria. Mgm1 regulates mitochondrial fusion and content mixing, as well as segregation of mitochondrial genomes. Therefore, this mechanism might favor the propagation of highly energetic mitochondria relative to mitochondria harboring mutations in their mitochondrial DNA.

Import by Folded Trap Mechanisms

Many proteins of the IMS are small, and their folding is stabilized by cofactors or disulfide bridges. The best-characterized representative of this class is cytochrome c. The apoform of cytochrome c is imported by the TOM complex into the IMS, where it is transiently bound by cytochrome c heme lyase (174–176). This enzyme inserts the heme cofactor into cytochrome c, which then is released in its soluble holoform into the IMS. Thus, the bidirectional movement of the apoform of cytochrome c across the TOM complex is converted to a vectorial import process. The enzymatic activity of cytochrome c heme lyase locks cytochrome c in its folded state and thereby traps it stably in the IMS.

A similar trapping mechanism is used by other IMS proteins, such as the small Tim proteins and proteins containing "twin Cx_9C motifs." This process relies on the formation of intramolecular disulfide bridges. Following their translocation across the outer membrane, the precursor forms are bound by the inner membrane protein Mia40 (177–179). In its active state, Mia40 contains intramolecular disulfide bridges that, upon interaction with precursor proteins, are converted to intermolecular disulfide bonds, covalently linking the precursors to Mia40. The imported proteins are finally released from Mia40, presumably in an oxidized state. The activity of Mia40 relies on the function of Erv1, a conserved essential sulfhydryl oxidase in the IMS (180–182). This FAD-binding protein directly interacts with Mia40 and maintains it in an ox-idized, active state. At present, it is not completely clear how Erv1 itself is oxidized, but the human homologue of Erv1 was efficiently oxidized in vitro by oxidized cytochrome c (183). Cytochrome c might connect the electron flow from oxygen and cytochrome c oxidase to Erv1 (181).

Cu,Zn-superoxide dismutase (Sod1) is another IMS protein that requires the binding of metal cofactors for uptake, and the formation of an intramolecular disulfide bridge in the IMS is critical for its uptake by mitochondria (184). Both steps are catalyzed by the Sod1-specific factor in the IMS, the copper chaperone of Sod1(CCS), which forms a transient disulfide bridge with Sod1. This reaction is highly reminiscent of that occuring between Mia40 and newly imported small Tim proteins.

SUMMARY POINTS

1. Mitochondrial preproteins are synthesized in the cytosol with specific targeting signals. In precursors of matrix-targeted proteins, these signals are mostly N-terminal presequences, which are proteolytically removed from the proteins by a protease in the matrix.

2. Receptors on the surface of mitochondria recognize preproteins.

3. The TOM complex mediates the transfer across the outer membrane.

4. β-barrel proteins are integrated into the outer membrane by the TOB complex.

5. The TIM23 complex contains the protein-conducting channel and the import motor, which together mediate the translocation of preproteins into or across the inner membrane.

6. The TIM22 translocase inserts carrier proteins and subunits of the TIM complexes into the inner membrane.

7. Some inner membrane proteins are initially completely translocated into the matrix and are inserted into the inner membrane from the matrix side.

8. On the basis of their import characteristics, three different classes of IMS proteins can be distinguished. Members of the first class contain bipartite presequences, members of the second are imported by a folding trap mechanism, and members of the third associate with binding sites on the outer or inner membrane, following translocation across the TOM complex.

FUTURE ISSUES

1. How are β-barrel proteins inserted into the outer membrane by the TOB/SAM complex? Do they assemble before, during, or after membrane insertion? Where does the energy for the insertion reaction come from?

2. How are the TIM complexes gated? What is the specific role of presequences in this process? Why is the membrane potential essential for gating?

3. How are membrane proteins inserted from the matrix into the inner membrane? What is the enzymatic activity of the Oxa1 complex? What are the molecular mechanisms that are responsible for establishing the correct topology of membrane proteins?

4. How are proteins folded in the IMS? Is the folding and oxidation of thiol groups mechanistically coupled in the IMS? Is there a protein with protein disulfide isomerase activity in the IMS?

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