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Mitochondrial protein-import machinery: correlating structure with function

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Most mitochondrial proteins are synthesized in the cytosol, translocated into the organelle and directed along specific sorting pathways. Over the past 20 years, >30 proteins have been identified as having key roles in mitochondrial protein import. Recently, the elucidation of the structures of several import components has provided fresh insight into the import process. Here, we review the different pathways involved in sorting proteins into mitochondrial subcompartments. Along the way, we highlight the available structural information about the protein-import machinery and discuss how these structures correlate with previously ascribed functions. Future challenges for the cell biologists will be to use this structural information to test specific hypotheses addressing the molecular mechanisms of mitochondrial protein import.

Introduction

In humans, only 13 proteins are encoded by mitochondrial DNA. Thus, complex protein-import machineries exist in mitochondria to ensure the correct import, membrane translocation and sorting of the 1000–1500 distinct mitochondrial proteins that are synthesized on cytosolic ribosomes. In the late 1980s and early 1990s, several of these mitochondrial import components were identified and functionally characterized, leading to the construction of a general model for how they are involved in the import of precursor proteins along the main sorting pathway, the matrix. These precursors are synthesized with an N-terminal positively charged presequence capable of forming a basic, amphipathic α-helix. They traverse membranes by crossing through protein translocases of the outer and inner membranes in an unfolded conformation. The mitochondrial membrane potential ($\Delta\psi$) and the action of matrix heat-shock protein (Hsp)70 (mtHsp70) are used to drive translocation. The presequence is cleaved by a matrix protease and the imported protein is folded, often with the aid of molecular chaperones. The basic outline of this model still remains, yet it has evolved to accommodate new components of the import machinery. In addition, the identification of novel import components has led to the elucidation of new protein-import pathways for the sorting of precursor proteins to the other mitochondrial subcompartments. Precursor proteins that follow these routes do not contain typical N-terminal targeting signals but instead harbor targeting information within their mature sequences.

At present, four major membrane protein translocase complexes are known and each contains one or more components that are essential for viability in yeast. The translocase of the outer membrane (TOM; see Glossary) is the universal entry gate for all proteins that are imported into mitochondria. The various protein-sorting pathways diverge at this point. Matrix-targeted precursors are sorted to the translocase of the inner membrane (TIM), which contains TIM23 as the central unit (the TIM23 complex). The presequence translocase-associated motor (PAM) regulates mtHsp70 action to drive precursors into the matrix. The TIM23 complex is also involved in the inner membrane sorting of precursors that contain hydrophobic stop-transfer signals. However, a separate translocase termed the TIM22 complex is required for the insertion of inner-membrane proteins that contain multispanning transmembrane domains and internal targeting signals. Finally, the outer membrane sorting and assembly machinery (SAM, also termed TOB) is involved in the insertion of β-barrel proteins into the outer membrane. A general overview of the different sorting pathways and the components involved is illustrated and detailed in Box 1.

Glossary

Presequence translocase-associated motor (PAM): Multiple subunits involved in tethering mtHsp70 to the TIM23 complex and regulating its ATPase activity. The PAM complex is involved in importing precursor proteins into the mitochondrial matrix. Some PAM subunits are listed alternatively as TIM subunits (see text).

Sorting and assembly machinery of the outer membrane (SAM): Involved in the biogenesis of β-barrel proteins. The complex consists of a number of subunits, including Sam50, which is conserved evolutionarily from bacteria to humans. Also referred to as TOB (topogenesis of outer membrane β-barrel proteins).

Translocase of the inner membrane (TIM): The TIM23 complex sorts precursor proteins containing N-terminal targeting signals that are destined for the inner membrane and matrix. The TIM22 complex is involved in the integration of polytopic inner-membrane proteins with internal targeting signals.

Translocase of the outer membrane (TOM): Universal entry gate for proteins translocating into mitochondria. It is a multisubunit complex that includes receptor components as well as the pore-forming subunit Tom40.

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Box 1. Sorting pathways for the import of precursor proteins into mitochondria

Cytosolically synthesized precursor proteins contain mitochondrial targeting signals that transport them to the TOM machinery at the outer membrane, often with the assistance of molecular chaperones. A matrix-targeted precursor typically contains an N-terminal targeting signal that can adopt a basic, amphipathic α-helix. These precursors translocate across the TOM channel in an unfolded conformation before engaging with the inner membrane TIM23 complex (Figure Ia). Matrix-sorted precursors are driven across the inner membrane through the action of the $\Delta\psi$ and the mtHsp70 motor system that involves the PAM machinery. Matrix-targeted precursors that contain a hydrophobic stop-transfer signal engage with a TIM23 complex that is tethered to the TOM complex by Tim21. These precursors exit laterally out of the TIM23 complex and into the inner membrane. Their import is facilitated by the $\Delta\psi$ and possibly complexes of the respiratory chain. On entry into the matrix, targeting signals are typically cleaved by the MPP.

Several additional pathways exist for the import of proteins into the other mitochondrial subcompartments (Figure Ib). 'Simple' outer-membrane proteins are targeted to the TOM complex where they are integrated into the membrane in a process that is poorly understood. β-barrel outer-membrane proteins translocate the TOM machinery and integrate into the outer membrane from the IMS side by the SAM (also termed TOB). Some precursors directed to the IMS pass through the TOM complex and interact with specific components involved in their folding and assembly, thereby trapping them in the IMS (Box 3). Precursors of polytopic inner-membrane proteins (e.g. metabolite carriers) with internal targeting information transit the TOM machinery and are then delivered to the TIM22 complex by the small TIM chaperones. These precursors are inserted into the inner membrane subsequently in a $\Delta\psi$-dependent fashion.

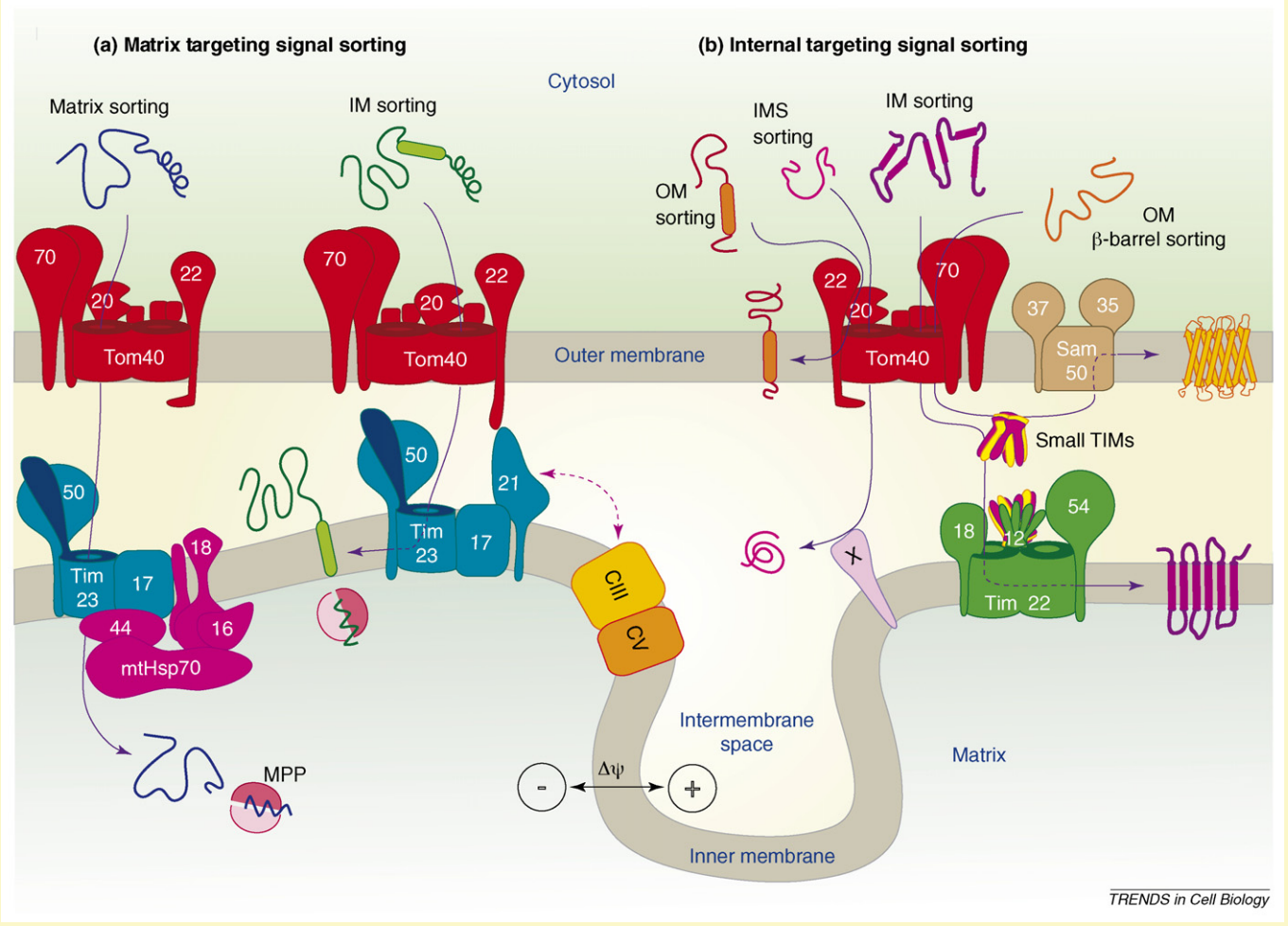


Figure I. Overview of mitochondrial protein-sorting pathways. Precursor proteins synthesized in the cytosol are targeted to one of four mitochondrial locations: outer membrane (OM), inner membrane (IM), intermembrane space (IMS) or matrix. All precursors interact with the TOM complex (red). (**a**) Matrix-targeted precursors are sorted to the TIM23 complex (blue), which contains the PAM–mtHsp70 complex (pink). MPP cleaves presequences. Tim21 associated with the TIM23 complex promotes IM sorting, possibly aided by respiratory-chain complexes III and IV (CIII and CIV). (**b**) Precursors with internal targeting signals follow different routes. 'Simple' outer-membrane proteins might interact with TOM components for their insertion. Some IMS precursors require cofactor attachment or disulfide-bond formation (mediated by protein labeled 'X') for import. The TIM22 complex (green) facilitates IM insertion of polytopic precursor proteins, whereas the SAM complex (brown) inserts OM β-barrel proteins. Small TIM proteins chaperone these precursors in the IMS. A membrane potential ($\Delta\psi$) is required for the insertion or translocation of precursors at the inner membrane.

The functions of most import components have been elucidated using the yeast *Saccharomyces cerevisiae* as a model organism. It has a pliable genetic system, which makes it readily amenable to gene deletions, mutations and genetic screens. These techniques have been used in conjunction with an *in vitro* protein-import assay, in which radiolabeled proteins are incubated with mitochondria isolated from normal and mutant cells [1]. More recently, proteomic approaches have been used to identify new proteins associated with known import complexes (e.g. [2,3]). This has been achieved through tagging a known import component (e.g. with a protein A or His tag) and performing affinity chromatography of the protein within its complex, followed by sequencing of co-purified

components. It is assumed that most components of the mitochondrial import machinery have now been identified. Although much information has come from genetic and *in vitro* approaches, structural data would enable us to redirect our efforts into understanding the fine mechanistic details of the import process. This would lead to the formation of novel hypotheses that could be pursued by cell biology studies. For example, based on structural insights, site-directed mutagenesis of yeast genes encoding protein-import components could be performed and their effects analyzed by *in vivo, in organello* and *in vitro* techniques. Unfortunately, such structural data have proved highly difficult to attain for several reasons: the major components are membrane-integrated proteins that are not overly abundant in mitochondria and can be difficult to isolate; recombinant proteins produced by heterologous expression have also had limited success in crystallization trials (particularly for the larger components); and the components often exist in complicated and dynamic complexes and success might therefore entail finding the right combination of binding partners for co-crystallization. Nevertheless, some of these hurdles have been overcome and several protein/domain structures of the protein import machinery have been solved recently (Table 1 and below). The structures of these components and the way in which they function at a cellular and molecular level can now be scrutinized.

At the outer membrane

Although proteins imported into mitochondria contain targeting signals specific for their subcompartment, their initial interactions with mitochondria take place at the TOM complex. This complex consists of the receptors Tom20 and Tom70, a central organizer Tom22, the translocation channel Tom40 and one or more low molecular-weight TOM subunits, whose functions are not well understood. Precursors with N-terminal targeting signals generally interact with the Tom20 receptor, whereas others that contain internal targeting signals appear to interact with Tom70, often in concert with cytosolic molecular chaperones. The structures of both receptors have now been determined and provide insights into the mechanism of precursor recognition at the mitochondrial surface.

Tom20 and presequence interactions

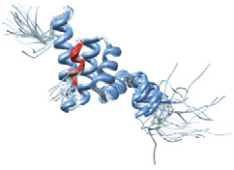
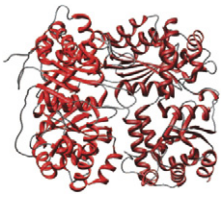
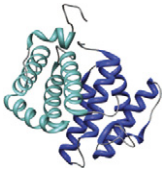
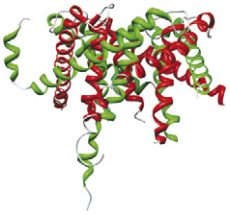
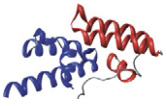
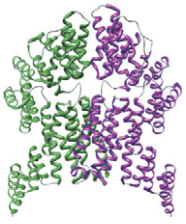
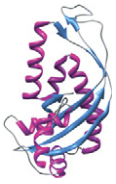
An NMR structure of the cytosolic portion of rat Tom20 (lacking its transmembrane anchor) in association with a presequence peptide revealed an all-helical secondary structure with five α-helices [4]. Helices 1 and 2 constitute a tetratricopeptide repeat (TPR) that forms one side of a surface groove in Tom20, whereas helix 3 makes up the other side (Table 1). The amphipathic α-helical presequence peptide binds within this surface groove and this interaction is mediated primarily by the hydrophobic side chains of a few residues. The positively charged side-chains of the presequence, although necessary for import, do not contact Tom20, thus leaving them available to face the aqueous solvent or to contact additional members of the TOM machinery. This would enable precursor proteins to interact dynamically with the import machinery and facilitate their stepwise transfer along the import pathway. Intriguingly, an NMR structure of the *Arabidopsis* Tom20 cytosolic domain resembles that of rat Tom20 but with its topology reversed, such that its C-terminal sequence is embedded in the membrane while the N-terminal end forms a cytosolically exposed receptor domain [5]. These findings led to the proposal that plant and animal Tom20 receptors are derived from separate ancestral genes, which had undergone convergent evolution [5], and they also highlight the important function of Tom20 in the recognition of a large set of precursor proteins.

Tom70 receptor: binding chaperones and precursors

Although Tom20 binds presequences, Tom70 acts as the main docking site for hydrophobic precursor proteins. Tom70 was first identified as a receptor for the precursor of the ADP/ATP carrier (AAC), which has six membrane-spanning segments and is targeted to the inner membrane [6]. AAC contains internal targeting information distributed throughout its length. Transit of the AAC precursor can be arrested at Tom70 by artificial depletion of ATP levels [7,8]. Tom70 itself does not hydrolyze ATP, thus suggesting that other ATPases, such as molecular chaperones, are involved in delivering the unfolded AAC precursor to the receptor. Indeed, the molecular chaperones Hsp70 and Hsp90 can bind precursor proteins and dock directly onto Tom70 [9]. However, substrates can bind to Tom70 directly as well. Cross-linking experiments with the precursor of AAC revealed that it associated with multiple Tom70 dimers. Furthermore, dividing the AAC precursor into three modules, each containing two transmembrane domains and a loop, showed that all could cross-link to a Tom70 dimer separately [8]. These observations were reinforced by the crystal structure of the yeast Tom70-receptor domain (Table 1). In the dimeric complex, each subunit contains 26 α-helices, arranged into 11 TPR motifs [10]. The first three TPR motifs form part of an N-terminal domain that is connected to the C-terminal domain by an unresolved linker. Previous studies suggested that the N-terminal domain interacts with Hsp70 [9,11] and, indeed, it resembles the clamp-like domain of the Hsp70/Hsp90-organizing protein (Hop), a cytosolic adaptor protein that binds these chaperones as part of steroid hormone regulation [12]. In addition, a large pocket in the C-terminal domain of Tom70 contains a mix of both hydrophobic and polar residues and might be involved in direct substrate binding [10]. The putative Hsp70- and substrate-binding sites are located on opposite sides of each Tom70 monomer. However, because the monomers are mirrored in the structure, both binding sites are found on each face. It was postulated that the arrangement might enable the dimeric Tom70 to interact with two Hsp70-bound precursor proteins at any one time [10]. Alternatively, as shown for AAC, the hydrophobic precursor might need to make multiple contacts with Tom70 and Hsp70 molecules to prevent its aggregation at the mitochondrial surface. Thus, Tom70 might also act as a molecular chaperone.

There is evidence to suggest that at least some precursors can use either Tom20 or Tom70 as a receptor. The structures of these receptors provide some insight into how this could be achieved. The hydrophobic face of the presequence could

Table 1. Structural details of mitochondrial protein-import components

Protein[a]	Structure	Function	Refs	PDB ID[b]	Description	Related structures
Rat Tom20-receptor domain and presequence		Outer membrane receptor for precursors with N-terminal-targeting signals	[4]	10M2	Five α-helices with α1–4 forming a defined hydrophobic core. Helices 1 and 2 form a TPR motif that is involved in protein–protein interactions. The α-helical presequence (red) sits in the hydrophobic pocket	*Arabidopsis thaliana* Tom20 [5]
Yeast mitochondrial-processing peptidase (MPP)		Matrix localized protease that cleaves presequences from preproteins	[38]	1HR2	Dimer of α- and β-subunits with a central cavity for presequence binding and cleavage	Core I and core II subunits of respiratory chain complex III [39]
Rat ALR (Erv1)		Sulfhydryl oxidase involved in disulfide-bond formation in the IMS	[67]	2HJ3	Homodimer connected by S–S bonds and hydrophobic packing. Each subunit forms a cone-shaped helical bundle with an attached FAD cofactor	–
Human Tim9–Tim10 complex		Chaperones some hydrophobic precursors in the IMS	[47]	2BSK	Hexamer of alternating Tim9 (red) and Tim10 (green) subunits. Forms α-propeller topology with central core stabilized by intramolecular S-S bonds and intermolecular salt bridges	Skp [48,49] Prefoldin [50]
Yeast Tim16-Tim14 (Pam16-Pam18) matrix domains		Regulates mtHsp70 function as part of presequence translocase-associated motor (PAM)	[35]	2GUZ	Each subunit contains three α-helices and a J-protein fold. Helices II and III form an antiparallel hairpin. In Tim14, the loop between these helices contains a HPD motif found in J-proteins. Tim16/Pam16 (blue) and Tim14/Pam18 (red)	J-proteins [36]
Rat Tom70-receptor domain		Outer membrane receptor for some hydrophobic precursor proteins	[10]	2GW1	Forms a homodimer. Each monomer contains 26 α-helices forming 11 TPR domains. N- and C-terminal domains might be involved in separate binding of chaperones and substrates	–
Yeast Tim21 IMS domain		Associates with TIM23 complex for sorting proteins to the inner membrane	[25]	2CIU	Contains two α-helices H-bonded to an eight-stranded β-sheet	–
Yeast Tim44 matrix domain		Tethers mtHsp70 to TIM23 complex	[26]	2BSK	Contains six α-helices and four β-strands. Forms a large hydrophobic pocket potentially involved in membrane binding	–

[a]Proteins are presented in the order that the structures were solved (most recent at bottom).
[b]RCSB Protein data bank ID (www.rcsb.org/PDB).

interact alternatively with the pocket in Tom70 because it is big enough to accommodate an α-helix. Similarly, the AAC precursor can be cross-linked to Tom20 [13], perhaps by interacting with its hydrophobic pocket.

The TOM complex

Tom40 is the pore-forming subunit and is the only TOM subunit essential for viability in yeast. However, Tom22 is also crucial for mitochondrial function. Similar to Tom20 and Tom70, Tom22 appears to have some receptor activity, however, it also holds the TOM complex together [13] and makes contact with components of the inner-membrane TIM23 complex [3,14]. Cryoelectron microscopy of the entire TOM complex purified from *Neurospora crassa* revealed that it is comprised of up to three stain-filled pits, each approximately 2 nm in diameter [15]. The general appearance varies according to the components present and the purification conditions [15–17]. A 2 nm pore is also consistent with the size deduced for an isolated Tom40 channel using electrophysiological methods [18]. The reason for the presence of multiple pores within a single TOM complex is not yet clear; however, this is not a unique phenomenon among the protein translocases (Box 2). The diameter of the Tom40 channel requires that precursor proteins translocate across it in a largely unfolded conformation, although there is evidence that loop-wise translocation events can be accommodated [8]. Although Tom40 acts as the membrane translocase, it is not just a passive player. For example, the inner-channel region of Tom40 and/or the face that projects into the intermembrane space (IMS) has affinity for unfolded proteins and can prevent protein aggregation [19]. Thus, Tom40 itself might facilitate the forward movement of unfolded precursor proteins into mitochondria and/or act as a molecular chaperone at this step along the import pathway.

Protein sorting at the TIM23 complex

Following translocation through the TOM complex, pathways diverge to sort precursor proteins to their respective mitochondrial destinations. The TIM23 complex sorts both inner-membrane and matrix proteins, although it appears that specific components regulate these two processes. The central components of this complex are: the translocase channel Tim23; a homologous subunit Tim17 whose function is not clear; and Tim50, which is involved in both precursor recognition in the IMS and channel gating [20–23]. Tim44, another component, is involved in linking the TIM23 complex to mtHsp70–PAM, which then drives the import of unfolded precursor proteins into the matrix (Box 1 and below). Tim21 was identified recently as an additional subunit of the TIM23 complex that lacks mtHsp70–PAM and shifts the role of the translocase to sorting precursor proteins into the inner membrane [3].

Tim21: switching the TIM23 complex to membrane sorting

Tim21 faces the IMS and makes contact with Tom22, thereby priming the TIM23 complex to contact incoming precursors for their sorting to the inner membrane [3,14].

Box 2. Translocases: just passing through?

The mitochondrial translocases (TOM, SAM, TIM23 and TIM22) are membrane-integrated assemblies that transport precursor proteins across or into membranes. The channel-forming subunits of the TOM and SAM complexes are predicted to assume a β-barrel topology, whereas those of TIM22 and TIM23 are likely to be α-helical. Detailed molecular structures would markedly improve our understanding of how they function in precursor translocation.

Thus far, low-resolution cryoelectron microscopy and electrophysiological techniques have illuminated several features of the channel subunits. However, many questions regarding pore structure, function and regulation remain unresolved. For instance, the holo–TOM complex appears to contain 1–3 stain-filled pits ~2 nm in diameter [15]. Although analysis of the channel-forming subunit Tom40 reveals that it forms a single channel, the variable number of channels depends on the presence of additional TOM components [16,17]. Similarly, single-particle analyses of the purified β-barrel subunit Sam50/Tob55 of the SAM (TOB) complex [2,57,58] reveals a donut-like structure with an internal diameter of approximately 4–5 nm [58]. As yet, it is unclear how SAM integrates proteins into membranes because it is unfavorable energetically for the β-strands within Sam50 to be disrupted to enable lateral release of precursors into membranes. SAM might simply supply a scaffold for precursor folding, masking the newly folded proteins from the aqueous environment of the IMS to facilitate membrane insertion.

Although the purpose for multiple channels in the TOM complex is unknown, it appears that other translocase complexes also possess a similar make-up. Cryoelectron microscopy of the isolated TIM22 complex revealed a twin-pore structure with each stain-filled pit being approximately 1.6 nm in diameter [59]. Currents measured from recombinant Tim22 reconstituted into vesicles indicate a single voltage-gated channel [60]. By contrast, two channels were measured from the purified endogenous TIM22 complex [59]. Likewise, purified Tim23 forms a single hydrophilic channel of approximately 1.3–2.4 nm wide [61], yet electrophysiology experiments on reconstituted inner membranes revealed that the entire TIM23 complex forms two voltage-gated pores [62]. Interestingly, these pores are regulated by Tim17, an integral part of the TIM23 translocase that displays sequence homology to Tim23 [62]. Given that Tim17 has roles in both protein integration into the inner membrane and translocation to the matrix [3], multiple channels might enable the translocases to 'multi-task' efficiently. Another possibility for multipore assemblies is that one channel is involved in protein translocation and the other is involved in binding regulatory proteins, as was shown recently for the bacterial translocase SecY [63].

Such a mechanism might prevent these precursors from inserting incorrectly into the outer membrane or aggregating in the IMS. Following precursor translocation, Tim21 dislodges from Tom22 and then appears to associate with complex III of the respiratory chain [24]. It has been proposed that complex III binding generates a localized increase in the transmembrane proton gradient around the TIM23 complex, however, it remains unclear how this would facilitate protein insertion into the membrane.

Given the function of Tim21, it is surprising that yeast cells lacking this protein are viable and only exhibit mild growth defects at high temperatures [3]. This is in contrast to all other known subunits of the TIM23 complex, which are essential for yeast-cell viability. It also indicates that additional factors might be involved in regulating the sorting activity of the TIM23 complex. The crystal structure of the Tim21 IMS domain was determined recently [25], revealing a novel fold of two α-helices secured to one face of an eight-stranded β-sheet by multiple hydrogen

bonds (Table 1). Because Tim21 associates with the Tom22 IMS domain by electrostatic interactions, it is possible that conserved patches of positively charged residues on the surface of Tim21 might interact with negatively charged residues on Tom22 [25]. However, until further work is done, key questions remain unresolved. These include the nature of Tim21 interactions with both the TIM23 complex and complex III and also how Tim21 might regulate the dual sorting roles of the TIM23 complex.

Regulation of mtHsp70 for matrix import

mtHsp70 is involved in the translocation of precursors across the Tim23 channel and into the matrix. Tim44 and PAM components are involved in tethering mtHsp70 to the TIM23 complex. Some of these subunits also regulate the mtHsp70 ATPase activity in concert with the nucleotide-exchange factor Mge1. Tim44 was one of the first components identified as regulating mtHsp70 action. The crystal structure of the membrane-binding, C-terminal domain of yeast Tim44 (residues 210–431) shows a central, four-stranded, anti-parallel β-sheet sitting between six α-helices, which are distributed on either side [26]. A cleft lined with conserved hydrophobic side chains is a prime candidate for mediating membrane association (Table 1). Although this provides us with information into the potential contacts that Tim44 makes with membranes, the structure of the entire molecule will provide information into how it interacts with other components of the TIM23 complex or with mtHsp70. Matrix protein import is regulated by other components that influence mtHsp70 action. Pam18 (Tim14) and Pam16 (Tim16) are members of the J-protein family. J-proteins typically bind and promote the ATPase activity of Hsp70 members, however, only Pam18 appears to perform this function for mtHsp70 [27–29]. Pam16 instead binds and regulates the function of Pam18 [30–33]. Another component, Pam17, is implicated in linking Pam16–Pam18 to the TIM23 complex [34]. A crystal structure of the essential domains of yeast Pam18 (residues 99–168) in complex with Pam16 (residues 54–119) [35] shows that both molecules adopt a fold like that of other J-proteins [36]. The His–Pro–Asp (HPD) consensus motif, which is required for J-protein activity, is located in Pam18 at the midpoint of an antiparallel hairpin formed by helices II and III (Table 1). Pam16 lacks this, explaining why it is unable to stimulate mtHsp70 ATPase activity. A positively charged surface on Pam18, which is believed to mediate mtHsp70 interactions, is also absent from Pam16 [35]. In complex, Pam16 might exert tight structural constraints on the Pam18 HPD-containing loop to regulate mtHsp70 interaction and ATPase activity. However, given that there are multiple components implicated in regulating mtHsp70 activity, analysis of the entire complex will be required to fully appreciate the molecular mechanisms involved in driving precursors into the matrix.

Processing matrix targeting signals

Typically, matrix targeting signals are removed by the mitochondrial-processing peptidase (MPP) on their entry into the matrix (Box 1). Although matrix-targeted presequences can form an α-helix when in contact with Tom20 [4], it appears that they can also adopt different conformations. Structural characteristics of presequence peptides in solution have shown that they can be extended but can also adopt helical conformations with membrane mimetics [37]. Indeed, two crystal structures of presequences trapped within yeast MPP displayed a largely extended conformation [38]. MPP is a heterodimer of homologous α and β subunits (Table 1). A conserved, highly flexible, glycine-rich loop in α-MPP has been implicated in substrate binding and release [38]. Prior to cleavage, the presequence is bound within a large cleft at the interface between the two MPP subunits. This cavity is rich in acidic residues, favoring ion-pairing and hydrogen-bonding interactions with the predominantly basic presequence. Presequences vary in sequence, and therefore different lengths can be accommodated inside the cavity by forming additional hydrogen bonds to available β-strands within the protease. MPP is similar structurally to the core I and II subunits of the ubiquinol–cytochrome *c* oxidoreductase (complex III) of the mitochondrial respiratory chain [39]. In mammals, these subunits have protease activity and process the Rieske Fe–S precursor to generate the mature subunit as well as the cleaved presequence, which is retained as subunit nine within the complex [39]. In plants, MPP is absent altogether and the core I and II subunits perform all presequence-processing functions [40]. Given that complex III associates with Tim21 in yeast [24], its protease activity could also be involved in processing presequences of those precursors sorted to the inner membrane, although this remains to be resolved.

Role of the small TIM proteins in divergent protein sorting pathways

Precursors of the metabolite-carrier family (e.g. AAC), which are directed to the inner membrane, use a separate sorting pathway that diverges from the matrix-targeted pathway following exit from the TOM machinery. Members of the small TIM family are involved in chaperoning these precursors across the IMS and delivering them to the TIM22 complex. Small TIM proteins also interact with precursors that form β-barrel proteins in the outer membrane. These precursors first translocate through the TOM complex into the IMS before their SAM-mediated insertion into the outer membrane (Box 1). In yeast, the most important members of the small TIM family are Tim9, Tim10 and Tim12 because all are essential for cell viability [41–43]. Tim9 and Tim10 form a stable hexamer that is soluble in the IMS. These proteins also associate with Tim12, which, along with Tim22, Tim54 and Tim18, constitutes the TIM22 complex [44]. During the functional characterization of the small TIM proteins, it was discovered that they contained intramolecular disulfide bonds [45,46]. By using an *Escherichia coli* strain with enhanced disulfide-bond formation, recombinant human Tim9 and Tim10 were produced, facilitating the successful reconstitution of the hexameric complex for structural analysis (Table 1). The crystal structure reveals that individual subunits have an α-hairpin topology, in which two intramolecular disulfide bonds brace a central loop connecting anti-parallel N- and C-terminal helices [47]. Tim9 and Tim10 subunits alternate within the assembly, resulting

Box 3. Trapping proteins in the IMS

The major driving forces involved in importing precursor proteins into the matrix and inner membrane are $\Delta\psi$ and mitochondrial mtHsp70. However, some proteins of the IMS and all outer-membrane proteins do not use these forces for their import. Although it is currently unknown how precursors of the outer membrane are integrated into the membrane, some information is now available for a subset of IMS proteins. Many of these proteins are small (7–16 kDa) and, following their translocation across the TOM machinery, become trapped in the IMS by factors that assist in their folding (Figure I).

One such mechanism is cofactor attachment. For example, the precursor of cytochrome *c* translocates across the TOM channel and into the IMS through its interaction with inner-membrane-bound cytochrome *c* heme lyase (CCHL) [64]. CCHL is involved in the covalent attachment of heme, which thereby traps cytochrome *c* in the IMS. CCHL might function specifically in cytochrome *c* biogenesis, whereas other proteins involved in disulfide-bond formation appear to act on a variety of substrates, including the small TIM proteins [54,56,65,66]. To initiate oxidative folding of the small TIMs, Mia40 captures them as they enter the IMS and forms transient 'mixed disulfide' intermediates through covalent S–S linkages, thus preventing any retrograde translocation. The mixed disulfide is resolved into an oxidized TIM subunit and reduced Mia40. Erv1, a FAD-dependent sulfhydryl oxidase, accomplishes re-oxidation of Mia40.

Although the function of Erv1 in the biogenesis of IMS proteins was only discovered recently, the structure of its human counterpart [first termed augmenter of liver regeneration (ALR)] depicts a cone-shaped helical bundle that binds a FAD cofactor at the mouth of the cone [67]. It forms a dimer linked by two intermolecular disulfides with considerable hydrophobic packing (Table 1). Two intramolecular disulfides are also found within each monomer. These disulfides and the bound FAD attest to its sulfhydryl oxidase activity – after oxidizing Mia40, cysteines in Erv1 are reoxidized by electron transfer to FAD. These electrons might then be fed into the electron-transport chain by cytochrome *c* [68,69]. Because the structure of both Erv1 and the small TIM proteins are known, it will be important to obtain structural and functional information on Mia40 to understand the cycling events involved in disulfide-bond formation in the IMS.

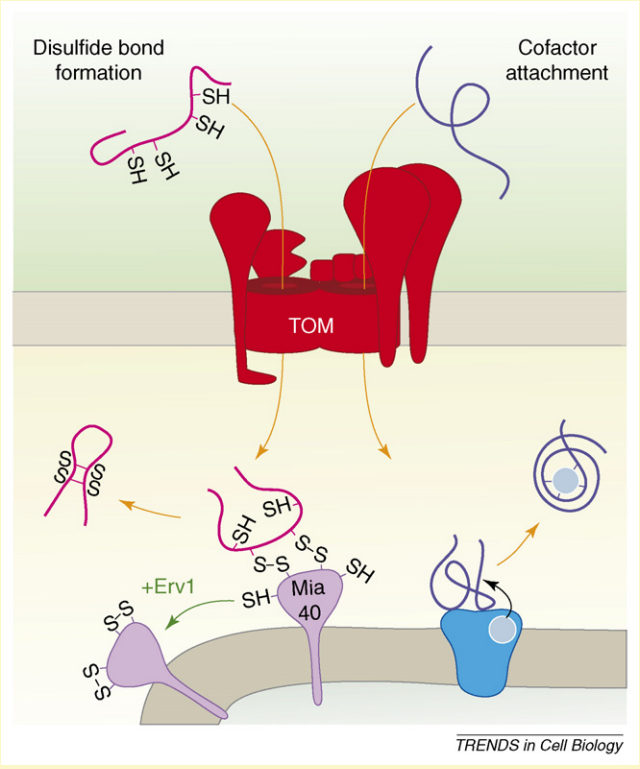


Figure I. Trapping precursors in the IMS. Some precursor proteins destined for the IMS translocate the TOM complex and become trapped within this compartment through facilitated protein folding events. For example, a set of precursor proteins become trapped in the IMS by a redox pathway that involves transient covalent interactions with Mia40. The oxidized precursor folds and is released from Mia40. The sulfhydryl oxidase, Erv1, recycles Mia40 into its active form. Another set of precursor proteins interact with assembly factors involved in cofactor attachment that results in their folding and hence their trapping in the IMS.

in the formation of a modular six-bladed α-propeller with a central pore of approximately 1.5 nm in diameter. Although the structure has been determined, the substrate binding sites can only be speculated upon. With small dimensions and a polar surface, it is unlikely that the pore is a binding site for the hydrophobic precursors. Therefore, the helices are the probable candidates for forming substrate contacts and would enable interactions with a variety of substrates, including both metabolite-carrier and β-barrel precursors. Indeed, the structure of the Tim9–Tim10 complex resembles that of the prefoldin and Skp chaperones of archaea and bacteria, respectively [48–50], and substrates have been visualized by cryoelectron microscopy contacting the α-helical tentacles of prefoldin [51]. Recently, peptide screens and complementation analysis in yeast were used to confirm that the N-terminal helix of Tim10 is involved in substrate binding [52,53]. Future cellular and biochemical studies will be required to ascertain the functions of the other regions within the structure. This will include identifying potential regions involved in docking of the Tim9–Tim10 complex with the TIM22 complex.

Disulfide-bond formation is important for maintaining the structure of the Tim9–Tim10 complex and it also has a crucial role in the import process of these proteins [45,54]. The disulfides are formed by a relay system involving the IMS proteins Mia40 and Erv1 [54–56]. Without the formation of disulfide bonds, the IMS-precursor proteins do not fold and can exit back into the cytosol through the TOM complex [54]. This mechanism of trapping by protein folding appears to be a specific pathway for the import of a subset of IMS proteins, the significance of which is only beginning to be realized (Box 3).

Concluding remarks

Although there has been considerable progress in gaining structural insights into the mitochondrial import machineries, many questions remain unanswered (Box 4). There are still seemingly intractable issues with obtaining

Box 4. Outstanding questions

- How do import components recognize precursor proteins efficiently, yet also facilitate their stepwise transfer along the respective import pathway in a directed and efficient manner?
- What is the mechanism for lateral release of precursor proteins from the translocases into the mitochondrial membranes?
- How does the mitochondrial $\Delta\psi$ act in regulating the function and sorting activities of the inner-membrane translocases?
- How do the multiple pores observed within the various translocases facilitate protein passage?
- What are the structural features of the multisubunit translocation complexes and how do these relate to their function in protein import?

structures of the larger and membrane-integrated components and precursor-bound translocation intermediates. Clearly, future analyses will need to focus on these issues. In addition, directed-mutagenesis approaches will help to authenticate potentially important features identified in already determined protein structures. This information will further clarify mechanisms involved in precursor recognition, translocation across channels, membrane insertion and the regulation of the import machineries. Such experiments will be crucially dependent on studies that incorporate phenotypic analysis of directed mutations in yeast (and other organisms that are amenable to such studies), along with classical approaches that use *in vitro* protein-import assays. Thus, structural insights will drive cell biologists to undertake new endeavors that will further our understanding of a long studied but complicated machinery.

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