Mechanisms of Protein Import into Mitochondria

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Apart from a handful of proteins encoded by the mitochondrial genome, most proteins residing in this organelle are nuclear-encoded and synthesised in the cytosol. Thus, delivery of proteins to their final destination depends on a network of specialised import components that form at least four main translocation complexes. The import machinery ensures that proteins earmarked for the mitochondrion are recognised and delivered to the organelle, transported across membranes, sorted to the correct compartment and assisted in overcoming energetic barriers.

Introduction
Protein trafficking is a vital cellular process in which proteins are transported from their site of synthesis to locations within cells where they function. In eukaryotes these locations are numerous, as cells are organised into several compartments formed from single or multiple membrane boundaries. Nuclear-encoded proteins, which are synthesised in the cytosol, are transported for example to organelles such as mitochondria, the endoplasmic reticulum (ER), peroxisomes, the nucleus and chloroplasts. A small number of proteins are encoded by the genomes of mitochondria and chloroplasts, and these add to the complexity of protein trafficking pathways. Here we shall discuss recent advances in our understanding of the mechanisms of import of nuclear-encoded proteins into mitochondria, and also the export pathway to the inner membrane that is used by mitochondrially encoded proteins.

Because of the way the mitochondrion is divided into four subcompartments — the two aqueous chambers (intermembrane space and matrix) and two membranes (the outer and inner membranes) — there are multiple pathways for protein translocation into this organelle. Most of the ~1,000 mitochondrial proteins are encoded by the nuclear genome, synthesised in the cytosol on free ribosomes as precursor proteins and then transported into or across mitochondrial membranes with the aid of a handful of distinct complexes (Figure 1). Targeting information resides within the protein itself; this may take the form of an amino-terminal extension or ‘presequence’, though for many membrane proteins it is contained within the mature protein [1–4]. The transfer of mitochondrial proteins from their site of synthesis in the cytosol to the surface of the mitochondrial outer membrane, where translocation begins, mainly occurs in a post-translational manner — that is, the fully synthesized protein contacts the mitochondrial import machinery [1–3,5,6]. However, a co-translational mechanism of translocation cannot be excluded for some precursor proteins [1–3,5,6].

To date, a single ‘translocase of the outer membrane’ (TOM) complex has been described, which is the only known entry point into mitochondria for nuclear-encoded proteins. After crossing the outer membrane at the TOM complex, precursor proteins segregate, as there are two structurally and functionally distinct ‘translocases of the inner membrane’ (TIMs): the TIM23 and TIM22 complexes, each of which guides the import of subsets of proteins to internal compartments of mitochondria.

All proteins with an amino-terminal presequence are imported via the TIM23 translocase [7]. The transport of presequences across the membrane occurs through an aqueous pore, while the membrane potential across the inner membrane provides a driving force for this energy-requiring process [8–11]. In addition, the ATP-dependent action of matrix heat shock protein 70 (mtHsp70), together with its helper proteins Tim44 and Mge1, drives to completion the unidirectional movement of precursor proteins into the mitochondrial matrix [12–15].

Some inner membrane proteins, which only contain internal targeting signals, are imported with the assistance of the pore-forming TIM22 complex in a membrane potential-dependent manner [16–21]. The mitochondrial export machinery is responsible for the insertion of proteins into the inner membrane from the matrix (Figure 1). The action of this machinery resembles protein translocation processes in bacteria.

Targeting Signals
Two types of mitochondrial targeting signal can currently be described: presequences and internal signals. Presequences direct precursor proteins via the TOM complex to the TIM23 translocase, where they are sorted to the matrix, inner membrane or intermembrane space (Figure 2). One would expect presequences to have a consensus amino acid sequence, but rather they consist of a handful of subtle distinguishing properties at the level of the primary and secondary structure. They generally have a high content of basic, hydrophobic and hydroxylated amino acids, and a length of about 10–80 amino acids [22]. An amphipathic α helix with one positive and one hydrophobic face is another common feature that is important for receptor recognition [1–3,23].

Once they are exposed to the matrix, presequences are often removed by a processing peptidase, as they are not necessary for protein function. In comparison, proteins which only contain internal targeting signals are directed to either the inner membrane, intermembrane space or outer membrane, depending on the nature of the signal — which, to date, has not been clearly defined (Figure 2). The
internal targeting information, best characterised for members of the carrier family of the mitochondrial inner membrane, is hidden in the mature protein amongst amino acid residues important for folding and function [2–4]. The targeting information seems to be spread throughout the length of the protein [4,24].

The Importance of Surface Receptors for Outer Membrane Translocation

Three proteins of the mitochondrial outer membrane TOM machinery act as receptors for the recognition of targeting signals: Tom70, Tom20 and Tom22 (Figure 3). Each has a large cytosolic domain anchored to the membrane, with a single transmembrane span. Tom22 also has a small, functional carboxy-terminal domain which is exposed to the intermembrane space. Tom22 forms part of a very stable general import pore (GIP) complex of the outer membrane, which additionally contains the pore-forming protein Tom40 and accessory proteins Tom5, Tom6 and Tom7 (Figure 3) [25–28]. Tom70 and Tom20 are the primary receptors: they are the first Tom proteins to make contact with precursor proteins, but show preferences for different precursor types. They are both only loosely associated with the GIP complex [25,26,28,29].

Tom70 is the major receptor for precursor proteins that contain internal targeting information, such as members of the metabolite carriers of the inner mitochondrial membrane [1–3]. It binds to multiple segments within the carrier proteins, though an amino acid recognition motif has not been identified [4,30]. The carrier proteins consist of three related modules, each containing two transmembrane domains connected by a hydrophilic loop, such that a carrier monomer contains six transmembrane domains. Each repeat module of a carrier contributes targeting information, so that a carrier precursor binds to several molecules of the Tom70 receptor simultaneously [24]. Interestingly, it has recently been shown that Tom70 is not only a receptor for precursor proteins, but also a docking point for cytosolic chaperones directly involved in the import pathway [31]. The transfer of newly synthesised Tom70-dependent precursor proteins from the cytosol to the receptor can now be described. After synthesis, the precursor protein forms a proteinaceous high molecular weight complex [32], which in mammals contains Hsp90 and Hsp70 and in yeast Hsp70 (Figure 3) [31]. This is the first time Hsp90 has been implicated in a mitochondrial import pathway. Previous studies indicated that Hsp70 and other cytosolic factors such as mitochondrial import stimulating factor (MSF) [33–36] can also stimulate protein import into mitochondria, although it remains open if MSF plays a role in protein import in vivo [6]. An association with chaperones probably prevents aggregation of the precursor and permits presentation of the protein in a conformation conducive for receptor recognition.

The cargo precursor protein is then transferred to the Tom70 receptor, an event that requires the docking of Hsp70 to the receptor [31]. Binding of Hsp90 and Hsp70 to Tom70 is mediated by a specialised tetra-tricopeptide repeat (TPR) domain in Tom70. The determinants for selective chaperone docking are apparently contained within Tom70. As has been previously described, the transport of carriers to subsequent import stages requires hydrolysis of ATP [32]; the authors propose that ATPase cycling, most likely by the chaperones, leads to release of the precursor proteins from Tom70.

The main function of Tom20 is to bind precursor proteins with presequences. The hydrophobic face of the helical presequence is predicted to interact with Tom20 [30]. The structure of a presequence in a complex with a soluble portion of Tom20 clearly shows that the helical presequence indeed fits into a
An aqueous pore through the ER translocon, the GIP complex, is located at the ER side of the mitochondrial outer membrane. The majority of the 100 kDa GIP complex is composed of Tom40 and small proteins, while the remaining components are found in association with the TOM complex or to be inhibited in their import when the Tom40 channel is blocked, indicating the broad capacity of this translocase for transporting all types of nuclear-encoded mitochondrial proteins [7,32,43–46]. Tom6 and Tom7 are integral membrane proteins that play a structural role in the organisation and stability of the TOM complex [47,48]. Tom6 facilitates interaction of the single TOM channel complex of 100 kDa with Tom22 to form the full-sized GIP complex [25,27,49]. Tom7 is at least partially antagonistic to Tom6. Tom7 favours dissociation of the TOM complex and also influences sorting of proteins to the outer membrane [48,49].

The multiple pore arrangement of the GIP complex is intriguing, but why has it evolved in this way? Several proteins of different types and locations have been found in association with the TOM complex or to be inhibited in their import when the Tom40 channel is blocked, indicating the broad capacity of this translocase for transporting all types of nuclear-encoded mitochondrial proteins [7,32,43–46]. Tom6 and Tom7 are integral membrane proteins that play a structural role in the organisation and stability of the TOM complex [47,48]. Tom6 facilitates interaction of the single TOM channel complex of 100 kDa with Tom22 to form the full-sized GIP complex [25,27,49]. Tom7 is at least partially antagonistic to Tom6. Tom7 favours dissociation of the TOM complex and also influences sorting of proteins to the outer membrane [48,49].

The multiple pore arrangement of the GIP complex is intriguing, but why has it evolved in this way when other translocases have only a single pore? It may relate to the fact that import into mitochondria occurs post-translationally: imported proteins are synthesised in their entirety before any portion is translocated. For polypeptide membrane proteins, a pair of transmembrane α helices separated by a hydrophilic loop (a helical hairpin) may insert into one pore while a second helical hairpin in the same protein inserts into a second TOM pore, allowing simultaneous import of multiple domains. Such a mechanism may promote efficient membrane insertion and subsequent assembly. An en bloc import mechanism would require considerable flexibility of the translocation complex in order to allow either lateral movement of the protein into the membrane or movement through the translocons to the intermembrane space.

With respect to its size a single TOM pore is wide enough (~20 Å) to carry two closely packed transmembrane α helices at the same time [26,41,50]. At least for the ADP/ATP carrier (AAC), import across the
outer membrane can occur in a partially folded state in which helical hairpins traverse the outer membrane [24,32]. In comparison, import into the ER translocon occurs largely co-translationally, so that the translocation machinery is presented with polypeptide segments step by step as they are synthesised. For polytopic membrane proteins, lateral release from the ER translocon may occur simultaneously once all of the transmembrane segments have gathered in the one translocon pore [51]. This may be necessary for correct intramolecular assembly. Indeed the active translocon is larger than the TOM pores, with an estimated diameter of 40–60 Å, and so could accommodate multiple transmembrane segments [52].

Assembly of Translocation Components
All mitochondrial translocation components are nuclear-encoded. This means that they must also be imported and assembled into multisubunit membrane complexes following synthesis in the cytosol on free ribosomes. Is a specialised machinery required for this process, or do the standard pre-existing translocation components suffice? At least for the essential pore-forming protein Tom40, the evidence indicates that a combination of these possibilities leads to its correct import and assembly [49].

The Tom40 precursor is first recognised by the TOM import machinery, as for any other precursor. It is targeted to the outer membrane mainly via recognition by the receptors Tom20 and Tom22. It is then transferred to a unique sorting and assembly complex which is distinct from the mature TOM complex [49]. Upon release from this assembly complex, the Tom40 precursor is found in a partial GIP complex in association with Tom6. Assembly to the mature GIP complex from this intermediate occurs upon incorporation of Tom22 and Tom7. The TOM components that join in the late assembly of Tom40 to form the GIP complex also occur as free endogenous components, indicating that the mature complex is structurally dynamic and can exchange its subunits. Tom7 also appears to play a role in the disassembly of the GIP complex.

The Import Pathway for Precursors with a Presequence
All precursor proteins that have a presequence are targeted to a specialised inner membrane translocation machinery — the TIM23 complex (Figure 4). The presequence is not only important for translocation of this subset of precursors across the outer membrane, but is also required for initiation of import at the inner membrane. The presequence is recognised by components of the TIM23 complex and also responds to a large electrical field at the inner membrane which actively promotes import [7–9,53].

What molecular events occur when the presequence emerges in the intermembrane space? A key player in the process is the multifunctional translocation component Tim23. Tim23 is a pore-forming integral protein of the inner membrane, which also has an amino-terminal hydrophilic receptor-like domain that extends into the intermembrane space [3,9–11,54]. Tim23’s hydrophilic domain incorporates a high-affinity binding site for presequence-containing precursor proteins, continuing the chain of precursor recognition events that begins with the outer membrane TOM receptors [9,54].

On its way to the matrix, a presequence-containing preprotein makes contact with several binding sites contributed by Tom20, Tom22 (cytosolic and intermembrane space domains), Tom5, Tom40 and then finally Tim23 (Figures 3 and 4). This chain of events was previously coined the ‘acid chain hypothesis’ because of the ionic nature of the interactions thought to be responsible for preprotein recognition and binding [54,55]. But as hydrophobic interactions have also been shown to be involved, the pathway has recently been renamed the ‘binding chain hypothesis’
Recent ly, it was reported that the extreme amino-terminal region of Tim23 also spans the outer membrane [58]. This could effectively bring the outer and inner membranes into close contact to promote efficient import. The missing link, however, is evidence for a direct contact between the Tim23 complex and the TOM machinery. With the recent discovery of a new translocation component, Tim50, we may be coming closer to understanding the true nature of TIM–TOM associations in organello.

The discovery of Tim50 came several years after the last novel member of the Tim23 translocon had been identified, and it had been thought that perhaps all the activities of the complex were performed by known components, even though there were indications that additional Tims exist [59–61]. But last year, two different biochemical strategies [62,63] brought to light Tim50, an essential and highly conserved component of the Tim23 translocon. In one approach [62], a tag was placed on the amino-terminal domain of Tim23 in vivo, and the intact translocon was isolated by affinity chromatography after mitochondrial solubilisation in a mild detergent. Isolated proteins were identified by mass spectrometry, revealing Tim50. In the second approach [63], a preprotein was arrested during import into mitochondria. Site-specific photocrosslinking was performed and cross-linked products were affinity purified via a tag on the preprotein. Identification of Tim50 forming part of the cross-link product was again achieved by mass spectrometry.

Tim50 is an integral membrane protein with a large carboxy-terminal domain protruding into the intermembrane space [62,63]. Selective depletion of Tim50 from yeast cells revealed that Tim50 plays a crucial role in the import of presequence-containing precursor proteins destined for the mitochondrial matrix [62,63], but Tim50 depletion had only a mild effect on the import of precursors with additional inner membrane sorting signals [62]. Precisely how Tim50 influences the presequence pathway has yet to be determined, but there are some tantalising possibilities.

Tim50 provides a functional link between the TOM machinery and the Tim23 channel. A key feature of Tim50 is that it binds to the part of Tim23’s amino-terminal domain exposed in the intermembrane space, and so it can guide precursor proteins directly to the channel protein. Tim50 also comes into direct contact with precursor proteins during their import. Tim50 may have chaperone-like properties and assist the classical matrix–targeted precursor proteins in the transfer across the intermembrane space and into the inner membrane channel.

Once precursors emerge in the mitochondrial matrix, their amino-terminal presequences are generally cleaved off by the dimeric mitochondrial processing peptidase, a zinc-dependent metalloendopeptidase [64,65]. The high-resolution structure of mitochondrial processing peptidase surprisingly showed that the presequence is in an extended conformation when bound to the peptidase, allowing access to the polypeptide backbone [66], as opposed to the helical conformation of the presequence when bound to the receptor Tom20 [23]. This indicates that conformational...
changes within the presequence itself are a way in which recognition by different translocation components can be accommodated.

Some precursors are additionally processed by a second matrix peptidase, mitochondrial intermediate peptidase, which removes an octapeptide behind the mitochondrial processing peptidase cleavage site [64]. A further processing peptidase, inner membrane peptidase, has its active site on the intermembrane space side. Both inner membrane peptidase subunits, Imp1 and Imp2, are homologous to the bacterial leader peptidase [67], and they remove the sorting sequences of some proteins that are directed to the inner membrane or intermembrane space [67,68]. Recently it was shown that, upon cleavage by mitochondrial processing peptidase, presequence peptides can be degraded by a novel mitochondrial zinc metalloendopeptidase, presequence protease [69].

Energetics of Import of Precursor Proteins with a Presequence

Two sources of energy are required for the translocation of precursor proteins across the mitochondrial inner membrane. The first to come into effect is the membrane potential (Δψ), which plays two roles in precursor import. It directly stimulates the channel protein Tim23 [9,10] and imparts an electrophoretic effect on presequences [8,53]. Most likely, the positively charged presequences are forced through the translocation channel as a result of an electrophoretic force originating from the mitochondrial membrane potential (Δψ) (negative on the matrix side) [8]. Thus it is not surprising that the magnitude of Δψ differentially influences the import efficiency of precursor proteins that vary considerably in the composition of their presequence. Interestingly, the membrane potential, by pulling upon the presequence, can even support the unfolding of precursor domains situated outside of mitochondria [70].

The second source of energy comes from matrix ATP. Completion of preprotein import into the mitochondrial matrix absolutely requires the ATP-dependent action of the molecular chaperone mtHsp70. Tim44, an adaptor protein located at the TIM23 translocase, is capable of switching the folding function of mtHsp70 to an essential role in protein translocation. Through binding to mtHsp70, Tim44 effectively localises a fraction of the total population of matrix mtHsp70 at the site of protein translocation [71–74]. The domains of mtHsp70 which are important for its folding function — an amino-terminal ATPase domain and a peptide-binding domain — are likewise essential for the translocation process. Positioned at the outlet of the import channel, mtHsp70 binds to both the presequence and mature segments of precursor proteins, which are presented in an unfolded state as they emerge from the translocation channel [75]. The energy derived from ATP hydrolysis by mtHsp70 drives protein translocation to completion. The co-chaperone Mge1 promotes the exchange of nucleotide from mtHsp70 after hydrolysis of ATP so that further reaction cycles may occur.

One of the most hotly debated topics in mitochondrial protein import has been the mechanism by which mtHsp70 action supports protein translocation. It is clear the precursor protein must be in an unfolded, linear conformation in order to pass through both the outer and inner membrane translocation channels [10,50,76]. But as translocation can occur post-translationally, some precursor proteins can first form folded domains. It is clear that mtHsp70 promotes the unfolding of these folded domains when the amino-terminal segment of the precursor protein is long enough to reach the matrix, but here the controversy begins.

It has been suggested that mtHsp70 actively pulls on translocating precursor proteins to facilitate the unfolding of protein domains on the outside of a mitochondrion, promoting active transport of precursor proteins into the mitochondrial matrix [13,77–79]. It is believed that an ATP-induced conformational change in mtHsp70 generates a pulling force on the preprotein, levering it into the matrix. This is the essence of the motor (pulling) model, one of two ideas describing mtHsp70 action (Figure 5). The alternative Brownian ratchet (trapping) model posits that mtHsp70 merely binds to the polypeptide chain, providing a bulky obstruction that effectively prevents the backsliding of the precursor protein, which moves by Brownian...
hydrophobic nature of the six transmembrane spans in of a presequence for targeting, and the highly most notable features of this protein family are the lack membrane. With respect to protein trafficking, the numerous metabolites across the inner mitochondrial of related proteins responsible for transport of mitochondrial proteins. It is a member of a large family The ADP/ATP carrier is one of the most abundant

The Carrier Import Pathway

The ADP/ATP carrier is one of the most abundant mitochondrial proteins. It is a member of a large family of related proteins responsible for transport of numerous metabolites across the inner mitochondrial membrane. With respect to protein trafficking, the most notable features of this protein family are the lack of a presequence for targeting, and the highly hydrophobic nature of the six transmembrane spans in each subunit. While the transport of carrier proteins through the cytosol and across the outer membrane is facilitated co-operatively by molecular chaperones and the TOM complex, as outlined in previous sections, their transport to the inner membrane depends entirely on a specialised translocase, the TIM22 complex (Figure 6).

When a carrier protein spans the TOM complex through the translocation pore formed by Tom40, it is still associated with the primary receptors Tom70 and Tom20 [45]. It is at this point that carrier proteins make contact with the first members of the TIM22 translocation machinery and thus diverge from the pathway taken by proteins with a presequence [18,19]. The carrier precursor interacts with a soluble 70 kDa hetero-oligomeric complex consisting of the essential subunits Tim9 and Tim10 [18,19,86,87]. This interaction occurs in a membrane potential-independent manner and is necessary to facilitate the release of the carrier from the primary receptors and hence permit subsequent movement of the protein across the outer membrane into the intermembrane space [18,19,45,88].

The purified Tim9–Tim10 complex has been shown to bind to the hydrophobic transmembrane spans of carrier proteins, rather than matrix exposed loops, and so it may act in a chaperone-like manner to prevent aggregation of these proteins when they are exposed to the aqueous intermembrane space [89]. But a carrier translocation intermediate entirely associated with the Tim9–Tim10 complex alone may not exist at any point in the translocation pathway as the translocating carrier has been found to make contact with the inner membrane while still associated with the outer membrane TOM machinery [90]. Thus transport from the outer membrane to the inner membrane occurs in a stepwise manner.

Contact of translocating carriers with the inner membrane is facilitated by the membrane-associated components of the TIM22 complex. This large complex consists of three integral membrane proteins, Tim22, Tim54 and Tim18, and a small percentage of peripherally associated Tim9 and Tim10 subunits as well as

![Figure 6. Import of carrier proteins into the inner membrane.](image-url)
Table 1. Mitochondrial export machinery.

<table>
<thead>
<tr>
<th>Component</th>
<th>Location and orientation</th>
<th>Proposed function</th>
<th>References</th>
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<tbody>
<tr>
<td>Oxa1</td>
<td>Integral inner membrane protein, Five TM segments, N_{out}–C_{in}</td>
<td>General insertion complex for export of mitochondrial and nuclear-encoded precursor proteins. Functions independently of Mba1</td>
<td>[105,107,109–114]</td>
</tr>
<tr>
<td>Mba1</td>
<td>Peripherally associated with inner membrane from matrix side</td>
<td>Required for the efficient membrane insertion of both mitochondrial and nuclear-encoded precursor proteins. A putative receptor that overlaps in substrate specificity with Oxa1</td>
<td>[105,114]</td>
</tr>
<tr>
<td>Cox18</td>
<td>Integral inner membrane protein</td>
<td>C-terminal export of cytochrome c oxidase subunit 2. Interaction partner of Mss2 and Pnt1</td>
<td>[116]</td>
</tr>
<tr>
<td>Pnt1</td>
<td>Integral inner membrane, Two TM segments, N_{out}–C_{in}</td>
<td>C-terminal export of cytochrome c oxidase subunit 2</td>
<td>[115]</td>
</tr>
<tr>
<td>Mss2</td>
<td>Peripherally associated with inner membrane from matrix side</td>
<td>C-terminal export of cytochrome c oxidase subunit 2</td>
<td>[117]</td>
</tr>
</tbody>
</table>

TM, transmembrane; N, amino terminus; C, carboxyl terminus; in, facing into matrix; out, facing into intermembrane space; Oxa1, oxidase assembly; Mba1, multi-copy bypass of AFG3; Cox18, cytochrome c oxidase subunit 18; Pnt1, pentamidine resistance protein.

The central player in this process is the Tim9/Tim10 protein family, Tim12 [16–19,21,86,87,91,92]. The mechanism by which these small Tims assist the translocation process at the inner membrane is far from understood, but their function is essential for insertion of hydrophobic proteins at the TIM22 complex [18,19,93]. While Tim22 forms the insertion pores (see below), the roles of Tim54 and Tim18 are not yet known, although Tim54 is required to maintain the structural integrity of the TIM22 complex, which remains unasssembled in its absence [17,20].

The insertion of membrane proteins facilitated by the TIM22 complex is absolutely dependent on the membrane potential. When there is no membrane potential, the carrier protein accumulates in the intermembrane space associated with the soluble Tim9–Tim10 complex and also weakly associates with the membrane associated TIM22 complex [16,18,21,90]. But under conditions of moderate to low membrane potential, the carrier protein arrests at the TIM22 membrane complex (stage IV) [Figure 6] [21]. It is only when the membrane potential is high that translocation proceeds to facilitate the completion of membrane insertion followed by the assembly of monomers to a functional dimer of carrier proteins (stage V) [21].

The central player in this process is the essential protein Tim22. Like Tim17 and the carboxy-terminal domain of Tim23, Tim22 has four predicted membrane spanning domains, and it exhibits sequence similarity to these other proteins [16]. In vitro reconstitution of purified Tim22 showed that it forms a cation-selective channel activated by both membrane potential and a targeting signal [20]. Greatest stimulation of the channel is achieved with high membrane potential in the presence of a carrier signal peptide [20], conditions that promote efficient import in intact mitochondria [21]. This was the first evidence that the translocation of these hydrophobic proteins requires a translocation pore for their insertion into the inner membrane, rather than direct insertion into the lipid phase assisted by translocation components.

Electron microscopy of the purified TIM22 complex revealed two stain-filled pits that looked likely to represent import pores. The cross section of each pit (∼16 Å) is consistent with the pore size determined for purified Tim22 [20,21]. Analysis of the purified TIM22 complex by electrophysiology showed that it indeed functions as a twin-pore translocase. A high membrane potential and an internal signal peptide together stimulate gating transitions of one pore in the complex while closing the second pore, indicating coordinated action and regulation of the pores. Thus, the two pores do not function as independent entities, but cooperate in the insertion of precursor proteins that contain multiple hydrophobic segments [21]. Under physiological conditions, the membrane potential most likely promotes membrane insertion through an electrophoretic effect on positively charged regions of the precursor, and also through direct activation of the channel.

In addition to carrier proteins, other polypeptide membrane proteins with internal targeting signals also use the TIM22 translocation machinery for their import. In the case of Tim23, import across the intermembrane space takes a slightly different route. Tim8 and Tim13, non-essential homologs of Tim9 and Tim10, assist the transport of Tim23 across the intermembrane space, particularly under conditions of low membrane potential [94–97]. The domains of Tim23 to which the Tim8–Tim13 complex binds requires clarification, as independent reports are not completely consistent [96–98]. The Tim9–Tim10 complex may also play a role in the import of Tim23 [97]. Although Tim8 is non-essential in yeast, mutation of its homologue in humans is responsible for the development of the severe genetically inherited disease referred to as deafness/dystonia syndrome [94,99,100].

The Export Machinery

Another major protein transport pathway in mitochondria involves the export of proteins from the matrix into the inner membrane. Proteins encoded by the mitochondrial genome and synthesised in the matrix, as well as a fraction of nuclear-encoded precursor proteins, take this route. Nuclear-encoded proteins must first be imported into the matrix via the TOM and TIM23 complexes before engaging the export machinery for
insertion into the inner membrane. In budding yeast, eight proteins are encoded by the mitochondrial genome, seven of which are integral membrane components of respiratory chain complexes.

The mechanism of protein export from the matrix to the inner membrane is poorly understood. It has become increasingly evident that membrane insertion from the matrix side is not a spontaneous event, but requires the assistance of specialised translocation components. The first component found to be involved in the biogenesis of mitochondrial respiratory chain complexes was Oxa1 (oxidase assembly 1) [101–103]. In fact, Oxa1 is a member of an evolutionary conserved protein family with homologs in the inner membrane of bacteria (YidC) and the thylakoid membrane of chloroplasts (Alb3) [104,105]. Each family member is known to play a role in the membrane insertion of proteins [105,106].

Oxa1 is a nuclear-encoded integral protein of the inner membrane [107,108]. Isolation from Neurospora crassa indicated that at least the stable core of the translocase consists only of Oxa1, possibly as a tetramer [109]. The translocase is believed to function as a general membrane insertion machinery, as it is not only required for the insertion of membrane proteins that undergo amino-terminal tail export, but also assists the import of other polytopic proteins in which the amino terminus is retained in the matrix [105,110–113]. It comes into direct contact with a translocating precursor [113], but so far there is no evidence to suggest that the translocase forms a pore for membrane insertion.

When Oxa1 function is compromised in yeast cells, the export of proteins is not completely blocked but rather the severity of the export impairment appears to be precursor-dependent [105]. This suggests that backup import pathways maintain protein export function in mitochondria. Indeed, additional proteins have recently been implicated in export pathways by genetic and biochemical evidence [114–117]. Table 1 summarises all proteins found, so far, to be involved in export pathways. The peripheral inner membrane protein Mba1 is involved in amino-tail export and can largely, but not completely, compensate for loss of Oxa1 [114]. The proteins Cox18, Pnt1 and Mss2, not parts of the Oxa1 translocase, together may constitute a second translocation complex [115,116]. Cox18, a weak homolog of Oxa1, has been implicated in carboxy-tail export [116]. The composition and mode of action of the export machinery is far from solved and thus should provide rapid developments in mitochondrial protein transport in the near future.

Perspectives

The basic principles currently defining precursor protein import into mitochondria are generally agreed upon. For example, there is a need for intrinsic targeting signals for protein recognition, molecular chaperones and receptors to mediate protein–protein interactions, aqueous channels to support transport across membranes and systems for generating protein movement from stored chemical or electrical energy. What is currently difficult to fathom is the way in which the import machinery collectively is able to sort the hundreds of different proteins to the correct compartment. Are the translocation channels highly regulated, capable of changing their interior surface in response to incoming precursors, thus leading to differential compartment sorting? Perhaps there are as yet to be discovered ‘sorting proteins’ or complexes. Protein sorting most likely results from a network of rapid and subtle changes in translocation components in response to targeting and sorting signals.

Furthermore, the molecular mechanisms by which translocation components such as molecular chaperones, receptors and channel-forming proteins mediate import are only partially understood. How are so many different proteins recognised by so few import components? Additional high resolution structures will certainly help our understanding of these processes. Finally further work is required to understand how proteins are assembled into multisubunit complexes. In some cases, specialised assembly components are required to assist and it seems that large complexes are structurally dynamic in order that new components can be incorporated.

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References


