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Review

Minor modifications and major adaptations: The evolution of molecular machines driving mitochondrial protein import ☆

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ABSTRACT

Bacterial endosymbionts gave rise to mitochondria in a process that depended on the acquisition of protein import pathways. Modification and in some cases major re-tooling of the endosymbiont's cellular machinery produced these pathways, establishing mitochondria as organelles common to all eukaryotic cells. The legacy of this evolutionary tinkering can be seen in the homologies and structural similarities between mitochondrial protein import machinery and modern day bacterial proteins. Comparative analysis of these systems is revealing both possible routes for the evolution of the mitochondrial membrane translocases and a greater understanding of the mechanisms behind mitochondrial protein import. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

1. Introduction

The conversion of ancestral α-proteobacteria to mitochondria involved the transfer of genes from the bacterial endosymbiont to the host cell genome [1–9]. Once relocated to the nucleus the gene products, translated in the cytosol, had to be recognized, targeted, translocated and assembled in mitochondria. An account of the evolution of such sophisticated molecular machinery should explain how the components could plausibly be established in a stepwise fashion with modifications to and the support of existing mechanisms.

Three main themes emerge when investigating the evolution of the mitochondrial protein transport machinery: (i) modifications of an existing system, wherein the ancestral function is conserved in bacteria and mitochondria; (ii) reorganisation and modification of bacterial proteins giving rise to machinery with new functions; (iii) use of structural or functional homologues to provide insight into components where sequence similarity does not illuminate the evolutionary path. In this review we examine the evolutionary implications of each of these cases, and the impact this has on our understanding of how the protein import machinery functions in mitochondria.

2. Mitochondrial protein translocation pathways

The majority of proteins targeted to mitochondria have a presequence, a short extension of the polypeptide which forms a positively charged amphipathic α-helix, and directs translocation across the outer and inner mitochondrial membranes [10,11]. The Translocase of the Outer mitochondrial Membrane (TOM complex) is

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a large multimeric machine, whose major subunit–the β-barrel Tom40–forms a channel across the membrane. With the exception of a few peripheral, outward-facing outer membrane proteins, all mitochondrial proteins are imported via the TOM channel. Translocation of matrix proteins is dependent on the presequence, which is drawn through the TOM complex by sequential interaction with negatively charged sites within the complex (acid chain hypothesis: [12–15]). The inner membrane presequence translocase, TIM23, receives the presequence as it exits the TOM channel and cooperates with the ATP-driven Presequence-Associated Motor (PAM) to complete translocation into the matrix (Fig. 1).

Alternative pathways exist to target proteins to the inner membrane, outer membrane and intermembrane space, often depending on as yet poorly defined targeting signals. In many organisms, a second inner membrane translocase, TIM22, assembles polytopic proteins into the inner membrane [16,17]. In all eukaryotes, the outer membrane Sorting and Assembly Machinery (SAM complex) assembles outer membrane β-barrels [18–20]. Both types of precursor proteins require assistance from the small Tim chaperones in the intermembrane space for delivery to TIM22 or SAM [21–23]. These chaperones are imported by another pathway, using the Mitochondrial Intermembrane space import and Assembly (MIA) machinery, which couples precursor import with oxidation [24–26]. A further translocase in the inner membrane, termed OXA (OXidase Assembly), inserts proteins from the mitochondrial matrix into the inner membrane [27–29]. As far as we know, the majority of OXA substrates are encoded in the mitochondrial genome, and inserted co-translationally.

The TOM, TIM23, TIM22, SAM and MIA molecular assemblies have been intensively studied in yeast, and we have a remarkable understanding of many of the mechanistic intricacies of this highly evolved system. By combining these insights with sequence and functional analyses of mitochondrial import systems in diverse eukaryotes, a picture is emerging of the minimal requirements of each import machine, and how the original, simplest versions of each might first have come to be.

3. Re-vamping bacterial protein translocases for continued function

The Oxa1 protein is the core component of the OXA translocase and is a direct descendant of the bacterial YidC translocase, which also inserts inner membrane proteins (Fig. 2) [27,30,31]. Like OXA, the mitochondrial SAM complex also has a direct counterpart in bacteria; the BAM complex, found in the outer membrane of all Gram-negative bacteria. The core component of the SAM complex, Sam50, is a member of the Omp85 family of proteins that also includes BamA, the core component of the BAM complex [4,18–20,32,33].

While Sam50 is clearly derived from BamA, and the SAM and BAM complexes are functionally homologous, significant evolutionary divergence is evident in the mitochondrial SAM complex (Fig. 2). Mitochondria have lost whole aspects of envelope biogenesis including the ability to synthesise lipoproteins, events that likely determined the loss of the lipoprotein partners, BamD and BamE, of the endosymbiont's BAM complex [34]. These have been replaced, either during or subsequent to this period of lipoprotein loss, by proteins of uncertain ancestry. From functional studies in several organisms we know of at least three types of these proteins: the metaxins, Mim1 and Mdm10. These “modules” of the SAM complex are not conserved across eukaryotes [35,36] and we anticipate that a better understanding of the precise function of these components will give insight into how this modular system evolved.

The metaxins are proteins with a predicted glutathionine-S-transferase type fold and are associated with the SAM complex in fungi (Sam35 and Sam37; [37–41]), animals (metaxin-1 and metaxin-2; [42,43]) and plants (metaxin; [44,45]). Given the divergence between these groups the metaxins may be found in other eukaryotic lineages too, but bioinformatics alone has not been able to resolve this issue. Work in the yeast system shows that the metaxin Sam35 is responsible for substrate docking/entry into the SAM complex [46,47]), while Sam37 is required for efficient release of substrates from the SAM complex [47]. Presumably, the metaxins associated with the SAM complex in animals and plants play similar roles.

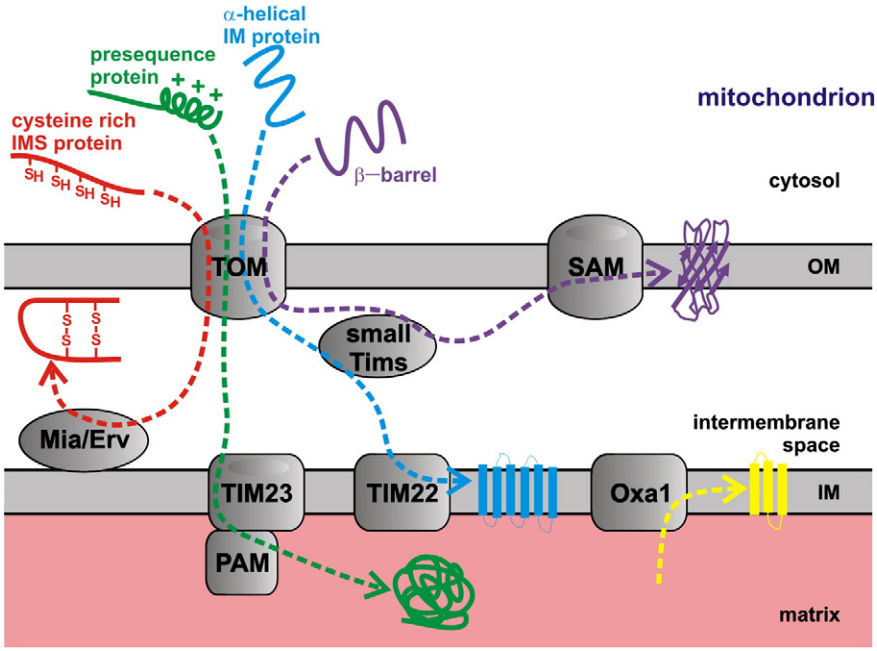

Fig. 1. An overview of mitochondrial protein import routes. Four classes of protein precursor are translocated across the TOM channel in the outer membrane (OM). Proteins with a presequence (green) are transferred from TOM to TIM23, and imported into the matrix with the help of the import motor, PAM. α-helical proteins (blue) are inserted into the inner membrane by TIM22 and β-barrel proteins (purple) are inserted into the outer membrane by SAM. Precursors of both β-barrel and α-helical membrane proteins are chaperoned in the intermembrane space by the small Tims. Small, cysteine-rich proteins (red) of the intermembrane space are imported by the MIA/Erv machinery, which also mediates their oxidation and folding. Oxa1 in the inner membrane inserts mitochondrially-encoded proteins into the inner membrane (yellow).

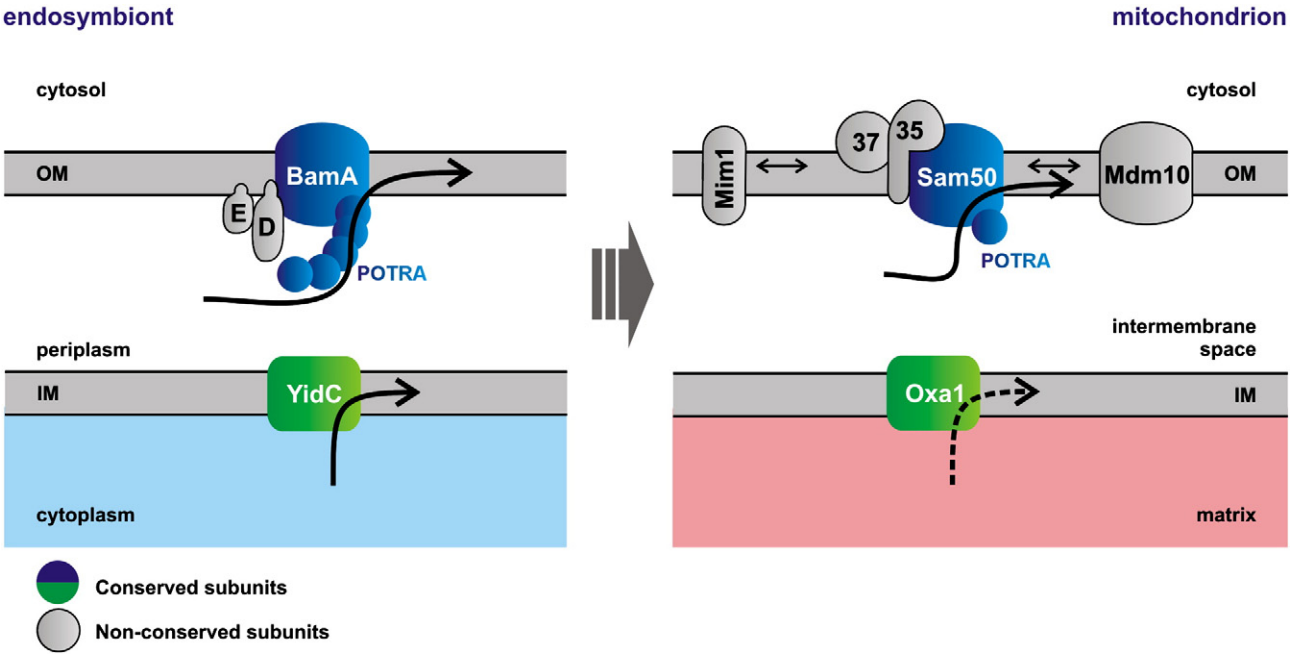

Fig. 2. Re-vamping the BAM and YidC translocases to function in mitochondria. The bacterial BAM complex consists of the core subunit BamA, and several lipoprotein partners, two of which (BamD and BamE) are conserved in all α-proteobacteria. BamA consists of a transmembrane β-barrel domain and five periplasmic POTRA domains. Sam50 is the core component of the SAM complex in mitochondria, and was derived from the endosymbiont BamA but has a truncated N-terminal domain with what is perhaps a single POTRA. However, the other components of these outer membrane complexes have been extensively remodeled. The SAM complex incorporates Sam35 and Sam37, and the modular subunits Mim1 and Mdm10. At least some of these SAM subunits may have analogous roles to the lipoprotein subunits of the BAM complex. Oxa1 inserts proteins into the inner membrane from the matrix, and evolved from the bacterial YidC. In mitochondria, relatively few proteins are assembled by Oxa1 (indicated by dashed arrow).

In addition, the SAM complex can engage with two outer membrane proteins found only in fungi: Mdm10 and Mim1. Mdm10 is a modular component of two complexes which seem to function in distinct pathways for assembly of outer membrane proteins. A SAM-Mdm10 complex assists in assembly of the TOM complex, while a second complex, containing Mdm10, Mdm12 and Mmm1, appears to function sequentially after the SAM complex in the β-barrel assembly pathway [48–50]. This second complex has also recently been described as ERMES (ER-Mitochondria Encounter Structure), a molecular tether between the endoplasmic reticulum and mitochondria, composed of Mdm10, Mdm12, Mmm1 and Mdm34 [51]. ERMES impacts on various aspects of cellular physiology including mitochondrial morphology, phospholipid and calcium homeostasis, and mitochondrial DNA replication [52]. This intriguing link between protein import and mitochondria-ER tethering machinery suggests a network of connections that might regulate mitochondrial biogenesis in response to higher-level cellular cues.

Mim1 is another modular subunit of the yeast SAM complex [53] which, like Mdm10, functions in assembly of the multimeric TOM complex [39,50,53–55]. Mim1 and Mdm10 are each required for integration of different subunits into the TOM complex. Despite their roles in assembling the TOM complex, neither Mdm10 nor Mim1 is directly involved in the import of β-barrel proteins [39,49]. Several subunits of the TOM complex evolved after the divergence of the eukaryotic lineages [56,57], consistent with the fungal-specific distribution of Mdm10 and Mim1. It seems highly likely that analogous, but non-homologous, proteins function in place of Mdm10 and Mim1 in other organisms.

4. The ultimate in evolutionary tinkering: a new machine for protein transport

The core of the TIM23 complex is the Tim23 subunit, a multi-topic membrane protein that forms the protein import channel [58,59]. It is widely accepted that Tim23 and Tim22 (the core of the TIM22 complex) are related to each other by sequence. While there is agreement that one was derived from the other by gene duplication and modification, which complex arose first has not been determined. By the principle of Occam's razor we favor the idea that the TIM23 complex was established first, and that it was cobbled together from existing bacterial proteins (Fig. 3).

Rassow et al. have suggested that the Tim23 channel was derived from an amino-acid transporter called LivH [60]. These transporters import large, bulky hydrophobic amino acids via an aqueous channel and might require relatively little modification in order to transport polymers of amino acids. Indeed, the OEP16 protein, found in the chloroplast outer envelope, is a member of the Tim23 protein family [60,61] and has been shown to transport amino acids when reconstituted in liposomes [62]. Not surprisingly, given the evolutionary distance and the sequence-based changes driven by interactions with multiple subunits in the TIM23 complex, pair-wise sequence conservation between LivH and Tim23 family proteins is low. However, a signature PReprotein and Amino acid Transporters (PRAT) motif found in the Tim23-type mitochondrial translocases and in OEP16 is also present in the LivH protein of bacteria.

The TIM23 import motor, mtHsp70, drives protein translocation across the inner membrane through successive rounds of ATP hydrolysis, and is derived from an Hsp70 (DnaK) protein found in extant species of α-proteobacteria [63]. The import motor is docked to the TIM23 translocase by the Tim44 subunit [64,65] and Pam18 (also known as Tim14) regulates motor ATPase activity [66–68]. Recent work has shown that α-proteobacteria carry inner membrane proteins with strong sequence similarity to the Tim44 (TimA) and Pam18 (TimB) [69]. Studies on the α-proteobacterium *Caulobacter crescentus* showed that these two proteins function distinctly, yet both are found in the same compartment and have the same topology as their mitochondrial counterparts. Furthermore, a single point mutation in the J-domain of an α-proteobacterial Pam18 homologue is sufficient to convert it to a functional TIM23 translocase subunit [69].

It is reasonable to infer from these findings that relatively little evolutionary tinkering would be required to derive a core TIM23

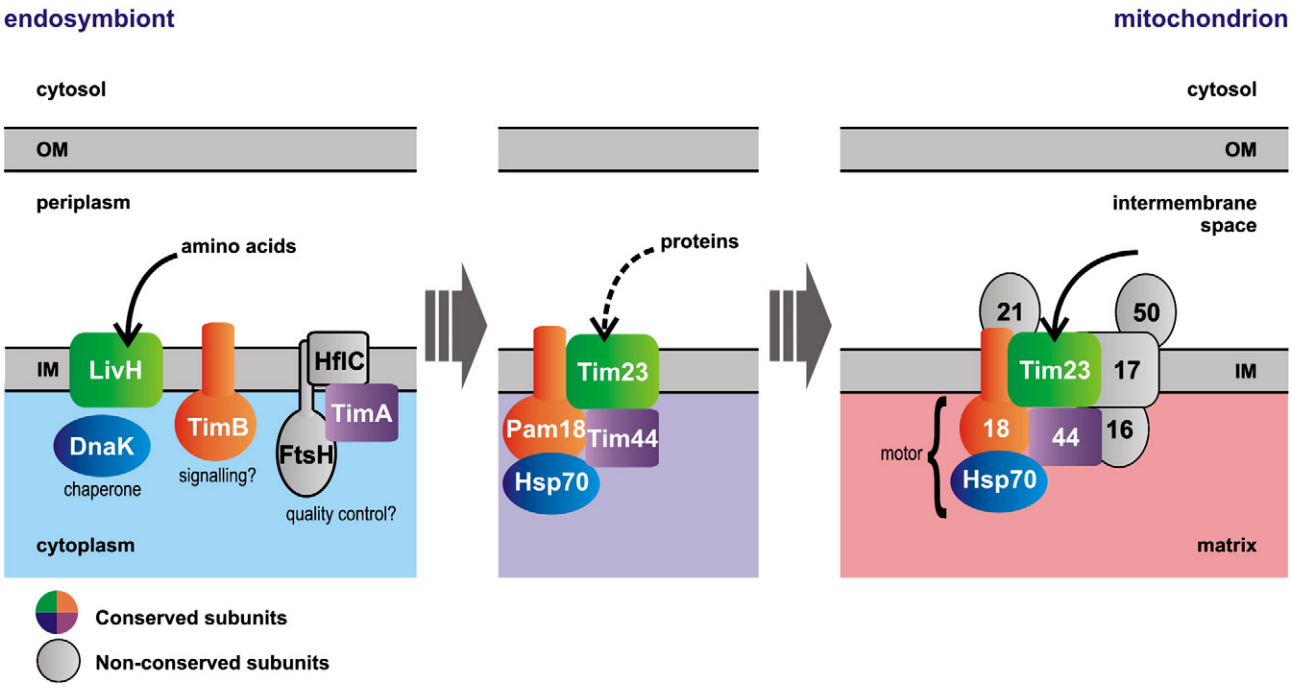

Fig. 3. A model for the evolution of the TIM23 complex. In many organisms, the TIM23 complex is composed from eight or more subunits and translocates all presequence-containing proteins (right panel). The core subunits of the TIM23 complex (Tim23, Pam18, Tim44 and mHsp70) are common to all lineages of eukaryotes [35]. Tim23 forms a transmembrane channel, and Pam18, Tim44 and mHsp70 are part of the presequence-assisted motor (PAM). Each of these components can be traced back to an ancestral protein in bacteria. The Tim23 subunit is related to LivH-type amino acid transporters [60]. The mitochondrial Hsp70 is clearly derived from α-proteobacterial DnaK [63] and the Tim44 and Pam18 subunits from the α-proteobacterial proteins TimA and TimB, respectively [35,69,97]. Harnessing the mitochondrial Hsp70 to the inner membrane provided a motive force to the transporter, providing a means to translocate proteins through the inner membrane. Initially, this would have been relatively inefficient (dashed arrow). Subsequent, lineage-specific evolution of some components (shown in grey) has provided further efficiency and sophistication to TIM23 function.

complex from components already present in the ancestral endosymbiont. With a rudimentary TIM23 translocase in place and the continued presence of both Sec and YidC translocases [35,69], the "proto-mitochondrion" would have had a functional system for import of both matrix and inner membrane proteins. A primitive system such as this would provide the basis for the evolution of the highly specialized, and diverse, TIM translocases in extant organisms.

5. The origin of the TOM complex: cultivating the endosymbiont–host interaction

It has been suggested that the first protein translocase system in the proto-mitochondrion would have involved a primitive set-up: a β-barrel protein in the outer membrane and substrates in the host cytosol predisposed for targeting to mitochondria [69,70] (Fig. 4).

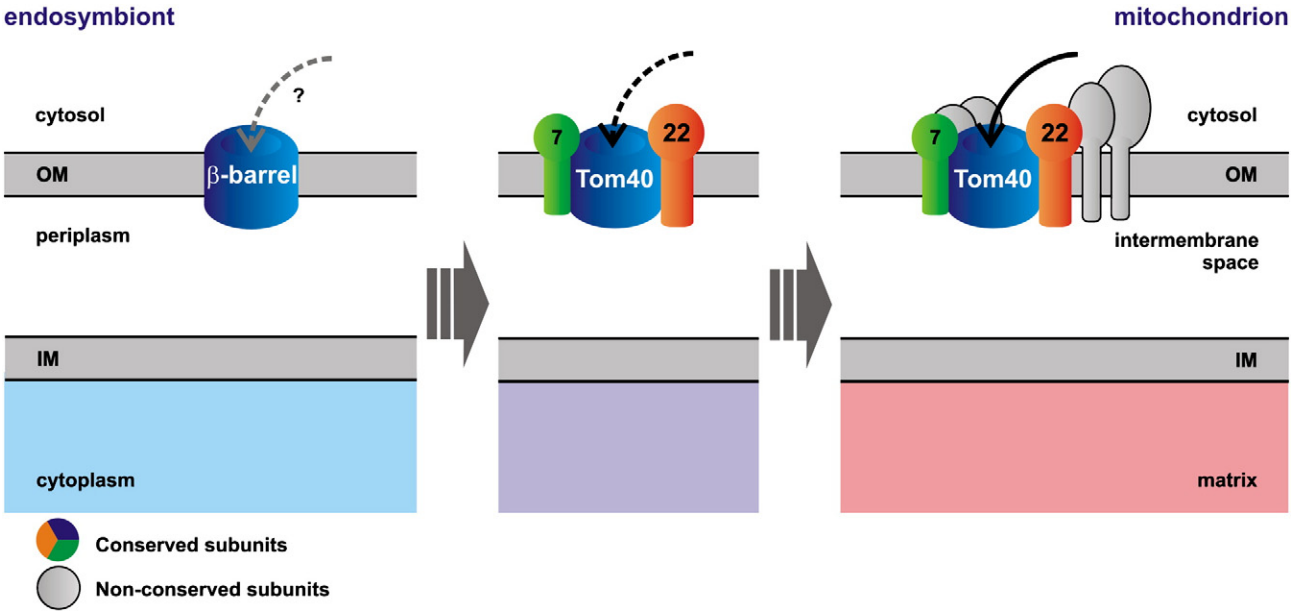

Fig. 4. Evolution of the TOM complex from an ancestral β-barrel. This model proposes a β-barrel protein in the outer membrane of the endosymbiont served as a binding site for proteins with basic, amphipathic N-termini [98]. Such a simple protein import system can be envisaged by analogy with the simplified, core complexes seen in some parasites. Tom7 and Tom22 are common to all lineages of eukaryotes suggesting they constitute the first partner proteins to have arisen in early eukaryotes [99]. Optimization of protein transfer, both in terms of efficiency and versatility, required the later addition of TOM complex subunits, after the divergance of some lineages.

Simple TOM complexes have recently been identified in both *Giardia* and microsporidians, consisting of Tom40 and perhaps a single partner subunit [71–73]. In the case of *Giardia*, as with many other organisms, it remains possible that additional, lineage-specific subunits of the TOM complex remain to be discovered. However, microsporidians provide a true proof-of-principle example of a simple TOM complex. Microsporidia are allied with fungi phylogenetically and in the case of the protein import machinery, sequence similarity of all TOMs and TIMs are extremely high [71,73]. Only two TOM proteins are encoded in the complete genome of *Encephilizoon cuniculi*: Tom70 and Tom40. Given the function of Tom70 as a receptor, acting prior to the translocation reaction, this says that Tom40 alone can form a functional protein translocase. While clearly a result of secondary gene loss in microsporidians, it demonstrates the feasibility of a primitive, "Tom40-only" TOM complex in the ancestral endosymbiont.

Phylogenetic analysis does not establish the ancestry of Tom40. Based on its β-barrel topology it is broadly accepted that Tom40 was derived from the genome of the endosymbiont. Like all bacteria with two membranes, the endosymbiont would have had a range of β-barrel outer membrane proteins [4,33,74]. Initially synthesised within the endosymbiont, a primitive TOM translocase could have been transported to the periplasm using the bacterial export pathway. Assembly of this β-barrel subunit into the outer membrane would then have been mediated by the endosymbiotic BAM complex. This primitive β-barrel would have been the founding member of the "mitochondrial porin" family of proteins, which includes both the protein translocation channel Tom40 and the mitochondrial outer membrane metabolite transporter VDAC [75]. Whether the first mitochondrial porins functioned in metabolite transport or protein translocation is as yet unknown.

What family of bacterial proteins gave rise to the first mitochondrial porins? There are numerous, divergent metabolite transporters in bacterial outer membranes that transfer charged substrates by virtue of "chains" of charged residues lining the inner surface of the pore. The acidic sugar-specific porin KdgM provides a beautiful illustration of how a "basic chain" of residues in the pore channel can lead a negatively-charged sugar through the outer membrane [76]. The transfer of a positively charged mitochondrial targeting sequence might likewise have followed an acid chain through a β-barrel protein in the outer membrane of the endosymbiont. The principles of the acid chain hypothesis were established based on acidic domains on Tom40's partner subunits [12,14,15,77,78], however a recent model of the structure of Tom40 shows such an "acid chain" of residues in the pore lining, with the net charge being greatest at the intermembrane space exit site [74].

An alternative proposition for the ancestry of Tom40 comes from a tantalizing observation of sequence signatures shared by the YdeK autotransporter (SwissProt accession P32051) and the Tom40 family of proteins [79]. Autotransporters are simple β-barrel protein translocation channels commonly found in the outer membrane of bacteria. The crystal structure of the β-domain of autotransporter EspP shows that the barrel pore can accommodate a positively charged α-helical segment, which is stabilised by complementary charged surfaces on the inside wall of the barrel [80]. If Tom40 was derived from such an autotransporter channel one caveat might be the directionality of substrate translocation, as the TOM complex imports proteins, rather than exports. However, this difference need not matter as biochemical analysis of purified mitochondrial outer membrane vesicles has shown that purified proteins can move in either direction through the TOM channel [81].

While the specific ancestry of Tom40 remains to be determined, it is reasonable to predict that with that first translocase subunit in place, additional subunits were sequestered from other activities to enhance the function of the primitive translocase. Both Tom7 and Tom22 are present in each of the major eukaryotic lineages, suggesting them to be the first partner proteins added into the primitive TOM complex (Fig. 4). There remains some uncertainty as to whether they are present or not in the Excavata (one of the six supergroups of the eukaryotes); the small size of the proteins makes their identification challenging (each protein is only ~50–70 residues in most lineages). In addition two other smaller proteins, Tom5 and Tom6, are present in many but not all lineages, and may have been added to the TOM complex later. Selection for further receptor subunits, Tom20 and Tom70, appears to have been a lineage-specific adaptation, with the "Tom20" receptor in opisthokonts (i.e. fungi and animals) being unrelated in sequence and ancestry to the functionally-analogous "Tom20" in plants [56]. These receptors would have enabled the evolution of an increasing diversity of substrate proteins and targeting sequences, enhancing efficiency of the import process and overall fitness of the host organism.

6. Replacing the old order in the intermembrane space

The protein transport reactions, signalling networks, structural peptidoglycan and redox conditions of the bacterial periplasm make it a radically different environment from the mitochondrial intermembrane space. The bacterial periplasm is a highly oxidising environment, reinforced with a thick peptidoglycan meshwork. Bacterial networks for monitoring and responding to a fluctuating extracellular environment have vanished from mitochondria, replaced with new systems for signalling and quality control in an intracellular context. Thus much of the bacterial periplasmic machinery has been replaced with eukaryote-specific proteins as new pathways evolved. There are now two examples where the periplasmic machinery of the endosymbiont seems to have been superseded by protein import apparatus: the small Tim chaperones that play a SurA-like role in mitochondria, and the MIA/Erv disulfide relay that has replaced the bacterial Dsb system (Fig. 5).

The assembly of β-barrel proteins into the bacterial outer membrane requires assistance from chaperones found in the periplasm, which fulfill three functions: precursor release from the inner membrane, molecular chaperone activity during transit, and targeting/hand-off to the BAM complex for outer membrane insertion. Periplasmic chaperones such as SurA, Skp, DegP and PpiD all play a role in this pathway in *Escherichia coli* [82–87] (Fig. 5, left panel). Bioinformatic analysis of SurA and Skp distribution revealed that both chaperones are present in diverse bacterial species, including all proteobacterial lineages, but are not detected in eukaryotes [88] [our unpublished data].

The small Tim chaperones are found only in eukaryotes [89], where they transfer precursors of both inner and outer mitochondrial membrane proteins from the TOM complex to the appropriate downstream machinery [21–23,90,91]. Comparative analysis of SurA with the small Tims shows that while both chaperones can bind similar substrates, SurA cannot transfer mitochondrial inner membrane proteins to the TIM translocase for insertion [88]. The small Tim family may therefore have arisen to enhance transport of inner membrane proteins, and also proved competent in transfer of outer membrane precursors, leaving bacterial chaperones like SurA redundant.

The bacterial Dsb proteins, which catalyse formation and isomerisation of disulfide bonds in the periplasm, are absent from mitochondria. The only redox proteins identified to date in the mitochondrial intermembrane space constitute the MIA disulfide relay machinery, which mediates the import of small, cysteine-rich proteins into the mitochondrial intermembrane space [24–26]. In yeast, substrates translocated through the TOM complex are bound by the oxidoreductase Mia40. Substrate and Mia40 together form a complex with the thiol oxidase Erv1, and electron flow from substrate via Mia40 to Erv1 is followed by release of the oxidised substrate [25,92–94].

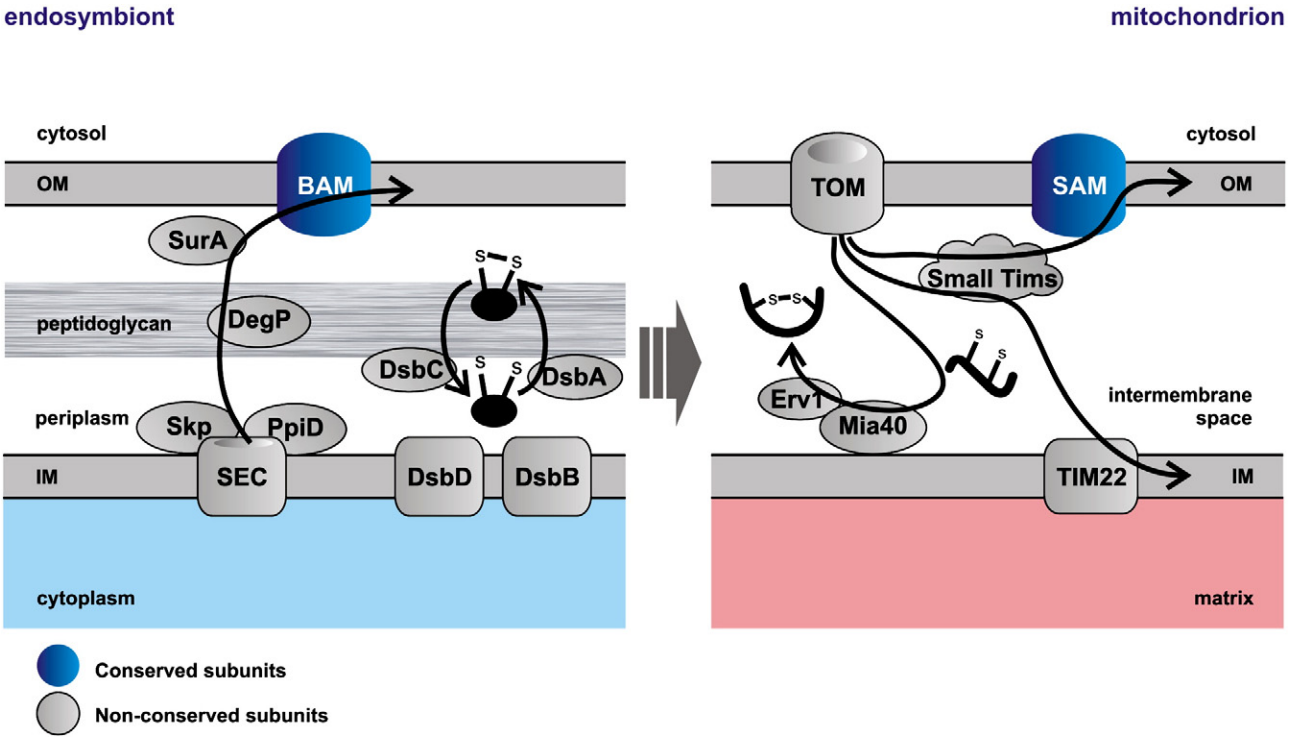

Fig. 5. Evolution of an intermembrane space in mitochondria. Molecular chaperones which translocate proteins across the periplasm (Skp, DegP, PpiD and SurA) also function in cellular stress responses. The Dsb redox system acts as a folding catalyst for several hundred predicted disulphide-containing periplasmic proteins [99]. These systems may have provided chaperone activity essential for the foundation of new protein import pathways, however it is likely that their inefficiency in these pathways meant they were replaced early in eukaryotic evolution. In mitochondria the small TIM chaperones fulfill a molecular chaperone function and transfer membrane protein precursors from the TOM complex to the TIM22 and SAM complexes. The MIA/Erv machinery couples a Dsb-like redox activity with import of precursors into the intermembrane space.

No homologue of either Mia40 or Erv1 has been identified in any prokaryotic genome, so it is difficult to determine the origin of this pathway. While some unicellular eukaryotes appear to lack both MIA pathway substrates and machinery, there are organisms, including the protozoan trypanosomatids, which do contain both classic MIA substrates and an Erv1 homologue, but seem to lack Mia40 itself [95]. Allen and colleagues suggest this minimalistic set-up reflects the ancestral pathway, where the redox cascade comprised only the substrate, Erv1 and molecular oxygen. This system would have used Erv1 to create disulfide bonds but might also have relied on the bacterial protein disulfide isomerase dsbC, homologues of which have been reported in α-proteobacteria. Mia40 could then have been added to the evolving eukaryote at a later date, making the bacterial isomerase dispensable and improving the efficiency and accuracy of the system.

7. Concluding remarks

Species of α-proteobacteria have conquered diverse environments, and show great breadth in the complexity of their genomes and proteomes [96]. We can assume that the endosymbiont that gave rise to mitochondria had a robust protein transport system for the assembly of proteins into both its outer and inner membranes. The existing bacterial protein folding and translocation pathways played a dual role in supporting the stepwise evolution of the mitochondrial machinery, providing both a source of building blocks for the evolution of new import systems and functional support to the fledgling translocases.

The mitochondrial SAM complex provides a prime example of the adaptation of a bacterial system to perform an equivalent role in mitochondria. The TIM23 complex appears to have been cobbled together from existing components to produce a sophisticated and versatile translocase, but is still assisted by the ancient inner membrane translocase Oxa1. Bacterial SurA-type chaperones, although unable to dock with newly established TOM and TIM machinery, might have provided chaperone activity essential for the passage of imported membrane proteins, until the later invention of the small Tim chaperones. Similarly, a rudimentary intermembrane space import pathway might have initially relied on the bacterial disulfide isomerase, DsbC [95]. With the current available evidence pointing towards proteins from the endosymbiont as progenitors for many of the translocase components, we suggest that these proteins played a central role in driving the evolution of the new protein transport pathways. The evolution of these import pathways eventually produced the mutually beneficial arrangement that became the first eukaryote.

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