Biochimica et Biophysica Acta 1818 (2012) 1085–1090

Contents lists available at SciVerse ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbamem

Mini-review

Unresolved mysteries in the biogenesis of mitochondrial membrane proteins ☆

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ARTICLE INFO

Article history:
Received 10 June 2011
Received in revised form 10 August 2011
Accepted 15 August 2011
Available online 27 August 2011

Keywords:
Mitochondrial import
Membrane protein
Mitochondrial translocase
Protein insertion into mitochondrial membrane

ABSTRACT

Mitochondria are essential eukaryotic organelles that are surrounded by two membranes. Both membranes contain a variety of different integral membrane proteins. After three decades of research on mitochondrial biogenesis five major import complexes with more than 40 subunits altogether were identified and characterized. In the current contribution we want to draw attention to some unexplored issues regarding the integration of mitochondrial membrane proteins and to formulate crucial questions that remain unanswered. This article is part of a Special Issue entitled: Protein Folding in Membranes.

Contents

1. Introduction

The first components mediating mitochondrial protein import were identified about three decades ago. Since these pioneering days five major protein complexes that are essential for protein insertion into the two membranes of the organelle were discovered (Fig. 1). The central entry gate for the vast majority of mitochondrial proteins is the translocase of the mitochondrial outer membrane (TOM complex). The second import machinery of the same membrane – the so called TOB (*t*opogenesis of *o*uter membrane β-barrel proteins) or SAM (*s*orting and *a*ssembly *m*achinery) complex – handles the insertion of a certain subset of membrane components, the β-barrel proteins. These are unique to the outer membranes of mitochondria, chloroplasts and Gram-negative bacteria. The mitochondrial inner membrane (MIM) contains three protein complexes that contribute to the biogenesis of integral membrane proteins. While the OXA1 (*o*xidase *a*ssembly) complex facilitates the insertion of proteins from the matrix into the inner membrane, the TIM22 and TIM23 (*t*ranslocase of the *i*nner *m*embrane) complexes integrate substrate proteins approaching from the intermembrane space (IMS) face of the membrane. The TIM23 complex mediates the insertion of mainly singlespan proteins into the MIM. In contrast, the TIM22 machinery mediates insertion of multispan proteins like the metabolite carrier type that contain six transmembrane helices. After passing the TOM complex these hydrophobic proteins are chaperoned in the IMS by the small Tim proteins that then hand over the precursor proteins to the TIM22 complex. The same small Tim chaperones are

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doi:10.1016/j.bbamem.2011.08.027

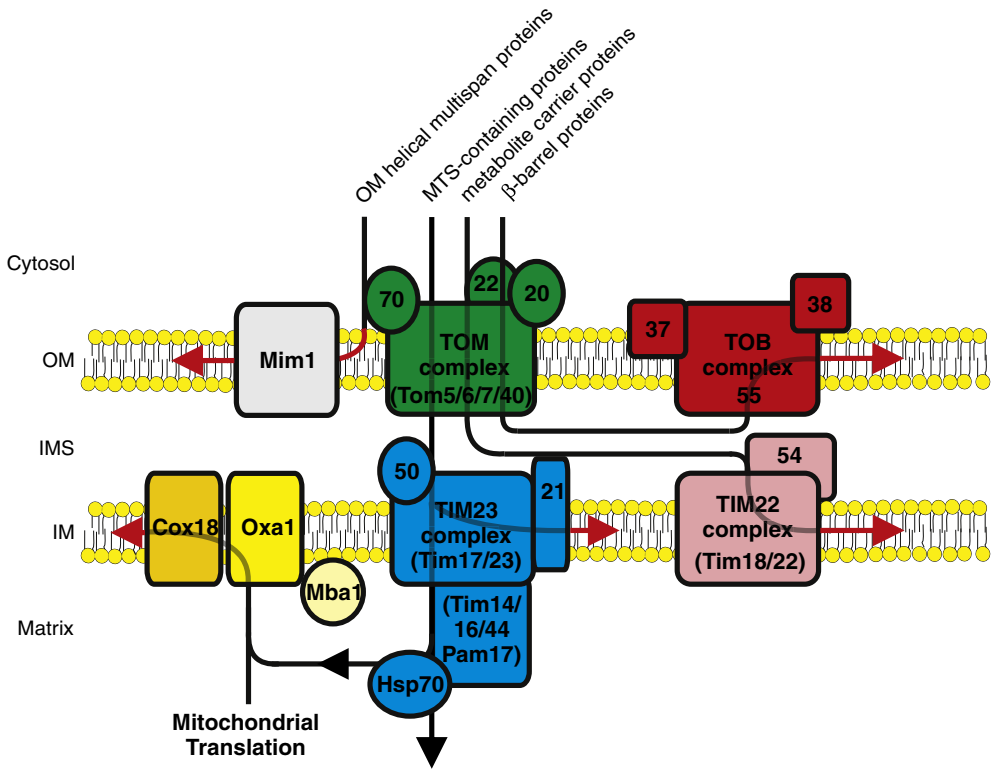

Fig. 1. Overview of insertion pathways of mitochondrial integral membrane proteins. The core complexes that mediate the various membrane integration processes are shown as larger entities with their components listed in parentheses. Discrete subunits are labeled by their "number", e.g. 70: Tom70 receptor of the TOM complex. Import complexes that are not directly involved in membrane insertion processes (e.g. Mia40 complex and the tiny Tims) are omitted for clarity. The actual insertion processes are depicted by red arrows.

also involved in relaying β-barrel precursors from the TOM to the TOB complex.

Accumulated studies from many groups resulted in quite detailed knowledge about the machineries and components required for proper insertion of proteins into both membranes. Yet many questions regarding the mode of action of these complexes and how they mediate the actual integration of proteins into their corresponding membranes still remain. Our intention in this contribution is not to provide a comprehensive overview on the various mitochondrial import machineries and pathways as these subjects were profoundly reviewed elsewhere (for example in the special issue of BBA Biomembranes in March 2011). We want to draw attention to some neglected issues regarding the integration of mitochondrial membrane proteins and to formulate crucial questions that remain unanswered even after thirty years of intensive research.

2. Do we know all mitochondrial proteins involved in protein import and membrane insertion?

Proteome studies of isolated mitochondria revealed that they consist of about 850 (in the unicellular *Saccharomyces cerevisiae*) [1] to 1500 (mammals) [2,3] proteins. Only ca. 80% of the identified proteins in yeast are functionally categorized and this number is even lower in mammals [4]. Hence, one fifth of the mitochondrial proteins await functional characterization. Considering our current knowledge of approximately 40 proteins that serve as import factors, there should be accordingly about ten additional components left to be discovered. Furthermore, it might well be that we still miss not only additional subunits in known machineries but rather complete novel insertase complexes.

An obvious example to such a putative stealth insertase is the one involved in the integration of helical MOM proteins. These integral membrane proteins come in different flavors. One has to distinguish among proteins that contain one transmembrane domain (TMD) close to the C-terminus (so-called "tail-anchored"), those harboring the single TMD at the N-terminus ("signal-anchored"), or proteins having it in the middle and thus expose soluble domains on both sides of the membrane. In addition, a small group of MOM proteins have several α-helical TMDs [5,6]. The membrane integration of some signal- and tail-anchored proteins was found to be independent of any known import component [7–12]. Instead the lipid composition of the membrane seems to be crucial (see below). The receptor Tom70 was reported to be involved in the insertion of multispan proteins without participation of other subunits of the TOM complex [13]. So far specialized machinery for the insertion of α-helical MOM proteins has not been reported. However, one cannot rule out that an unknown insertase for MOM helical proteins still awaits its identification.

It appears that the picture is even more complicated as there are reports, that TOM and/or TOB complex are involved in the embedding of helical Tom subunit proteins in the MOM [12,14–16]. Some tail-anchored TOM components (Tom5, 6, 7) and signal-anchored ones (Tom20 and 70) need the TOM complex itself for efficient assembly. Yet it is unknown whether this necessity results from an active insertion process mediated by the TOM complex or these proteins rather only get stabilized upon their proper assembly into the complex.

A new player in this field is the MOM protein Mim1. This protein, which is present in a higher molecular weight complex of unknown composition [17–22], could function as an insertase for α-helices into the outer membrane. Accordingly, the biogenesis of some signal-anchored (Tom20, Tom70) and tail-anchored proteins (Tom5, 6, 7) depend on Mim1 [19,22,23] and the protein plays a role in the assembly of the TOM complex [20,21]. Mim1 seems to be also a component of a minor subpopulation of the TOB complexes although the functional importance of this interaction is still not clear [22]. Recent studies identified a new insertion route for helical multispan MOM proteins in

which Mim1 is playing a key role [24,25]. This pathway involves initial docking of chaperone-associated precursor proteins to the import receptor Tom70, followed by their transfer to Mim1-containing complex and finally their insertion into the membrane in a process that is facilitated by Mim1.

Considering the size and importance of the Mim1 complex, deciphering its composition might contribute to the identification of new import components.

New insertion pathways are also expected in the integration of proteins into the mitochondrial inner membrane. These novel routes can be mediated by either unknown insertases or new components of the known complexes. One report shows that subunit e (Sue) of the F_1F_o-ATPase is inserted into the inner membrane from the IMS side even if the mitochondrial membrane potential ($\Delta\psi$) is severely reduced [26]. Since TIM22 and TIM23 complexes strictly depend on $\Delta\psi$, it is not clear how this is achieved. Furthermore several observations suggest an additional insertion pathway from the matrix side independent of the OXA1 complex. In yeast the assembly of the F_1F_o-ATPase is not exclusively dependent on Oxa1. For example, the Atp9 oligomer is still present after deletion of *OXA1* and Cytb, Atp4 and Atp6 maintain their insertion capacity into the MIM in this deletion strain [27,28]. Supporting this assumption are reports that cytochrome c1 mutations and deletion of mitochondrial AAA protease compensate for loss of Oxa1 [29,30]. Thus, at least for the F_1F_o-ATPase subunits another insertion pathway can be predicted.

3. What are the mechanisms by which the known complexes insert proteins into membranes?

There are currently five major known multi-subunit complexes that contribute to the insertion of mitochondrial membrane proteins (Fig. 1). Yet the actual mechanisms by which these complexes mediate the membrane integration of substrate proteins are ill defined. One major problem is the lack of high resolution structural data. Although there is NMR and X-ray structure analysis data for many of the soluble import components and for soluble domains of the membrane embedded components [31], the atomic structures of the important membrane-embedded segments remain obscure.

Currently, there is no evidence that the TOM core complex itself inserts proteins into the lipid environment. Rather, it is involved in some cases in recognition of substrate proteins as well as in the assembly of newly-synthesized TOM components. In mammals, the biogenesis of MOM proteins with several transmembrane domains (like mitofusins, mitochondrial ubiquitin ligase and benzodiazepine receptor) depend on the receptor Tom70 but not on any other TOM component [13]. This dependency reflects probably the ability of Tom70 to recognize these substrate proteins and/or to function as an anchoring partner to cytosolic chaperones that stabilize them in the cytosol [32]. Irrespective of the actual role of Tom70, the actual membrane insertion step is independent of the TOM-complex and thus involves most likely, a yet to be identified, new insertase.

In contrast to the lack of information regarding the biogenesis of multispan helical proteins, it is widely accepted that the other major complex of the outer membrane, the TOB machinery is the insertase for the β-barrel proteins [5,33]. The core component of this latter complex is Tob55/Sam50, by itself a β-barrel protein [34–36]. Despite extensive work, it is not clear how the TOB-mediated insertion of substrate proteins into the lipid environment takes place. One possible scenario is based on the observation that Tob55 can create ion conductive channels *in vitro* [35]. Thus, it can be envisaged that after insertion of the β-barrel substrate into the lumen of the pore and partial folding there, the substrate is released into the lipid bilayer by a lateral opening of the barrel structure. However, since lateral opening of a single Tob55 β-barrel is thermodynamically unfavorable, a more popular scenario suggests that the substrate is inserted into and released from a central pore that is formed by several copies of Tob55. Yet, a third alternative is that the pore formed by the TOB complex has no relevance for the membrane integration of β-barrel proteins and the complex serves as a scaffold on which the barrel structure is formed. Future experiments will help us to discriminate among these options. Regardless of the correct mechanism, it is assumed that the thermodynamically favorable transfer of hydrophobic residues into the lipid bilayer provides the driving force for the membrane integration process.

The inner membrane complexes TIM22, TIM23 and OXA1 insert precursor proteins into the inner membrane (Fig. 1). The mechanisms of these insertion processes still have to be elucidated. Lateral opening events are postulated in all these cases to allow the release of a substrate protein, but in the absence of high-resolution structure-function analysis this proposal awaits experimental support.

In addition to the import of presequence-harboring polypeptides into the matrix, the TIM23 complex mediates the insertion of transmembrane α-helices into the MIM. The core components Tim23, Tim17 and Tim50 are required for both tasks. Insertion of proteins into the inner membrane additionally depends on the subunit Tim21 [37–40]. Furthermore, the TIM23 complex associates with the matrix import motor (containing Tim44, Tim14/Pam18, Pam17, Tim16/Pam16, mtHsp70 and Mge1) which is essential for import of soluble proteins into the matrix [41,42]. It is still a matter of debate whether there are different TIM23 complex subtypes for the two different tasks or a single structure mediates both insertion and translocation. There is evidence that TIM23 complexes that insert proteins into the inner membrane lack the entire matrix import motor and consist only of Tim23, 17, 50 and 21 [39]. On the other hand, other studies propose that the import motor is permanently attached to the TIM23 core complex although it is non-functional during insertion of transmembrane domains [43]. The changes during rearrangement of the TIM23 complex into its functional subtype are still poorly understood. Essential for the rearrangement is the presence of a hydrophobic sequence in the precursor shortly after the canonical mitochondrial presequence [41,42]. There are indications that this hydrophobic stretch stalls the import machinery in the process of translocation and leads, by a so far unknown reorganization of the TIM23 subunits, to lateral opening of the complex and insertion of the hydrophobic domain itself into the lipid core. It is therefore referred to as the stop-transfer mechanism. Although the actual insertion mechanism is unknown, it was reported, that the hydrophobicity and flexibility of the transmembrane segment play an important role. TMDs with high hydrophobicity are preferably inserted into the MIM, whereas those with moderate hydrophobicity and higher flexibility (containing proline residue(s)) are first completely transferred into and then inserted from the matrix [44]. The energy requirements for the process are met by three different sources: (i) ATP is consumed by the import motor during translocation of the peptide until the stop by the hydrophobic stretch, (ii) the membrane potential provides an electrophoretic force, and (iii) the final release into the lipid core is driven by the interactions of hydrophobic residues of the helix with the phospholipid molecules.

The TIM22 machinery inserts multispan proteins with four or six TMDs. Known substrates of this pathway are Tim22, Tim23 and the carrier-type proteins [45–47]. The precursor proteins transverse the IMS while they are bound to the small Tim chaperones [48]. Next, they are probably handed over to Tim54 that has a large domain in the IMS. Finally, the substrates are relayed from Tim54 to the central subunit Tim22. The latter protein forms pores in the IM and it is believed that substrate proteins insert loop-wise in the pores of the TIM22 core [49]. The subsequent steps are a matter of speculation, but lateral opening and integration of the hydrophobic helices into the bilayer seem plausible. For this process the membrane potential of the MIM is essential. Also in this case the insertion of hydrophobic residues into the membrane probably provides part of the driving force for the insertion process.

Finally, the insertion mechanism by the OXA1 complex is the least known among the IM insertases. The main component is Oxa1 that is homologous to the bacterial YidC and the chloroplast protein Alb3 [50]. Oxa1 has different functions. At least in some cases, like in the process of F_1F_o-ATPase assembly, Oxa1 has a scaffold function for proper arrangement of the subunits after their insertion into the inner membrane [28]. Additionally, its C-terminal domain binds to mitochondrial ribosomes to ensure co-translational insertion of hydrophobic mitochondrial-encoded MIM proteins [51,52]. Finally, in some cases like in the biogenesis of CoxII it acts together with Cox18/Oxa2 as translocase and insertase [53–55]. However, nearly nothing is known about the actual insertion mechanism by which Oxa1 substrates are integrated into the membrane.

Taken together, we still do not know the precise molecular mechanisms for all the insertases of mitochondria. To understand the transfer process of the hydrophobic domains into the lipid bilayers structural information on the different insertion machineries is crucial. High resolution atomic structures, ideally with import precursors stalled in transit, would be the best source to solve these questions. Obtaining such information presents a real challenge for structural biologists.

4. Is there a direct cooperation of the complexes in different insertion pathways?

In many cases the import complexes do not function alone but rather functionally cooperate in assuring membrane integration of substrate proteins. Such cooperation can be between two complexes in the same membrane like OXA1 and TIM23 in the inner membrane or between the TOM and TOB complexes in the outer membrane. Moreover, even complexes in two separate membranes can work together as is the case with TOM and TIM23 complexes. Several reports identified interactions of subunits of the two complexes with each other in presence but also in absence of import substrates. These contacts are crucial for the import and insertion for precursors carrying the canonical presequence that are inserted by the stop transfer-mechanism [37,56–62].

An example for functional cooperation between insertion complexes of the inner membrane was provided recently regarding the insertion of the six TMDs containing protein Mdl1 [63]. The first two helices of this protein were shown to be inserted by the TIM23 machinery, thereafter the third and fourth segments are incorporated into the membrane by the OXA1 complex [63]. The last two membrane domains probably are embedded by the TIM23 complex according to the stop transfer mechanism. Despite this clear functional cooperation, a direct physical interaction of components of the two insertion complexes has not been shown so far.

The biogenesis of β-barrel proteins provides an example for functional cooperation between the complexes of the outer membrane. Since β-barrel proteins are imported and inserted sequentially by the TOM and the TOB complex speculations about the formation of a super-complex during insertion are tempting. Yet physical interactions of subunits of the two MOM complexes have not been shown until today and no such supracomplex has been observed so far. However, some evidence like the genetic interactions among components from these two complexes favors such a model [64,65]. Interactions of the TOB complex with additional two MOM proteins (Mim1 and Mdm10) have been observed. First, trace amounts of TOB components can be pulled down with Mim1 although the functional significance of this rather transient interaction is not clear [22]. Second, Mdm10 was shown to be a component of a subpopulation of the TOB complex. The function of the Mdm10-containing so called TOB-holo complex is not entirely understood, yet it seems to be important for the efficient biogenesis of β-barrel proteins [66,67].

5. Are specific lipids important for the membrane assembly process?

The interaction of TOB complex with Mdm10 brings other aspects of cell biology into play. Mdm10 was shown to be a component of the Mdm10/Mmm1/Mdm12 complex that is crucial for proper mitochondrial morphogenesis [68]. Only recently the same components were identified together with Mmm2 [69] as essential factors for mitochondria-ER juxtaposition at so called contact sites (ERMES-complex; *ER-m*itochondria *e*ncounter *s*tructure) [70]. This intimate interorganellar contact is further important for the transport of lipids to and from mitochondria [70]. One can easily anticipate that proper insertion of transmembrane domains requires a special environment suggesting that the correct lipid composition of the two mitochondrial bilayers is of importance.

One classical example is the “mitochondria only” phospholipid cardiolipin. It was long known, that several complexes of the electron transport chain depend on this lipid for proper assembly and optimal function [71–73] and that cytochrome *c* is partially bound to the inner membrane by interaction with cardiolipin [74]. Recently, it has been shown that the inner membrane complexes TIM22 and TIM23 as well as the TOM complex in the MOM require cardiolipin for their assembly [75,76]. Using different approaches it was shown that efficiency of protein insertion by TIM23 depends on cardiolipin and the deletion of certain genes involved in cardiolipin homeostasis (*CRD1*, *TAM41*, *UPS1*, *UPS2/GEP1*) affects protein import and the stability of the TIM23 translocase [75,77,78]. Furthermore, the assembly of some outer membrane proteins is compromised in yeast strains with altered cardiolipin levels [76].

Ergosterol provides another example for a lipid with an important role in protein biogenesis. Low levels of this lipid in the MOM were found to be important for specific insertion of tail-anchored proteins into this membrane rather than into the ER bilayer. The outer membrane has the lowest content of ergosterol among all membranes facing the cytosol [79,80]. The low sterol content should lead to a higher fluidity of the MOM in comparison to other organellar membranes, which in turn is advantageous for spontaneous membrane insertion of TMDs of tail-anchored proteins. Indeed it could be shown that direct insertion of the tail-anchored MOM protein, Fis1, into lipid vesicles is diminished by increased ergosterol content [7].

6. Does mRNA localization play a role in the biogenesis of mitochondrial membrane proteins?

Another unexplored aspect of mitochondrial biogenesis and protein insertion is the specific localization of mRNAs to the organelle. Ribosomes could be detected on the surface of mitochondria [81–83] and mRNAs that encode for mitochondrial proteins have been shown to be enriched on the organelle [84–86]. One key player seems to be the RNA binding protein Puf3 [87] that was found to interact with the ERMES complex component Mdm12 [88]. The presence of both mRNA and ribosomes on mitochondria encourage speculations on a cotranslational import mechanism and indeed this mechanism has been proposed for some substrates. A cotranslational mechanism would have several advantages especially for mitochondrial membrane proteins: i) mitochondrial proteins would be targeted efficiently and the problems of misstargeting and molecular crowding inside the cell could be minimized; ii) hydrophobic sequences could be imported/inserted while the protein is still translated therefore missfolding and aggregation of these proteins could be omitted; iii) the process of translation could contribute to the driving force for insertion. Despite these theoretical advantages, there are so far only very few examples in yeast of mitochondrial proteins that are exclusively imported by a cotranslational mechanism, such as the dually localized fumarase (cytosol and mitochondria) [89]. Further investigations are definitely necessary to understand the importance

of mRNAs localization and cotranslational protein import to the biogenesis of mitochondrial membrane proteins.

7. Resume

Although five major mitochondrial import machineries and over 40 different import components are currently known, we probably have not discovered yet all the players that contribute to the import and assembly of the variety of mitochondrial membrane proteins. Many observations lead to the assumption that we not only miss components of the known machineries but rather even new insertases are waiting to be identified. The mechanisms by which membrane proteins are inserted into the two different membranes are only at the verge of being understood. Lateral opening and release into the lipid core seem to be a preferred model for the mode of action of mitochondrial insertases, yet profound data like high resolution atomic structures are still lacking for all the membrane embedded domains of the known import and insertion complexes.

An enormous progress has been achieved in the last 30 years in identifying and characterization of import components and their function. Still the second major components of biological membranes, the lipids have suffered so far a step-child-like fate. To obtain a comprehensive understanding of the membrane-integration process, it will be crucial to determine the lipids' function in insertase complex stability, insertion mechanisms and assembly of new protein complexes. Since mitochondria should function in the context of the whole cell, the cross-talk with other cellular compartments and structures like the ER and cytosolic translation system has to be deciphered. These unresolved issues will keep us busy in the future and assure many more challenging years of research in the field of mitochondrial biogenesis.

Acknowledgements

Our work is supported by a grant from the Deutsche Forschungsgemeinschaft (RA1048/4-1 to D.R.).

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