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Review

The mitochondrial protein import machinery has multiple connections to the respiratory chain ☆

Bogusz Kulawiak [a], Jan Höpker [a,b], Michael Gebert [c], Bernard Guiard [d], Nils Wiedemann [a,e,*], Natalia Gebert [f]

[a] Institut für Biochemie und Molekularbiologie, ZBMZ, Universität Freiburg, 79104 Freiburg, Germany
[b] Fakultät für Biologie, Universität Freiburg, 79104 Freiburg, Germany
[c] Department of Neurobiology, Harvard Medical School, Boston, MA 02115, USA
[d] Centre de Génétique Moléculaire, CNRS, 91190 Gif-sur-Yvette, France
[e] BIOSS Centre for Biological Signalling Studies, Universität Freiburg, 79104 Freiburg, Germany
[f] Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA 02138, USA

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ABSTRACT

The mitochondrial inner membrane harbors the complexes of the respiratory chain and protein translocases required for the import of mitochondrial precursor proteins. These complexes are functionally interdependent, as the import of respiratory chain precursor proteins across and into the inner membrane requires the membrane potential. *Vice versa* the membrane potential is generated by the proton pumping complexes of the respiratory chain. Besides this basic codependency four different systems for protein import, processing and assembly show further connections to the respiratory chain. The mitochondrial intermembrane space import and assembly machinery oxidizes cysteine residues within the imported precursor proteins and is able to donate the liberated electrons to the respiratory chain. The presequence translocase of the inner membrane physically interacts with the respiratory chain. The mitochondrial processing peptidase is homologous to respiratory chain subunits and the carrier translocase of the inner membrane even shares a subunit with the respiratory chain. In this review we will summarize the import of mitochondrial precursor proteins and highlight these special links between the mitochondrial protein import machinery and the respiratory chain. This article is part of a Special Issue entitled: Respiratory complex II: Role in cellular physiology and disease.

1. Introduction

According to the endosymbiotic theory mitochondria originated when a respiring α-proteobacterium was enclosed by a host cell and kept as an endosymbiont [1,2]. This was of great benefit for the host since the engulfed prokaryote became mainly responsible for the energy metabolism of the eukaryotic cell. To date mitochondria also fulfill many other important functions in eukaryotic cells and play a role in amino-acid metabolism, calcium storage, iron–sulfur cluster synthesis, lipid metabolism and programmed cell death [3,4]. Similar to its ancestor, mitochondria are surrounded by a double membrane and consist of four different compartments, the outer membrane, the intermembrane space, the inner membrane and the mitochondrial matrix in the interior. The inner membrane can be divided into the inner boundary membrane adjacent to the outer membrane and the cristae membrane which forms the large invaginations harboring predominantly the respiratory chain [5]. In the mitochondrial respiratory chain electrons from $NADH + H^+$ are transported through the proton pumping complexes I, III (cytochrome bc_1) and IV (cytochrome *c* oxidase) of the inner membrane to reduce molecular oxygen. Complex II named succinate dehydrogenase similarly delivers electrons from the oxidation of succinate to fumarate via ubiquinone to complex III and therefore indirectly contributes to the proton gradient across the inner membrane. This proton gradient is mainly used to drive the ATP production by complex V (F_1F_o-ATP-synthase), which occurs in the matrix of mitochondria. On the one hand ADP as well as inorganic phosphate have to be imported before the reaction and on the other hand ATP has to be exported after synthesis to support the host cell's metabolism. The import and export of these metabolites are also driven by the proton gradient across the mitochondrial inner membrane and are facilitated by proteins of the metabolite carrier protein family [6].

As during evolution the majority of genes were transferred from the prokaryotic genome into the nucleus of the eukaryotic host cell, nearly all of the roughly 1000 mitochondrial proteins are synthesized

Abbreviations: MIA, mitochondrial intermembrane space assembly; MIM, mitochondrial import complex; mtHsp70, mitochondrial heat shock protein 70; PAM, presequence translocase-associated motor; SAM, sorting and assembly machinery; SDH, succinate dehydrogenase; TIM22, carrier translocase of the inner membrane; TIM23, presequence translocase of the inner membrane; TOM, translocase of the outer membrane

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* Corresponding author at: Institut für Biochemie und Molekularbiologie, ZBMZ, Universität Freiburg, 79104 Freiburg, Germany. Tel.: +49 761 203 5280; fax: +49 761 203 5261.
E-mail address: Nils.Wiedemann@biochemie.uni-freiburg.de (N. Wiedemann).

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in the cytosol [7,8]. To avoid mislocalization into other cellular compartments and to ensure precise targeting to and within mitochondria, the mitochondrial precursor proteins contain specific signal sequences. The precursors are recognized by receptors at the mitochondrial surface, imported by the translocase of the outer membrane (TOM) and sorted to the different mitochondrial subcompartments (Fig. 1). This is facilitated by several import machineries, which recognize and deliver the incoming precursors to their final destination [9–12]. Most mitochondrial precursor proteins contain an N-terminal presequence, which is required and sufficient for mitochondrial protein import. Their import across or into the inner membrane is mediated by the presequence translocase (TIM23). Another class of inner membrane proteins, the metabolite carrier proteins, are also synthesized in the cytosol and recognized by internal targeting signals. Their insertion into the inner membrane is promoted by a dedicated carrier translocase (TIM22). In both cases the import of proteins across or into the inner membrane requires the membrane potential ($\Delta\psi$), which is generated by the respiratory chain. In contrast, the other import pathways are not dependent on the membrane potential. These are the import and insertion of β-barrel proteins of the outer membrane by the sorting and assembly machinery (SAM), the biogenesis of many α-helical outer membrane proteins mediated by the mitochondrial import complex (MIM) and the oxidative folding by the mitochondrial intermembrane space assembly machinery (MIA). The mitochondrial protein import machineries are conserved through eukaryotes and unless stated otherwise we refer to the model organism *Saccharomyces cerevisiae*, since mitochondrial protein import is best studied in yeast.

2. Mitochondrial protein import across and insertion into the outer membrane

2.1. TOM — the general import pore for mitochondrial precursor proteins

After the synthesis at cytosolic ribosomes mitochondrial precursor proteins first encounter the general import pore in the outer membrane called translocase of the outer membrane (TOM). The central subunit of the TOM complex is the pore forming β-barrel protein Tom40, which is essential for the survival of the cell [13,14]. The cytosolic domains of the three receptor subunits Tom20, Tom22 and Tom70 recognize and bind the respective signal sequences of mitochondrial precursor proteins. Tom70 and Tom20 are considered as loosely associated subunits of the TOM complex and are both N-terminally anchored proteins exposing hydrophilic receptor domains to the cytosol [15–18]. Although they differ in their substrate specificity, both proteins show overlapping function and can substitute for each other. Additionally, metazoan Tom20 was found to cooperate with Tom70 to trigger the release of the precursor for subsequent import [19]. Tom20 preferentially recognizes the classical N-terminal amphipathic presequence of mitochondrial precursor proteins [20], which are cleaved off after import. By contrast the cytosolic domain of Tom70 interacts with Hsp90 and

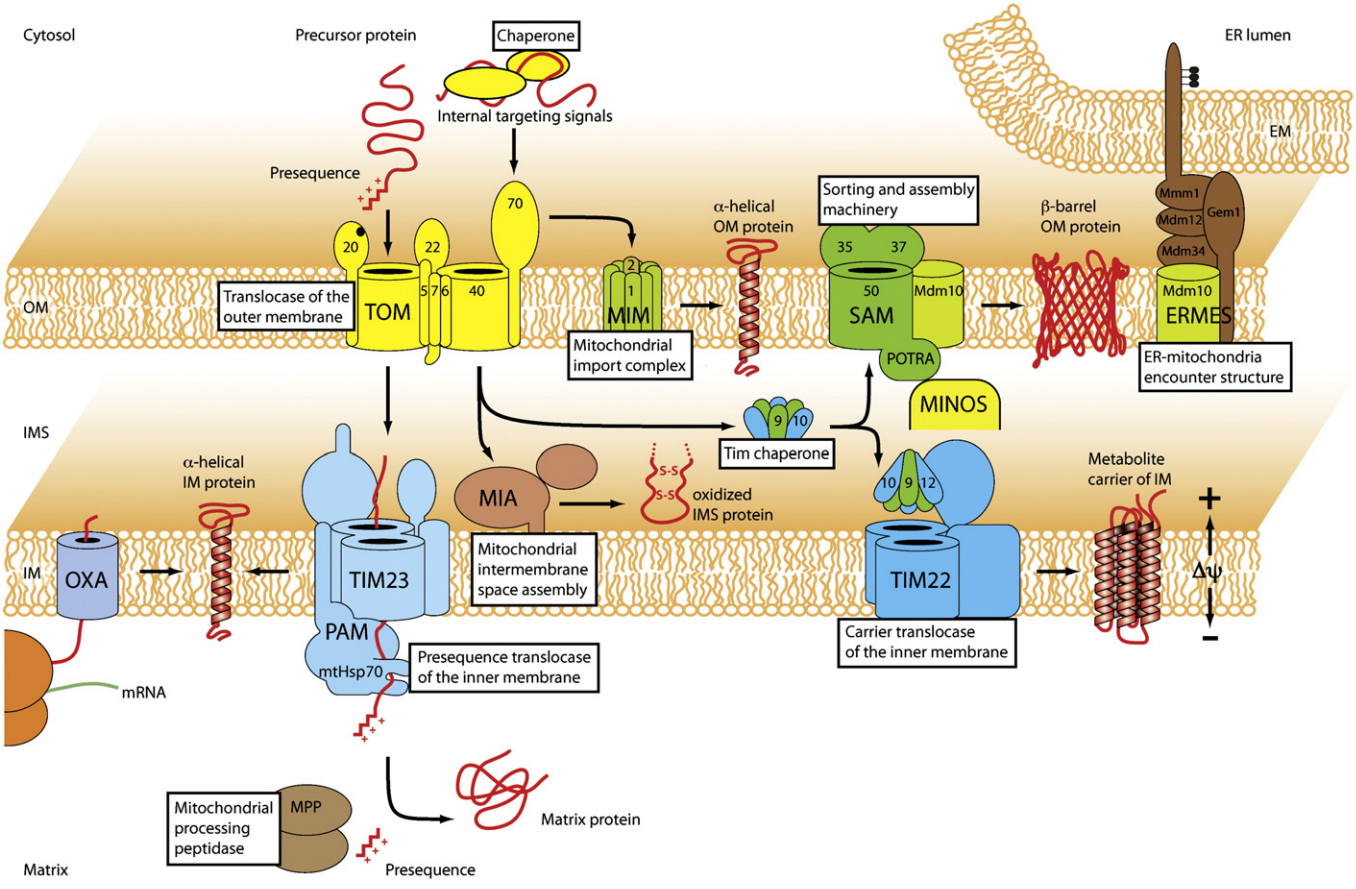

Fig. 1. Protein translocation pathways in mitochondria. Most mitochondrial precursor proteins are translated in the cytosol and imported with the help of the translocase of the outer membrane (TOM). β-Barrel proteins are chaperoned to the sorting and assembly machinery (SAM) and inserted into the outer membrane. Intermembrane space (IMS) proteins with cysteine motifs are oxidized and folded with the help of the mitochondrial intermembrane space assembly machinery (MIA). Metabolite carrier proteins are inserted with the help of the translocases of the inner membrane (TIM22). The presequence translocase (TIM23) inserts proteins into the inner membrane or imports proteins in cooperation with its associated motor (PAM) into the matrix. Presequences are cleaved off by the mitochondrial processing peptidase (MPP). Mitochondrially encoded proteins are directly inserted into the inner membrane with the help of OXA machinery. Some α-helical outer membrane proteins are imported with the help of the mitochondrial import complex (MIM). Membrane contact sites are formed between the inner and the outer membrane with the help of the mitochondrial inner membrane organization system (MINOS). The endoplasmic reticulum membrane (EM) is connected to the outer membrane by the ER–mitochondria encounter structure (ERMES).

Hsp70 proteins that deliver hydrophobic precursor proteins to the TOM complex [21–23]. Tom22 contains an N-terminal cytosolic receptor domain as well as a C-terminal domain facing the mitochondrial intermembrane space. In addition to its receptor function Tom22 is required for assembly of the 440 kDa TOM complex with multiple Tom40 pores [24,25]. This TOM core complex also contains the three small proteins Tom5, Tom6 and Tom7, which also play a role in stabilization and organization of the TOM complex [26–28]. After the passage through the TOM complex the precursor proteins are further sorted to their respective mitochondrial subcompartment.

2.2. SAM — import and insertion of β-barrel proteins into the outer membrane

In eukaryotic cells β-barrel membrane proteins are exclusively found in the outer membrane of endosymbiotic organelles like mitochondria, which derived from the outer membrane of Gram-negative bacteria. Besides their barrel-like structure formed by multiple β-strands, mitochondrial β-barrel precursors share a conserved β-signal at their C-terminus which is recognized by the sorting and assembly machinery (SAM) [29,30]. After the passage through the TOM complex the soluble hexameric Tim9–Tim10 chaperone complex guides the hydrophobic β-barrel proteins through the aqueous intermembrane space to the SAM complex in the outer membrane [31,32].

The central essential subunit of the SAM complex is Sam50 (Tob55, Omp85), which is a β-barrel protein itself and related to BamA of the bacterial β-barrel assembly machinery [33–35]. In the SAM complex Sam50 acts together with another essential subunit Sam35 (Tob38, Tom38) and two further subunits Sam37 (Mas37, Tom37) and Mdm10. The two peripheral subunits Sam35 and Sam37 and the polypeptide transport associated domain (POTRA) of Sam50 help to recognize incoming precursors and to release folded β-barrel proteins into the lipid phase [29,30,36–40]. Mdm10 is specifically required for the biogenesis of the TOM complex, where the β-barrel protein Tom40 has to associate with the α-helical subunit Tom22 to form the stable core of the TOM complex [41–43]. In addition to its localization at the SAM complex Mdm10 also forms a complex with Tom7 and is also a crucial subunit of the ER mitochondria encounter structure (ERMES), which tethers the membrane of the endoplasmic reticulum to the outer mitochondrial membrane [44–46]. In ERMES the mitochondrial outer membrane proteins Mdm10 and Gem1 are connected with the help of Mdm12 and Mdm34 to the membrane integral ER protein Mmm1 [47–49]. Mutations of SAM and ERMES subunits lead to outer membrane import and assembly defects [41–44,50]. In addition, the ERMES was proposed to facilitate ER mitochondria lipid transport, which is crucial for mitochondrial biogenesis [47,51,52].

The SAM complex also physically interacts with the mitochondrial inner membrane organization system (MINOS, MICOS, MitOS), which is required for the maintenance of the mitochondrial cristae architecture [53–56]. In order to build cristae junctions the inner boundary membrane must be connected to the outer membrane. This connection is mediated by the inner membrane protein Fcj1 (mitofilin), which mediates the formation of cristae junctions [57,58]. The specific interaction between the POTRA domain of Sam50 and the intermembrane space domain of Fcj1 is crucial for the formation of contact sites between the outer and the inner membrane of mitochondria [59–62]. In the absence of Fcj1 the β-barrel precursor of Tom40 is only imported with minor efficiency, indicating that a distinct proximity of the mitochondrial membranes is required for efficient import and assembly [60].

2.3. MIM — import and insertion of α-helical outer membrane proteins

Even though α-helical outer membrane proteins are targeted to mitochondria by receptors of the TOM complex, an involvement of the TOM channel was not shown for the insertion of natural α-helical outer membrane proteins [63]. Instead, a separate mitochondrial import complex (MIM) was identified in the outer membrane, which contributes to the efficient import of many single- and multi-spanning α-helical outer membrane proteins [64–69]. The 200 kDa MIM complex contains an oligomeric assembly of the two small single-spanning outer membrane proteins Mim1 (Tom13) and Mim2 [70]. Mim1 was found to associate with the SAM complexes and therefore the Mim proteins might also contribute to membrane protein complex assembly [40,64,71].

3. Matrix import and inner membrane insertion of presequence proteins

3.1. TIM23 — the presequence translocase of the inner membrane

The majority of proteins imported into mitochondria carry an N-terminal presequence comprising an amphipathic α-helix with hydrophobic residues on the one side and polar residues on the other side. Most presequences have a length of 10 to 55 amino acids and a net charge between +3 and +6 [72]. After translocation through the TOM complex the incoming precursors with N-terminal targeting sequences are handed over to the presequence translocase of the inner membrane (TIM23). The precursor proteins are either transported into the matrix or inserted into the inner membrane (Fig. 2). The membrane potential is thought to exert an electrophoretic effect on the positively charged presequence to initiate the import by the TIM23 complex.

The core of the presequence translocase consists of three membrane-integrated components Tim23, Tim17 and Tim50 [73–76]. Tim23 forms four transmembrane helices, as well as an N-terminal domain in the intermembrane space and is thought to be the channel of the complex [77]. Tim17, a homolog of Tim23, was demonstrated to play a multifunctional role in the TIM23 complex. It is crucial for stabilization of the translocase complex and was suggested to act as a voltage sensor of the TIM23 complex [78]. Tim17 functions in lateral sorting into the inner membrane as well as in import of preproteins into the matrix [79]. Tim50 is a single spanning membrane protein and exposes a large C-terminal domain into the intermembrane space. This domain is involved in closing the Tim23 channel in the inactive state to preserve the membrane potential across the inner membrane [80]. Furthermore the intermembrane space domains of Tim23 and Tim50 are responsible for binding and handover of incoming precursors [81–86]. Depending on the precursor, the three core components of the TIM23 complex associate with further proteins to facilitate the import of presequence proteins.

3.2. PAM — the presequence translocase-associated motor for matrix import

Import of precursor proteins into the mitochondrial matrix requires the additional action of the presequence translocase-associated motor (PAM). The central component of PAM is mtHsp70 (Ssc1), which has a binding domain for the incoming precursor as well as an ATPase domain [87–91]. By multiple rounds of substrate binding mtHsp70 proteins are able to import the precursor proteins into the matrix. This essential function of mtHsp70 is highly regulated by other subunits of the PAM complex. Tim44 is a central organizer of the PAM complex and is peripherally attached to the inner mitochondrial membrane [92]. It displays binding sites for both mtHsp70 as well as for Tim23 and was shown to recruit the so-called J-complex to the TIM23 translocase [93,94]. The two J-proteins Pam16 and Pam18 influence mtHsp70 function by stimulating its ATPase activity [95–102]. Pam17 promotes the association of Pam16 and Pam18 to the TIM23 complex [103]. The nucleotide exchange factor Mge1 catalyzes the ADP to ATP exchange on mtHsp70 to promote another cycle of precursor binding for efficient precursor protein import into the mitochondrial matrix [104–106]. Mutations in the human ortholog of the J-co-chaperone Pam18 cause cardiomyopathy indicating that matrix import is a crucial process [107].

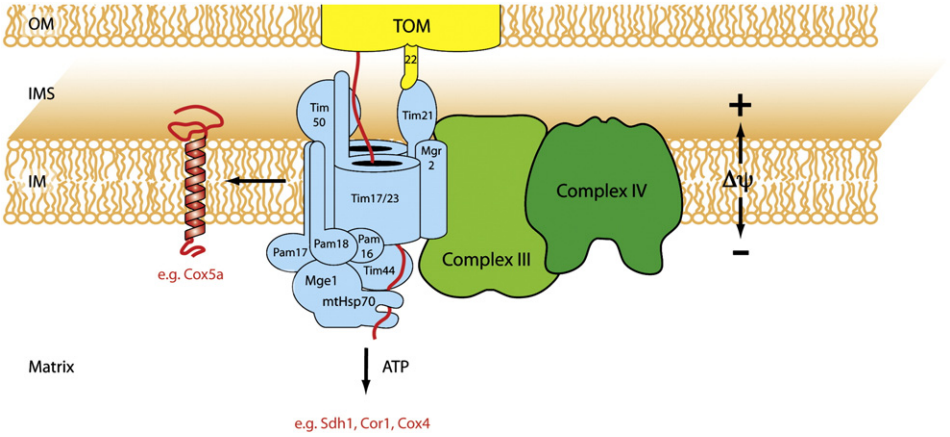

Fig. 2. The presequence translocase of the inner membrane (TIM23): precursor proteins with positively charged amphipathic presequences are imported with the help of the translocase of the outer membrane (TOM) and are handed over to the TIM23 complex. Association with the respiratory chain supercomplex III + IV (bc_1-COX) supports the membrane potential ($\Delta\psi$) dependent translocation of the presequence across the inner membrane. The presequence translocase-associated motor (PAM) is required for ATP dependent translocation into the matrix. Alternatively precursors can be laterally inserted into the inner membrane.

3.3. The presequence translocase TIM23 of the inner membrane is connected to the respiratory chain

Membrane insertion into the inner membrane depends on the presence of a sufficiently hydrophobic transmembrane segment in the precursor [108]. In contrast to matrix import membrane insertion into the inner membrane can be driven by the membrane potential alone [109–111]. For this membrane potential dependent step the TIM23 complex associates with another subunit called Tim21. Tim21 is a single spanning membrane protein and exposes its C-terminal domain to the intermembrane space. This domain was shown to bind to the intermembrane space domain of Tom22 to facilitate precursor transfer from the TOM complex in the outer membrane to the TIM23 complex in the inner membrane [79,112,113]. Furthermore, Tim21 was described to couple the TIM23 translocase to the cytochrome bc_1-cytochrome *c* oxidase (bc_1-COX) supercomplexes of the respiratory chain via a direct interaction with Qcr6 (a subunit of complex III) [114–116]. This interaction with the respiratory chain supercomplex supports the import of presequence proteins under membrane potential limiting conditions. This observation is consistent with the idea of a localized higher membrane potential in the direct vicinity of proton pumping complexes of the respiratory chain [117–120]. Therefore, Tim21 dependent coupling of the presequence translocase to the respiratory chain might be crucial, when the membrane potential is reduced. Indeed, when the *tim21*Δ strain is grown in the presence of low amounts of protonophores it shows a considerable growth defect compared to wildtype [114]. Recently, it was shown that binding of Tim21 to the TIM23 core complex requires Mgr2, which is an additional subunit of the Tim21-containing TIM23 complex [121]. As such Mgr2 is also required for efficient coupling of the TIM23 complex to the TOM translocase in the outer membrane and the bc_1-COX supercomplexes in the inner membrane. Furthermore, the two regulatory subunits Pam16 and Pam18 of the import motor are independently associated with the respiratory chain supercomplex [115]. This might initiate the association of the import motor when a matrix precursor emerges from the TIM23 channel.

The subunits Tim23 and Tim17 are also related to the subunit B14.7 of bovine complex I [122]. They all belong to a preprotein and amino acid transporter family (PRAT) [123,124]. In plants the TIM23 complex does not only interact with the bc_1-complex, in addition, Tim23 isoforms were found to associate with complex I (NADH dehydrogenase) of the respiratory chain [125]. However, so far it is not known if the interaction with complex I serves as alternative connection to proton pumping complexes of the respiratory chain, as regulatory mechanism for presequence protein import or for the biogenesis of complex I precursor proteins imported from the cytosol [125]. Since mitochondria from yeast *S. cerevisiae* contain only a type II NADH dehydrogenase instead of complex I, other organisms have to be used to reveal the role of complex I for the presequence translocase [126].

4. Processing of mitochondrial presequences

4.1. MPP — the mitochondrial matrix peptidase cleaves the presequences

The cleavage of the N-terminal presequences by the mitochondrial processing peptidase (MPP) in the matrix is crucial for the subsequent folding and the catalytic activity of the imported precursor proteins (Fig. 3). MPP consists of two homologous subunits Mas1 (Mif1, β-MPP) and Mas2 (Mif2, α-MPP) [127,128]. The zinc-metalloprotease Mas1 is the active subunit, which cleaves the precursor in an extended confirmation during or shortly after the transport through the TIM23 complex [129]. Cleavage takes place at a specific site, preferentially with arginine at position minus 2 relative to the mature N-terminus (R-2 motif: R-X↓X) [72].

After MPP cleavage some imported proteins are even further processed by the matrix aminopeptidases Icp55 or Oct1. The intermediate cleaving peptidase Icp55 removes the most N-terminal amino acid whereas the mitochondrial intermediate peptidase Oct1 (MIP) removes an N-terminal octapeptide. What is the function of this additional processing step? Vögtle et al. [72,130] show that both peptidases remove destabilizing amino acid residues at the N-terminus of the import intermediates to generate mature proteins with stabilizing N-terminal residues according to the bacterial N-end rule of protein degradation. Therefore, Icp55 and Oct1 are crucial for protein stability of newly imported substrate proteins. *Oct1*Δ cells were shown to have respiratory defects, since some respiratory chain proteins are Oct1 substrates [131].

4.2. The mitochondrial processing peptidase MPP is related to the core subunits of the bc_1-complex

Interestingly in *Rickettsiae*, which are intracellular bacteria closely related to mitochondria, a protease with homology to MPP was found that is able to cleave mitochondrial presequences [132]. Therefore, it is widely accepted that a bacterial homolog was the progenitor for MPP and took over the new function to process the presequences of mitochondrial precursor proteins. However this does not explain, why the two subunits Mas1 and Mas2 of MPP are homologous to the core subunits of the mitochondrial bc_1-complex named Cor1 and

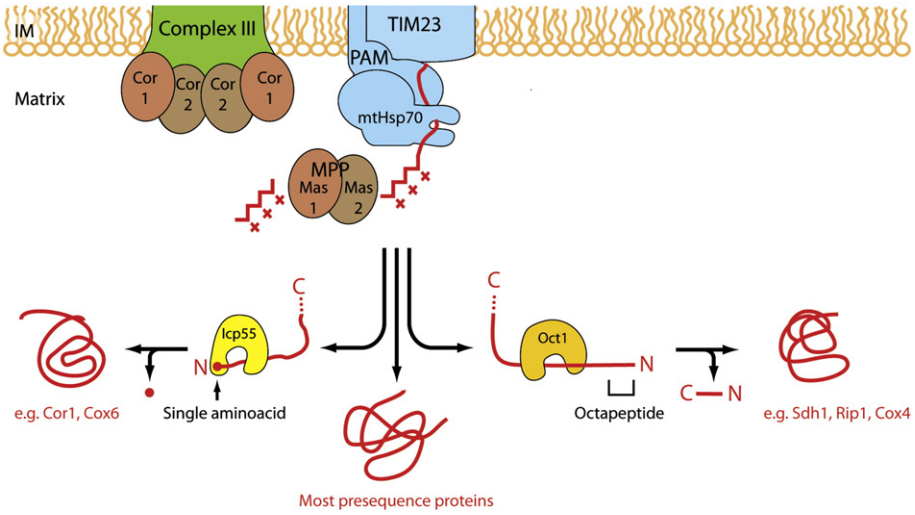

Fig. 3. Processing of mitochondrial presequence proteins. When the N-terminal presequences emerge in the matrix, they are cleaved off by the mitochondrial processing peptidase (MPP). In case the N-terminal amino acid is destabilizing the intermediate cleavage peptidase (Icp55) can cleave off a single amino acid. Similarly mitochondrial intermediate peptidase (Oct1) can cleave an N-terminal octapeptide to generate a stable N-terminus of imported protein. The subunits Mas1 and Mas2 of MPP are homologous to the core proteins Cor1 and Cor2 of the complex III (bc_1).

Cor2 (Fig. 3). In *Neurospora crassa* it was found that β-MPP (Mas1) and Cor1 are identical proteins encoded by a single gene [133]. Even more astonishingly in plants it was found that Mas1 and Mas2 replace the core proteins and the MPP-activity is exclusively integrated into the bc_1-complex [134,135]. Therefore it was argued that the bc_1-proteins Cor1 and Cor2 are relics of the MPP subunits [136]. We speculate that the TIM23 complex coevolved with the bc_1-complex and both complexes were associated with each other, to enable efficient translocation of presequence proteins across the inner membrane. The additional association of the MPP activity to the bc_1-complex would have coupled the translocation across the inner membrane with the processing of the presequences. The potential advantage of a localized high membrane potential at the TIM23-bc_1-supercomplex for the translocation of positively charged presequences might have been a disadvantage for the import of negatively charged domains in the C-terminal part of presequence proteins. Therefore it seems reasonable that later on the reversible association of TIM23 with the bc_1-complex on the one hand and with the import motor PAM on the other hand evolved to make the import of individual presequence proteins as efficient as possible. Finally, the MPP genes might have been duplicated to generate a soluble matrix version of MPP to efficiently process PAM dependent precursors. The bc_1-associated MPP subunits remained structurally important for the complex III but degenerated in some species to processing-inactive core proteins.

5. Biogenesis of intermembrane space proteins

Even though it is assumed that all intermembrane space proteins are translocated through the TOM complex across the outer membrane [137–139], multiple differing biogenesis pathways exist in parallel (Fig. 4) [140–145]. All of these import mechanisms are related to the biogenesis of the respiratory chain, because either structural subunits or assembly factors use the respective pathways.

5.1. IMP — the cleavage of inner membrane precursors releases proteins into the intermembrane space

Mitochondria harbor an inner membrane protease complex (IMP), which is dedicated for the cleavage of specific soluble domains of inner membrane precursor proteins. The two major membrane embedded subunits Imp1 and Imp2 both have catalytically active intermembrane space domains with different specificity [146,147]. The third subunit Som1 directly interacts with Imp1 and supports Imp1 activity [148]. The inner membrane protease is required to cleave the membrane anchor of inner membrane precursor proteins to generate soluble intermembrane space proteins. Such precursors consist of a presequence, an MPP cleavage site, an α-helical hydrophobic transmembrane segment and an IMP cleavage site prior to a soluble C-terminal domain. These precursors are classical TOM/TIM23 substrates, which are laterally sorted into the inner membrane and upon IMP cleavage their C-terminal domain is released as mature protein into the intermembrane space.

5.2. Complex formation and cofactor insertion trap proteins in the intermembrane space

Soluble proteins can also be trapped in the intermembrane space by cofactor insertion. The apo-form of cytochrome *c* (Cyt. *c*, Cyt1) is imported through the TOM complex [138,139]. In the mitochondrial intermembrane space heme lyases catalyze the covalent attachment of heme to conserved cysteine residues of *c*-type cytochromes via two thioether bonds. The binding of the apo-protein to the heme lyase and the subsequent folding of the holo-protein trap the precursor in the intermembrane space [149–152]. Other intermembrane space proteins, like the precursor of cytochrome *c* heme lyase itself (holocytochrome *c* synthase, Cyc3), are also imported by the TOM complex and subsequently trapped in the intermembrane space by specific interactions with the membrane [153–155]. This mechanism is plausible for peripheral membrane proteins, which can form a membrane bound complex with the intermembrane space domain of inner or outer membrane proteins.

5.3. MIA — oxidative folding traps proteins in the intermembrane space

A prerequisite for the import of the hydrophobic outer membrane β-barrel precursors as well as for the inner membrane metabolite carriers is the presence of the small Tim chaperone complexes in the intermembrane space. It was debated whether the cysteine motifs of these intermembrane space proteins are required for zinc binding or for disulfide bond formation in the intermembrane space until the crucial subunits of the mitochondrial intermembrane space assembly machinery (MIA) were identified. Both subunits Mia40 (Tim40) and Erv1 are essential for respiration and viability and form a disulfide relay system in the intermembrane space required for oxidative folding of cysteine motif containing intermembrane space precursors

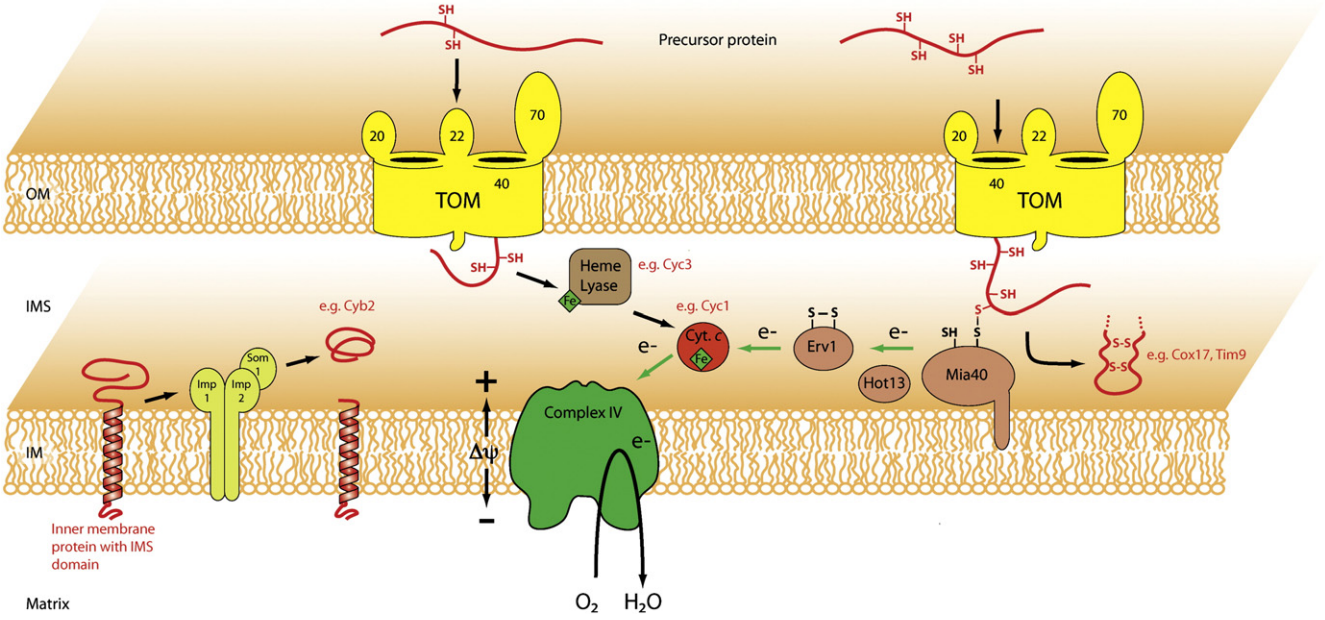

Fig. 4. Multiple import pathways are used by intermembrane space proteins. On the one hand precursors can be imported and inserted into the inner membrane along the presequence pathway and subsequently cleaved by inner membrane protease (IMP) to release a soluble intermembrane space domain. On the other hand proteins are directly imported with the help of the TOM complex and trapped in the intermembrane space by high affinity interactions, cofactor insertion or by oxidative folding catalyzed by the mitochondrial intermembrane space assembly machinery (MIA). Electrons released by disulfide bond formation of cysteine motif containing precursor proteins are donated via cytochrome *c* (Cyt. *c*, Cyc1) to the cytochrome *c* oxidase (complex IV).

[156–160]. Initially the oxidoreductase Mia40 recognizes the substrate protein and forms an intermolecular disulfide bond with the precursor. The mitochondrial intermembrane space sorting (MISS) or targeting signal (ITS) of these proteins consists of a docking cysteine (C) for the initial disulfide bond formation with Mia40, two hydrophobic (Hy) and a single aromatic (Ar) residue on the same face of an α-helical segment of the precursor like $(Ar)X_2(Hy)_2X_2C$ and is sufficient to import proteins into the intermembrane space [161,162]. Mia40 has a dual function as receptor for binding the MISS/ITS-signal proteins as well as oxidoreductase to promote correct folding of the substrate proteins by introduction of intramolecular disulfide bonds. Mia40 binds the import signal in the precursor protein when it emerges from the TOM channel into the intermembrane space. In the absence of the MINOS subunit Fcj1, which is also required for the formation of contact sites between the inner and the outer membrane, Mia40 dependent import into the intermembrane space is diminished [53]. In order to promote the folding of the substrates Mia40 has a chaperone like folding activity [163]. The substrates usually contain two cysteine motifs with either 3 or 9 amino acids between two cysteines (CX_3C or CX_9C) [156,164,165]. Protein structures at atomic resolution have revealed that usually two interhelical disulfide bonds are formed between the two $CX_{3/9}C$ motifs of the substrates in an antiparallel orientation [166–168]. After formation of the first disulfide bond in the substrate protein the sulfhydryloxidase Erv1 binds transiently to Mia40. A likely explanation for the formation of this ternary complex is, that Erv1 reoxidizes Mia40 to enable the formation of additional disulfide bonds in the precursor protein [169–171]. The conserved zinc binding protein Hot13 improves Erv1-dependent oxidation of Mia40 [172,173]. After the introduction of two intramolecular disulfide bonds by the MIA machinery, Tim9 and Tim10 form a hexameric ring-like chaperone complex with three molecules of each protein arranged in alternating order [166,174–177]. The disulfide bonded helix-loop-helix structure of the Tim9–Tim10 complex results in a tertiary structure where the N- and C-termini resemble tentacle arms on the same side of the complex which were suggested to bind hydrophobic patches of incoming carrier or β-barrel precursors [166,175,178,179]. In addition, a second small Tim8–Tim13 chaperone complex was discovered, which is not essential in yeast [180]. The Tim8–Tim13 complex seems to be an intermembrane space chaperone important for the precursor of Tim23 [168,181–183]. Mutations in the human gene ortholog of TIM8 lead to a severe neurodegenerative disease called Mohr–Tranebjaerg syndrome or deafness dystonia syndrome [180].

5.4. Electrons released by oxidative folding of imported intermembrane space proteins can be transferred to the respiratory chain

Before Erv1 was functionally characterized it was already known that the homolog Erv2, which is involved in disulfide bond formation in the endoplasmic reticulum, is able to donate its electrons to molecular oxygen. Therefore it was likely that oxygen would be the terminal electron acceptor of Erv1, however the interesting question was if Erv1 could donate its electrons to the respiratory chain. Multiple groups could show that mitochondrial Erv1 can use cytochrome *c* (Cyc1) as an electron acceptor [184–187]. During the import of mitochondrial intermembrane space proteins, electrons are transferred from the substrate protein to Mia40 and further via Erv1 to cytochrome *c* [188], which donates the electrons to complex IV. Prior to their final transfer to molecular oxygen, these electrons are used by the respiratory chain to pump additional protons across the inner membrane to maintain the membrane potential. In that way the oxidative folding of intermembrane space proteins is coupled to the respiratory chain to conserve energy from the formation of disulfide bonds.

6. Biogenesis of metabolite carrier proteins of the inner mitochondrial membrane

6.1. Import and assembly steps of carrier precursor proteins

Metabolite carriers like the ADP/ATP carrier or the phosphate carrier are hydrophobic proteins with 6 transmembrane helices [6]. In contrast to mitochondrial precursor proteins with a cleavable N-terminal presequence, carrier proteins contain hydrophobic, hydrophilic, charged or uncharged sequence motifs distributed throughout

the whole polypeptide chain acting as signal sequences [175,189,190]. On the way to their final destination they pass the TOM complex and the intermembrane space before they are inserted into the membrane by the carrier translocase of the inner membrane (TIM22). This import and assembly pathway is divided into five stages (Fig. 5) [191–194]. After the synthesis in the cytosol (stage I), the hydrophobic carrier precursors are guided to mitochondrial Tom70 receptors with the help of cytosolic Hsp90 and Hsp70 chaperones (stage II) [21,193,195]. The carrier precursor is transferred from Tom70 to Tom22 and is subsequently translocated through the membrane by the TOM complex [24,25]. Unlike proteins with an N-terminal signal sequence, which cross the channel as linear polypeptides, precursor proteins of the carrier pathway can be translocated through the TOM pore in a loop conformation [183,196]. After passage through the TOM complex the carrier proteins reach the aqueous compartment of the intermembrane space and bind to the Tim9–Tim10 chaperone complex (stage III) to prevent aggregation of the hydrophobic precursors [175]. The Tim9–Tim10 complex guides the carrier precursor to the TIM22 translocase where it is inserted into the inner membrane (stage IV). This step is fully dependent on the membrane potential across the inner membrane. Finally the carrier precursor is released from TIM22 into the membrane and forms a homodimer representing the mature form of carrier proteins (stage V). The crystal structure of the ADP/ATP carrier shows that the homodimer is stabilized by tightly associated cardiolipin molecules [197,198]. The mitochondrial signature lipid cardiolipin, which is synthesized in the inner membrane, is also involved in organization of respiratory chain supercomplexes in the inner membrane and plays a role in assembly and function of the TOM complex in the outer membrane [199–203].

6.2. TIM22 — the carrier translocase of the inner mitochondrial membrane

All members of the metabolite carrier family are exclusively inserted into the inner membrane by the carrier translocase. The first subunit was identified when Sirrenberg et al. (1996) discovered Tim22, which is homologous to the two subunits of the presequence translocase Tim17 and Tim23. Tim22 is not required for the import of proteins with N-terminal signal sequences, but it is essential for the insertion of polytopic inner membrane precursor proteins [204]. Tim22 is the pore-forming core subunit of the complex, which is therefore also called TIM22 complex [205]. Tim22 is a hydrophobic protein with internal signal sequences and similar to Tim17 and Tim23 also itself a substrate of the carrier translocase [206]. The TIM22 complex was extensively studied and five further subunits Tim9, Tim10, Tim12, Tim18 and Tim54 were discovered within the next four years. Tim9 and Tim10 are not only subunits of the TIM22 complex, but also form a separate soluble hexameric Tim9–Tim10 chaperone complex in the intermembrane space.

Tim12 is a third essential subunit of the small Tim protein family, which is exclusively peripherally associated with the TIM22 complex [207–209]. It is the only representative of the small Tim family, which is not found in soluble complexes. The C-terminal part of Tim12 was shown to interact with lipids and thus supports the peripheral membrane association with the carrier translocase [210]. Tim9, Tim10 and Tim12 form a stable subcomplex, which subsequently assembles with the other carrier translocase subunits to form the TIM22 complex [211,212]. Tim12 is closer related to Tim10 and therefore it is likely that Tim12 replaces Tim10 to form a similar hexameric small Tim chaperone complex together with Tim9, which is associated with the carrier translocase [213,214].

Tim54 is an integral single transmembrane spanning protein carrying an N-terminal signal sequence and a large C-terminal intermembrane space domain [215]. Cells with a *tim54* deletion show severe growth defects and the import of carrier proteins is dramatically reduced [205,215]. Furthermore, it was demonstrated that the absence of Tim54 leads to the loss of the interaction between the Tim9–Tim10–Tim12 complex and Tim22 suggesting that its role is the recruitment of the small Tim proteins to Tim22 [205]. Indeed, Tim54 can be chemically crosslinked to a Tim10 from the Tim9–Tim10–Tim12 subcomplex [206]. Tim54 is not only required for membrane protein insertion, but is also required for

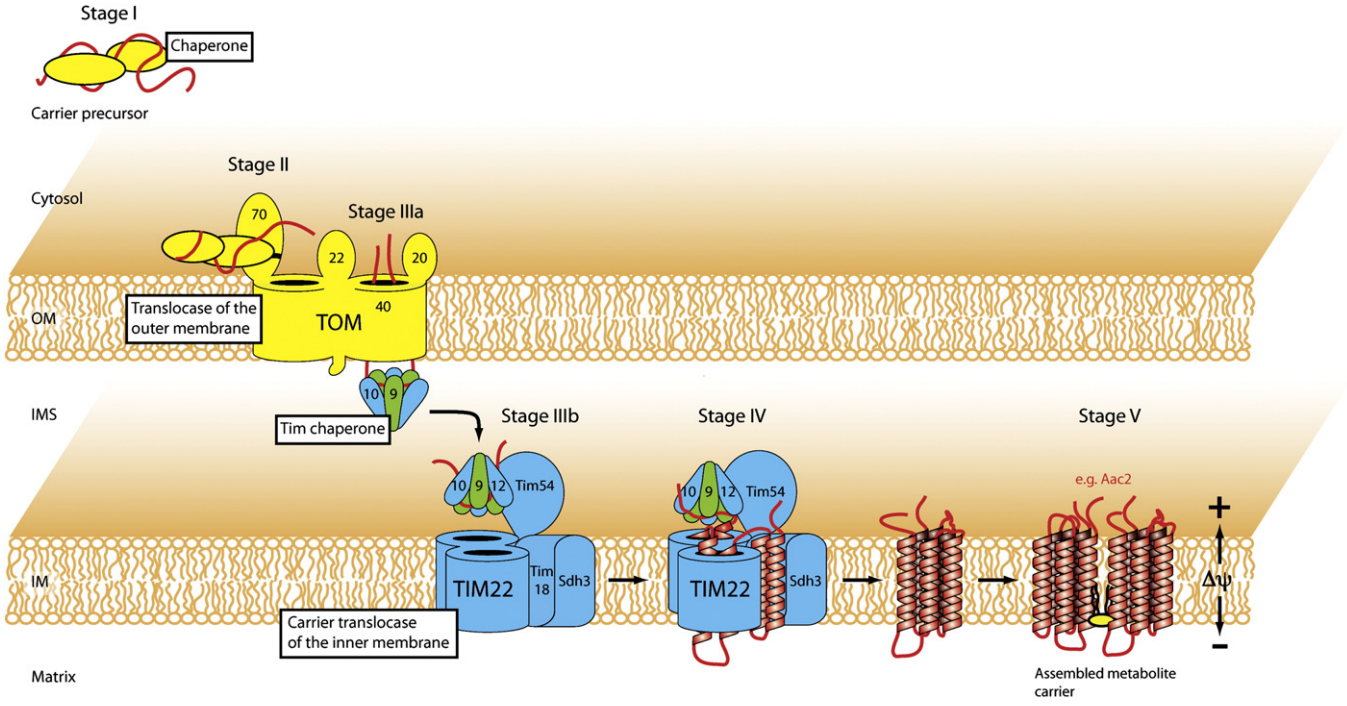

Fig. 5. Import of metabolite carrier proteins by the carrier translocase of the inner membrane (TIM22). After synthesis the hydrophobic precursors of the metabolite carriers are bound by chaperones in the cytosol (stage I). The precursor protein–Hsp90 complex is recognized by the Tom70 receptor in the outer membrane (stage II). After translocation through the Tom40 channel the carrier precursors are bound by the hexameric Tim9–Tim10 chaperone complex in the intermembrane space and transferred to the carrier translocase (TIM22) in the inner membrane (stage III). The membrane potential ($\Delta\psi$) is required for insertion and lateral release of the metabolite carrier proteins into the inner membrane (stage IV). Finally, metabolite carrier proteins form dimers (stage V), which are stabilized by the mitochondrial membrane lipid cardiolipin (yellow).

the assembly of inner membrane protein complexes as shown for the assembly of mitochondrial i-AAA protease Yme1 [216].

Tim18 was discovered independently by co-precipitation of the TIM22 complex as a multi copy suppressor of a *tim54* mutant [217,218]. Additionally, deletion of TIM18 is synthetically lethal with mutants of *tim9*, *tim10* and *tim54*. In contrast to the other subunits of the TIM22 complex, which are all required for the integrity of the 300 kDa translocase complex, deletion of Tim18 results in the formation of a 250 kDa TIM22 subcomplex [206,212,217–219]. Interestingly, Tim18 is an inner membrane protein with homology to the membrane subunit Sdh4 of the succinate dehydrogenase; however overexpression of Sdh4 did not rescue a *tim54* mutant [217,218]. It is likely that Tim18 has a structure similar to Sdh4 with three transmembrane-helices [220,221]. However, it is not known why a homologous protein of the succinate dehydrogenase would be part of the carrier translocase [222]. It was speculated that it serves as an auxiliary protein, since it apparently does not directly participate in the membrane insertion of carrier metabolite precursors [218].

6.3. The respiratory chain protein Sdh3 is also a stoichiometric subunit of the carrier translocase

In order to reveal the relation between the succinate dehydrogenase (SDH) and the carrier translocase Gebert et al. (2011) analyzed the suppression of a temperature sensitive *tim22* mutant by overexpression of other carrier translocase and succinate dehydrogenase subunits. Overexpression of Tim54 and Sdh3 rescued the growth of the *tim22* mutant, but not overexpression of Sdh4, which is related to Tim18 [219]. Many *S. cerevisiae* mutants can grow without mitochondrial DNA, including strains with a deletion of SDH1, SDH2 and SDH4. Such respiratory deficient yeast strains form small colonies on glucose medium, called petite. Nuclear mutations which are lethal for respiratory deficient yeast strains are consequently called petite negative. Gene deletion mutants of the ADP/ATP carrier (AAC2) and of the β-subunit of the F_1F_o-ATP synthase (ATP2) are petite-negative because they are not able to grow without mitochondrial DNA. Their gene products seem to be required in respiratory deficient yeast to maintain the membrane potential by the electrogenic exchange of cytosolic ATP^{4-} for ADP^{3-}, which is produced by hydrolysis in the mitochondrial matrix. The petite-negative phenotype of TOM70 and TIM18 can be explained, because their gene products are required for the import of the ADP/ATP carrier [223]. In contrast to the other genes of complex II, only deletion of SDH3 causes a petite-negative phenotype highlighting its special behavior [219]. Deletion of SDH3 causes a phenotype similar to *tim18*Δ, showing an assembly defect of Tim22, Tim54 and metabolite carrier proteins. In addition it was shown, that Sdh3 is required for the assembly of Tim18 into the carrier translocase. Moreover, by quantitative mass spectrometry and antibody shift of the translocase Sdh3 was shown to be a stoichiometric subunit of the TIM22 complex. The presence of Sdh3 in two different complexes explains the specific phenotypes of its deletion strain compared to the other subunits of the succinate dehydrogenase [219].

6.4. The succinate dehydrogenase of the respiratory chain

The succinate dehydrogenase or complex II of the inner mitochondrial membrane is well known for its dual role as Krebs cycle enzyme and complex of the respiratory chain. Electrons from the oxidation of succinate to fumarate are transferred using the prosthetic FAD group. The SDH complex is structurally related to the fumarate reductase, which catalyzes the opposite reduction of fumarate to succinate [220,221,224,225].

The SDH complex is comprised of two subcomplexes, a catalytic soluble module in the matrix containing Sdh1 and Sdh2 and a hydrophobic module in the inner membrane built by Sdh3 and Sdh4. The two electrons from the oxidation of succinate to fumarate reduce the prosthetic group FAD, which is covalently bound to Sdh1, and the electrons are subsequently transferred to ubiquinone by the three iron–sulfur clusters localized in the Sdh1–Sdh2 module [221]. Sdh3 and Sdh4 form a membrane embedded heterodimer in which each protein contributes three transmembrane helices for the formation of a six-helix bundle [221]. The Sdh3–Sdh4 module binds ubiquinone and is crucial for the proper electron transfer as well as the stable assembly of the SDH complex [221,226]. According to crystal structures of the SDH complex a *b*-type heme is present in the Sdh3–Sdh4 module, but does not play a role in catalytic activity of SDH complex [220,221,227–229]. The membrane integral Sdh3–Sdh4 module in the succinate dehydrogenase is required for the recruitment of the catalytic subunits Sdh1 and Sdh2 to inner membrane. Without a functional Sdh3–Sdh4 module serving as membrane anchor the two catalytically active subunits Sdh1 and Sdh2 reside in the matrix and electron transfer from the Krebs cycle to the respiratory chain is blocked [226,230].

6.5. Role of Sdh3 for succinate dehydrogenase and carrier translocase assembly

Sdh3 is a moonlighting protein with a function in two different mitochondrial membrane protein complexes, the succinate dehydrogenase and the carrier translocase. In both complexes Sdh3 forms a membrane integral heterodimer with its partner proteins Sdh4 and Tim18, respectively [219–222,231]. Tim18 is a close homologue of Sdh4 and modeling based on the crystal structure of the succinate dehydrogenase suggests a high structural similarity of the Sdh3–Sdh4 and the Sdh3–Tim18 module [219,221,232]. Upon deletion of TIM18 the protein levels of Sdh3 are slightly affected, whereas deletion of SDH4 leads to a dramatic reduction of Sdh3. This reflects most likely the ratio of the carrier translocase subunit Tim18 to the succinate dehydrogenase subunit Sdh4 in the cell. In contrast, upon deletion of SDH3 the protein levels of both partner proteins Tim18 and Sdh4 are virtually undetectable. One can conclude that only the assembled membrane integral subunits are resistant to degradation. When ^{35}S-labeled mitochondrial precursor proteins of Tim18 and Sdh4 are imported into isolated mitochondria they assemble into the mature carrier translocase [206,217–219] and the succinate dehydrogenase complexes (Wiedemann N., unpublished observation). Unlike when radiolabelled Sdh3 is imported into wildtype mitochondria neither the assembly into the SDH nor into the TIM22 complex is observed. We speculate that a small fraction of Sdh3 is available in mitochondria to enable heterodimer formation with Sdh4 and Tim18 and subsequent assembly into their mature complexes. Assembly of radiolabelled Sdh3 is only efficient when the respective partner proteins Sdh4 or Tim18 are co-imported to ensure formation of the heterodimers. In this case the available partner protein determines the complex in which Sdh3 is assembled [219].

It is controversial as to whether the Sdh3–Sdh4 module in *S. cerevisiae* contains a *b*-type heme like SDH complexes from *Escherichia coli*, and mitochondrial SDH complexes from higher eukaryotes [220,221,231,233,234]. Since the heme *b* is dispensable for catalytic activity of SDH complex this raises the question if a heme is required for the stability of Sdh3–Tim18 module or even for the function of the TIM22 complex [227–229]. Mutational analysis of heme *b* binding residues in Sdh3–Tim18 module could answer these questions.

6.6. Hypothetical model of carrier translocase evolution and function

Whatever the initial driving force for the maintenance of the endosymbiotic relationship between the host cell and the α-proteobacterium was, the ultimate benefit for the host cell was the presence of confined organelles which support the energy metabolism of the host cell by export of ATP. This requires the presence of an efficient ADP/ATP carrier system and the corresponding import pathway to insert the carrier proteins into the inner membrane. Therefore it was speculated that the

carrier translocase was developed before the presequence pathway evolved [235]. What could be the evolutionary origin for the carrier translocase of the inner membrane? The carrier translocase contains a heterodimeric membrane protein module consisting of Tim18 and Sdh3 together with the essential channel forming protein Tim22. This module is homologous to the membrane modules of the succinate dehydrogenase and fumarate reductase. In the evolving mitochondrion with an oxidative metabolism, the fumarate reductase with an associated fumarate/succinate transporter channel was not required and might have served as a template for carrier translocase evolution (Fig. 6). The channel forming core subunit of the carrier translocase Tim22 belongs to the preprotein and amino acid transporter family (PRAT), which includes OEP16 a chloroplast outer envelope protein forming an amino acid selective channel [123]. The reconstituted OEP16 is selective for multiple amino acids including glutamate and *in vivo* analysis of OEP16 mutants suggest that aspartate is also a major substrate of the outer envelope channel of chloroplasts [236,237]. The dicarboxylic amino acids glutamate and aspartate are structurally similar to succinate and fumarate. Even though a sequence based ancestry for Tim22 is lacking, this functionally supports our hypothesis that Tim22 might have evolved from a bacterial fumarate/succinate transporter channel, which was associated with the ancestor of the Tim18/Sdh3 module. In *Trypanosoma brucei* only a single simplified inner membrane translocase is found which is putatively capable of matrix translocation and carrier insertion [238]. It is therefore feasible that a rudimentary TIM22 translocase might have cooperated with the bacterial Hsp70 chaperone system in the matrix to mediate also the matrix import of the first nuclear encoded presequence proteins. To increase the efficiency of the protein import and to cope with the increasing number of nuclear encoded substrate proteins the two specialized inner membrane translocases for carrier precursors (TIM22) and for presequence proteins (TIM23) might have developed from the simple ancestral TIM22 translocase [235]. A connection between the two different translocases was shown for the ADP/ATP carrier and the phosphate carrier which have the ability to be sorted via the TIM22 or the TIM23 pathway [239,240].

Although the small Tim chaperones of the intermembrane space are very conserved in mitochondria, no bacterial proteins have been found which could be considered as an ancestor, suggesting a *de novo* development [213,241,242]. However the Tim9–Tim10 complex is structurally and functionally related to the periplasmic chaperones Skp and SurA. The small Tim complexes with their N- and C-terminal tentacles bear structural similarity to the jellyfish like chaperone Skp [166] and they share a common binding specificity for their substrates with SurA [243]. Initially, the periplasmic chaperones of the bacterial endosymbiont could have facilitated the transport of the hydrophobic carrier precursor across the intermembrane space. The periplasmic machinery for oxidative folding was likely the ancestor for the MIA import pathway and disulfide bond formation was established to specifically import the small Tim precursors from the cytosol. Afterwards the genes for the periplasmic chaperones on the endosymbiont's genome were superfluous. This contributed to the massive gene loss to form the current mitochondrial DNA with only about a dozen remaining genes primarily required for the formation of the respiratory chain.

What features could be missing to built an even more efficient carrier translocase? First, a dedicated receptor subunit for the recognition of the substrates and second, a mechanism for optimal steric docking of the substrates to the channel of the insertase. Both of these requirements were likely fulfilled by the evolution of Tim54. As a single spanning membrane protein Tim54 is associated with membrane embedded core of the translocase. The large C-terminal intermembrane space domain of Tim54 might bind to the Tim9–Tim10–Tim12 chaperone receptor complex in a way, that the carrier precursor is optimally oriented for the membrane potential dependent insertion of the positively charged carrier protein matrix loops [244–246].

The presence of the evolutionary conserved Tim18–Sdh3 module in the carrier translocase certainly reflects a relict of evolution and is therefore interesting *per se*, but what could be the function of the Tim18–Sdh3 module? In contrast to the Tim22 channel, which is required for membrane insertion of carrier proteins, the whole Tim18–Sdh3 module is dispensable, since deletion of either gene leads to the formation of a 250 kDa TIM22 subcomplex lacking both Tim18 and Sdh3. What are the consequences? The import of carrier precursors is affected *in vitro* and yeast strains with deletions of TIM18 or SDH3 are petite negative, which can be correlated with a carrier import defect *in vivo*. Overexpression of Sdh3 rescued a temperature sensitive *tim22*

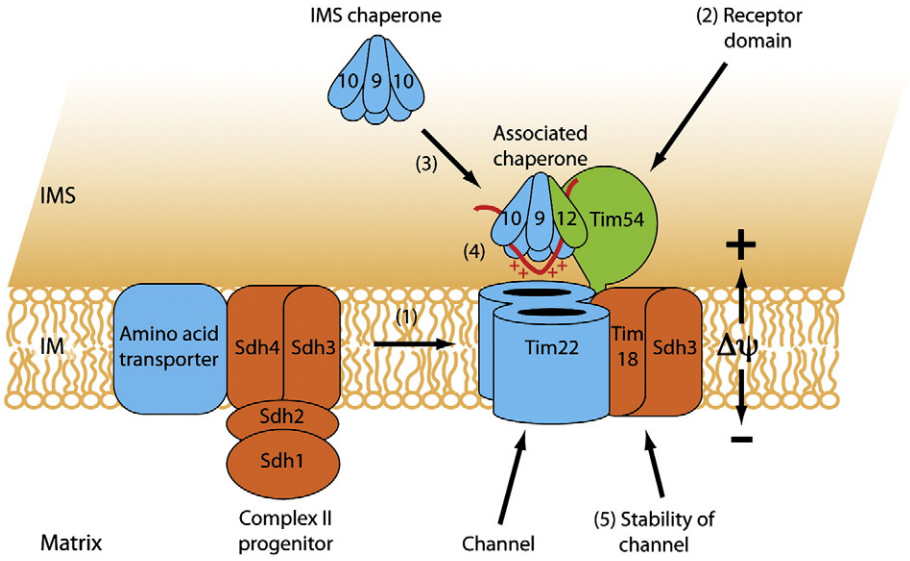

Fig. 6. Hypothetical model for carrier translocase (TIM22) evolution and function. (1) The membrane module of a complex II progenitor with an associated amino acid transporter might have served as a template to create a simple carrier translocase consisting of proteins with homology to Tim18, Sdh3 and Tim22. (2) An intermembrane space receptor domain associated with the translocase was created by evolution of Tim54. (3) Due to partial replacement of Tim10 with the translocase specific Tim12 subunit Tim54 recruited the hexameric Tim9–Tim10 chaperone complex from the intermembrane space permanently to the TIM22 complex. (4) The Tim54–Tim9–Tim10–Tim12 complex might be sterically optimal oriented to facilitate the membrane potential dependent insertion of the positively charged matrix loops of the carrier precursor proteins into the Tim22 channel. (5) The Tim18–Sdh3 module might stabilize the lateral gate of the Tim22 channel, which must be flexible to allow the membrane insertion of carrier proteins.

mutant strain and conversely deletion of SDH3 causes an assembly and stability defect of the essential channel subunit Tim22 [219]. Due to these phenotypes we speculate that Tim18 and Sdh3 form a membrane module, which binds to the lateral gate of Tim22 to enhance the stability of this dynamic protein insertion channel.

7. Assembly of the respiratory chain requires multiple protein import and export pathways

Nowadays there are still about a dozen core subunits of the respiratory chain, which are encoded on mitochondrial DNA, synthesized in the matrix and exported into the inner membrane by the OXA machinery (Fig. 1) [247]. This conservative insertion pathway is mediated by Oxa1 and Cox18, which constitute protein translocation export pores in the inner membrane [248–251]. Both proteins are homologous to YidC, which is also required for membrane protein insertion in bacteria [252,253]. For the import and insertion of mitochondrial inner membrane proteins with an N-terminal presequence, two conflicting models were proposed. In the 'conservative sorting' model it was assumed that the inner membrane proteins synthesized in the cytosol are first imported into the mitochondrial matrix and only subsequently reexported into the inner membrane. This is well established for the Oxa1 itself [254]. In contrast, the vast majority of the inner membrane proteins with N-terminal presequence, including respiratory chain subunits and assembly factors, are imported with the help of the TOM complex and are laterally released into the inner membrane by the TIM23 complex [255]. The other respiratory chain subunits and assembly factors, which are not membrane integral, are either localized in the mitochondrial matrix or in the intermembrane space. Matrix import is achieved by the presequence translocase with the help of its associated import motor. Transport to the intermembrane space is mediated by inner membrane import and subsequent proteolytic release into the intermembrane space by cleavage through the inner membrane peptidase (IMP) [256,257]. Alternatively intermembrane space proteins are directly imported through the TOM complex and either trapped through complex formation, cofactor insertion or oxidative folding by the MIA pathway [164,165,258]. Mutations in the human MIA pathway ortholog of Erv1 cause myopathy and combined respiratory chain deficiency, indicating the importance of this import pathway for the biogenesis of the respiratory chain [259]. Another twist was added by Wagener et al. (2011), proposing a molecular function for the AAA-ATPase Bcs1. The Rieske iron–sulfur apo-protein (Rip1) is first imported into the mitochondrial matrix, where it acquires its special iron–sulfur cluster [3]. The C-terminal holo domain is subsequently exported from the matrix into the intermembrane space. This process seems to be mediated by Bcs1, which is presumably also required for lateral release of the transmembrane segment [260,261]. The fact that mutations in the human BCS1 gene cause complex III deficiency underlines the importance of this special biogenesis pathway for the respiratory chain [262].

8. Conclusions

Multiple evolutionary, functional and structural connections have been observed between the mitochondrial import machineries and the respiratory chain. The subunits Sdh3/Tim18 of the carrier translocase are identical/homologous to the membrane integral module Sdh3/Sdh4 of the succinate dehydrogenase (complex II) of the respiratory chain [219]. Additionally the core proteins of the cytochrome bc_1 complex (III) are homologous to the mitochondrial processing peptidase (MPP) [133–136]. Since the respiratory chain derives from the endosymbiont, it is evident, that the different protein import pathways into mitochondria developed afterwards. These homologies indicate evolutionary connections between the mitochondrial protein import pathways and the respiratory chain. A functional connection was observed for the MIA-dependent oxidative folding in the intermembrane space and the respiratory chain. Electrons released by precursor protein oxidation can be donated from the sulfhydryloxidase Erv1 via cytochrome *c* to the respiratory chain [184–188]. In addition, a physical connection exists between the presequence translocase (TIM23) and the proton pumping complexes of the respiratory chain [114–116,125]. Since all subunits of the respiratory chain have to be imported or exported in order to assemble the respiratory chain, a physical connection between the translocase and the respiratory chain complexes may be beneficial for efficient assembly of the precursor proteins to ensure proper maintenance and function of the respiratory chain. On the other hand the protein translocases in the inner membrane require the membrane potential. It is likely that links to the respiratory chain create a strong local membrane potential to facilitate efficient matrix protein import and inner membrane protein insertion [117–120].

The dependence of the mitochondrial import system on the membrane potential generated by the respiratory chain is also used to regulate nuclear expression of mitochondrial genes and mitochondrial degradation. In order to activate the mitochondrial unfolded protein response *Caenorhabditis elegans* has a transcription factor called ATFS-1, which not only contains a nuclear localization signal, but also a mitochondrial presequence. ATFS-1 is usually imported into mitochondria and subsequently degraded. However, in case the mitochondrial import is not sufficient, ATFS-1 is also imported into the nucleus to activate the transcription of specific mitochondrial genes like chaperones and translocase subunits to improve mitochondrial function [263]. Also the PTEN-induced putative kinase 1 (PINK1) is usually imported into mammalian mitochondria and subsequently degraded [264,265]. However, in case the membrane potential is diminished, PINK1 accumulates at the TOM complex and recruits Parkin to induce the organelle specific degradation by autophagy [266,267]. This PINK1/Parkin pathway seems to be conserved in metazoans and dysfunction of PINK1 causes Parkinson's disease [268,269]. Both examples highlight the important interdependence between the mitochondrial import system and the respiratory chain.

In summary subunits and assembly factors of the respiratory chain are imported via all known import pathways for targeting to the intermembrane space, the inner membrane and the matrix. In addition all internal mitochondrial import machineries are either functionally linked to the respiratory chain or contain homologous subunits. This underlines the important connection between mitochondrial protein import and the respiratory chain and suggests even more interconnected functions to be discovered.

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