The Tightly Regulated and Compartmentalised Import, Sorting and Folding of Mitochondrial Proteins

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Abstract: Mitochondria are eukaryotic intracellular organelles that still bear the signatures of their prokaryotic ancestor and require nuclear assistance. They generously dispense energy to cells, but are also involved in several biosynthetic processes, as well as in cell signalling pathways and programmed cell death.

Mitochondria are partitioned into four intra-organelle compartments: the outer membrane, the inner membrane, the intermembrane space and the matrix. Each compartment contains a unique set of proteins and a personalised system for guaranteeing protein homeostasis.

What follows is a survey of the function and topology of the multiple systems that operate the concerted action of protein sorting and folding in the four mitochondrial compartments.

Keywords: Mitochondrial proteins, mitochondrial protein folding, mitochondrial compartments, mitochondrial protein sorting.

1. INTRODUCTION

Mitochondria are organelles that contribute essential functions to cellular metabolism. They are the energy power-houses of the cell, as they are the major sites of ATP production, which in turn derives from oxidative phosphorylation; moreover, they are involved in other metabolic processes, such as the biosynthesis of amino acids, vitamin cofactors, fatty acids, and iron-sulfur clusters. Additionally, they play an important role in cell signalling pathways and in programmed cell death [1, 2].

Mitochondria are peculiar organelles because they are surrounded by a double membrane system that forms four intra-organelle compartments: the Outer Mitochondrial Membrane (OMM), the Inner Mitochondrial Membrane (IMM), the InterMembrane Space (IMS) and the Mitochondrial Matrix (MM). Each membrane contains a unique set of proteins that defines the specific functions of that membrane, and each mitochondrial compartment has specific features. In addition, these organelles bear their own genome, the mitochondrial DNA (mtDNA), and their own translational machinery; both features recall mitochondria's ancient bacterial origin.

The mitochondrial genome encodes only a small portion of mitochondrial proteins. They are subunits of the respiratory chain complexes, which are translated by mitochondrial ribosomes and inserted by dedicated machinery into the IMM. In mammalian mitochondria, for instance, 13 subunits of the respiratory chain complexes are encoded by mtDNA [3]. The vast majority of mitochondrial proteins, including those of the mitochondrial membranes, are by contrast nuclear-encoded, and synthesised as precursor proteins in the cytosol. Subsequently, they are targeted to the mitochondria and sorted to the correct sub-mitochondrial destination. Therefore, maintenance of mitochondrial activities depends on the coordinated expression of the two genomes. Research has identified a number of regulatory circuits that allow the adjustment of nuclear gene expression to specific demands within mitochondria and thus ensure a balanced accumulation of nuclear- and mitochondrial-encoded proteins [4, 5].

The biogenesis of mitochondrial proteins relies on several coordinated steps: protein synthesis in the cytosol, targeting of the precursor protein toward the organelle, protein import, sorting in the appropriate compartment and folding into the native protein conformation. Chaperone proteins and translocases participate and assist proteins during their folding and routing to the final destination [6, 7]. This review offers a detailed analysis on the mechanisms of mitochondrial protein folding with respect to their subcompartment localisation, and gives particular emphasis to the coupled-mechanisms of import and folding. The introductory sections recapitulate the key steps from mitochondrial protein synthesis in the cytosol to import within the organelle. The main body of the review describes mitrochondrial protein sorting and folding relative to each mitochondrial subcompartment. In the interests of a comprehensive overview of mitochondrial protein homeostasis, we conclude with two sections that respectively regard a) those organellar systems that are devoted to the surveillance of protein quality control, and b) recent findings on mitochondrial unfolded protein response.

1.1 Mitochondrial Protein Synthesis and Import

Nuclear-encoded mitochondrial proteins are translated by cytosolic ribosomes as preproteins and then imported into the organelle. Targeting signals on precursor proteins are necessary and sufficient both to direct the proteins to mitochondria and to determine their import. Most targeting signals are contained within N-terminal segments (prese-

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quences) and are cleaved upon import into the mitochondria. Since they direct preproteins, at least partially, into the matrix space, presequences are also called matrix-targeting sequences/signals. Many preproteins, on the other hand, contain targeting sequences that do not reside at the N-terminus but more internally in the proteins [6, 8].

Most mitochondrial preproteins are released from the ribosomes as completed chains, but some start importation into the organelle while translation is still ongoing. Preproteins that are released in the cytosol are generally more loosely folded than are their mature counterparts, and they may lose translocation competence, undergo aggregation or be degraded by cellular proteases. There are cytosolic chaperones that can prevent these adverse reactions. The requirement for such factors is not general, because some purified preproteins are efficiently imported into isolated mitochondria *in vitro*.

A number of cytosolic components have been reported as interacting with nascent polypeptide chains, *i.e.* even before they are released from the ribosome, to mediate stabilisation and (partial) folding [6, 9]. In mammals, the cytosolic chaperone Heat Shock Proteins (Hsp) Hsp90 and Hsp70 dock onto a subunit of the Translocase of the Outer mitochondrial Membrane (TOM) complex, the Tom70 import receptor. This interaction allows the delivery of a set of preproteins to the receptor for subsequent membrane translocation. This is a novel mechanism in which chaperones are recruited for a specific targeting event by a membrane-bound Tom70 receptor that acts as a membrane-localised co-chaperone, one that integrates the Hsp70/Hsp90 chaperones with mitochondrial preprotein targeting and translocation [10]. In yeast, Hsp70 but not Hsp90 is involved in mitochondrial import, and Hsp70 docking is required for the formation of a productive preprotein/Tom70 complex [10].

The described mechanisms refer to proteins that are post-translationally imported into mitochondria. Interestingly, translocation could also occur in a co-translational fashion, on the basis that preproteins have N-terminal targeting signals that direct them into the organelle. The example of fumarase import into mitochondria while the ribosome is still attached to the nascent chain has been described [11].

Once mitochondrial preproteins have been synthesised in the cytosol, they have to be recognised by receptors on the surface, or inside, mitochondria. These receptors are translocases that localise in the outer and inner mitochondrial membrane of mitochondria. They are membrane-embedded protein complexes that mediate the translocation of polypeptides from one side of the membrane to the other and their subsequent sorting. These processes rely on ATP and mitochondrial membrane potential ($\Delta\psi_m$) for the necessary energy and on several chaperone proteins and co-factors for the folding and assembly of the proteins into their native conformation. The first component of the import pathways is the TOM complex. Other major components of the system are the Translocase of the Inner Membrane 23 (TIM23), which mediates translocation of preproteins across and into the IMM (the TIM23 complex is also termed inner membrane presequence translocase) and the Translocase of the

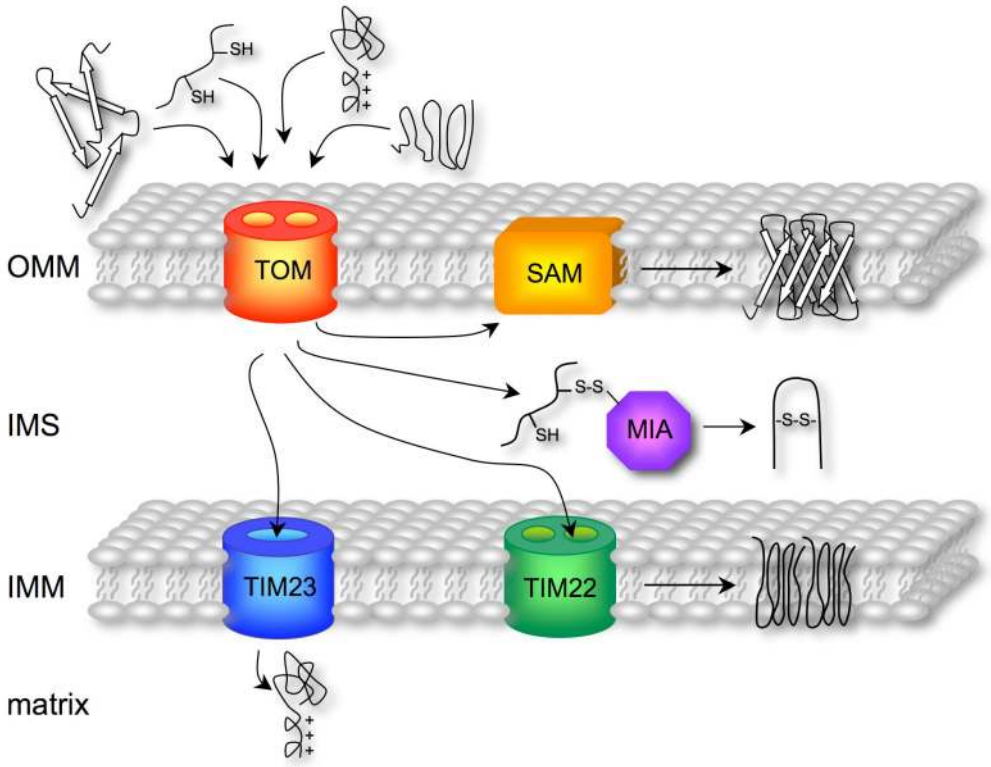

Fig. (1). Main protein import and sorting pathways in mitochondria. Nuclear-encoded mitochondrial proteins enter the organelle through the TOM complex. Outer membrane proteins with a β-barrel topology are inserted into the OMM by the SAM complex. Proteins containing characteristic cysteine motifs are sorted to the MIA machinery in the IMS. Precursors with a mitochondrial targeting presequence are transferred to the TIM23 complex, whereas carrier proteins are inserted into the inner membrane by the TIM22 complex.

Inner Membrane 22 (TIM22), which is also known as the inner membrane carrier translocase (Fig. **1**).

The TOM complex provides an entry gate into the mitochondria for virtually all mitochondrial precursor proteins studied to date. Once precursors have passed through the TOM complex, import pathways and precursor entry separate into specific biogenesis routes that enable their delivery to the correct mitochondrial sub-compartment. Specific targeting sequences at the N-terminus or within the polypeptide chain drive the precursors to their sorting route. In brief, outer membrane proteins with a transmembrane β-barrel topology and a specific internal targeting signal are transferred from the TOM complex to the Sorting and Assembly Machinery (SAM) complex on the intermembrane space side of the OMM; this transfer is assisted by soluble chaperone complexes of the IMS (small Tim proteins). Precursor proteins containing characteristic cysteine motifs are sorted to the IMS by means of the Mitochondrial Intermembrane space Assembly (MIA) machinery, which catalyses the formation of disulfide bonds and their attachment to precursor proteins, and additionally determines the IMS import pathway. Polytopic inner membrane proteins with internal targeting elements are transferred from the TOM complex *via* small Tim proteins to TIM22 translocase for integration into the IMM. Finally, presequence-containing precursor proteins are directly transferred from the TOM complex to the inner membrane TIM23 complex (Fig. **1**) (for reviews, see [6, 7, 12, 13]). Details on each of these sorting options will be introduced in the following sections.

1.2 The TOM Complex: The Mitochondrial Entry Gate

Precursors of mitochondrial proteins enter the organelle through the TOM complex, the starting point of all mitochondrial sorting routes.

This complex consists of 8 subunits and involves a total of 7 different proteins (Fig. **2**). Tom20, Tom22 and Tom70 function as receptors for the various classes of mitochondrial precursor proteins. Tom40, the core of the complex, forms the protein-conducting channel across the outer membrane. The small Tom protein Tom5 supports the insertion of precursor proteins into the Tom40 channel, while two other small Tom proteins, Tom6 and Tom7, influence the stability of the TOM complex. The precursors of all Tom proteins are recognised by pre-assembled TOM complexes in the OMM [14-17]. The driving force for translocation through the TOM complex does not come from ATP or $\Delta\psi_m$ across the IMM. According to the binding-chain hypothesis, the translocating preproteins interact with several binding sites of increasing affinity. The sequence of events starts at the *cis*-binding site in the cytosolic region of the TOM complex, and ends at the *trans*-binding site on the IMS-exposed region of the TOM complex. The *trans*-binding site is thought to hold the preproteins until further sorting to the mitochondrial subcompartments takes place [18].

2. IMPORT AND FOLDING OF OUTER MEMBRANE PROTEINS

The mitochondrial outer membrane represents the first barrier that newly synthesised proteins have to pass to reach their final destination within mitochondria; it is also one of the compartments of the organelle. There are resident proteins that are imported and targeted to this location (for reviews, see [12, 19]). These proteins can be divided into two main classes: β-barrel proteins and α-helix-containing proteins. The mechanisms whereby these proteins are imported to and inserted in the OMM, as well as the macromolecular complexes that participate in these processes, are described in the following sections.

2.1 β-Barrel Proteins

β-Barrel membrane proteins consist of amphipathic, antiparallel β-strands that are connected by loops of varying size; thus arranged, the β-strands form cylindrical, barrel-like structures that cross the bilayer. The number of β-strands varies from 8 to 22, but it is invariably an even number. On average, a β-strand consists of 8–11 amino acid residues, a number that is sufficient to span a biological membrane.

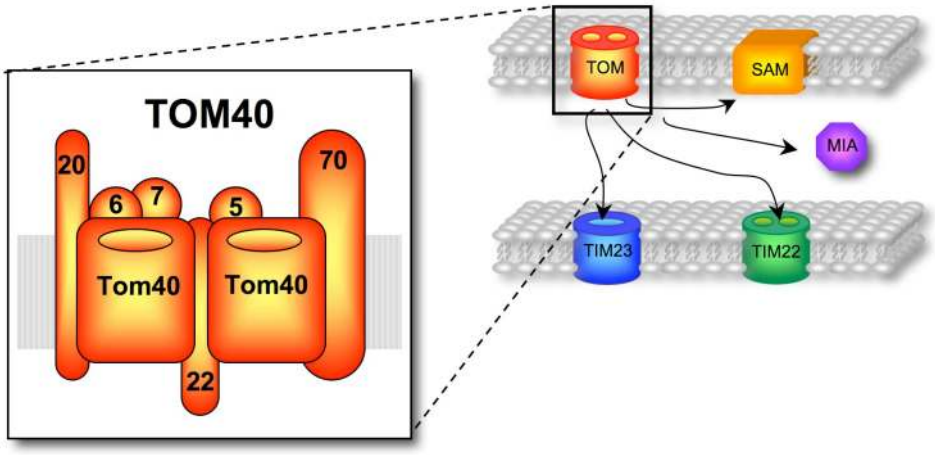

Fig. (2). Composition of the TOM complex. Eight subunits are present in the complex. Tom40 is the protein-conducting channel. Tom20, Tom22 and Tom70 act as receptors for precursor proteins. Tom5 participates in the insertion of precursors into the Tom40 channel, while Tom6 and Tom7 stabilise the whole TOM complex.

The presence of these proteins in the OMM is believed to reflect the bacterial origin of mitochondria, since β-barrel membrane proteins are exclusively found in the outer membrane of bacteria and in the outer membrane of the two endosymbiotic derived organelles, plastids and mitochondria. Interestingly, a recent study demonstrates that bacterial outer membrane proteins can be properly assembled within the OMM. This finding supports the notion that β-barrel protein assembly is conserved from bacteria to eukaryotic cell organelles of endosymbiotic origin [20]. In bacteria, β-barrel proteins act as metabolite and protein transporters, as receptors, and as enzymes or virulence factors. In eukaryotes, the mitochondrial proteins that adopt a β-barrel topology are porin, which is also termed Voltage-Dependent Anion-selective Channel (VDAC), Tom40, Sam50, Mitochondrial Distribution and Morphology 10 protein (Mdm10) and Mdm34 [21-23].

β-Barrel protein import and sorting begins with the interaction between the precursor protein and the TOM complex. Precursor outer membrane proteins are recognised by Tom receptors on the mitochondrial surface and translocated through Tom40 channel to the IMS. Here, intermembrane space components are involved in the transfer of the β-barrel precursors to the OMM [24, 25]. These components are the small Tim proteins, which are known also for their role in transfer inner membrane precursors from the TOM complex to the TIM22 complex. Small Tim proteins, *i.e.* the essential Tim9-Tim10 complex and the non-essential Tim8-Tim13, exert chaperone-like functions by shielding hydrophobic segments of preproteins against aggregation in the aqueous IMS and thus keeping β-barrel precursors in a competent state for insertion into the OMM by a second outer membrane complex. This is named the Sorting and Assembly Machinery (SAM) or the Topogenesis of mitochondrial Outer membrane β-Barrel complex (TOB) (Fig. **3**) [22, 23].

The SAM complex comprises three subunits: the Sam50 protein (also named Tob55), which is itself a β-barrel protein, and two hydrophilic subunits, Sam35 (Tob38) and Sam37 (Mas37), which are located on the cytosolic surface of the OMM. Sam50 is the core component of the SAM complex and consists of two domains. The N-terminal hydrophilic region is exposed to the IMS and forms a characteristic structure called the POlypeptide TRanslocation Associated domain (the POTRA domain); the C-terminal domain forms a β-barrel domain, with what are presumed to be 14-16 transmembrane β sheets [8]. In yeast, Sam50 and Sam35 are essential for cell viability, whereas the loss of Sam37 is only lethal at high temperatures. Upon purification of the SAM complex, an additional β-barrel protein, Mdm10, was found to be a fourth component of the complex [26]. This protein had previously been identified by its role in the maintenance of mitochondrial distribution and morphology. It has now been demonstrated that Mdm10 plays a specific role in the assembly of the TOM complex, while the three subunits of the SAM core complex are required for the biogenesis of all OMM β-barrel proteins.

SAM machinery has been studied mainly in yeast, but it is conserved in higher eukaryotes. Human Sam50 has been

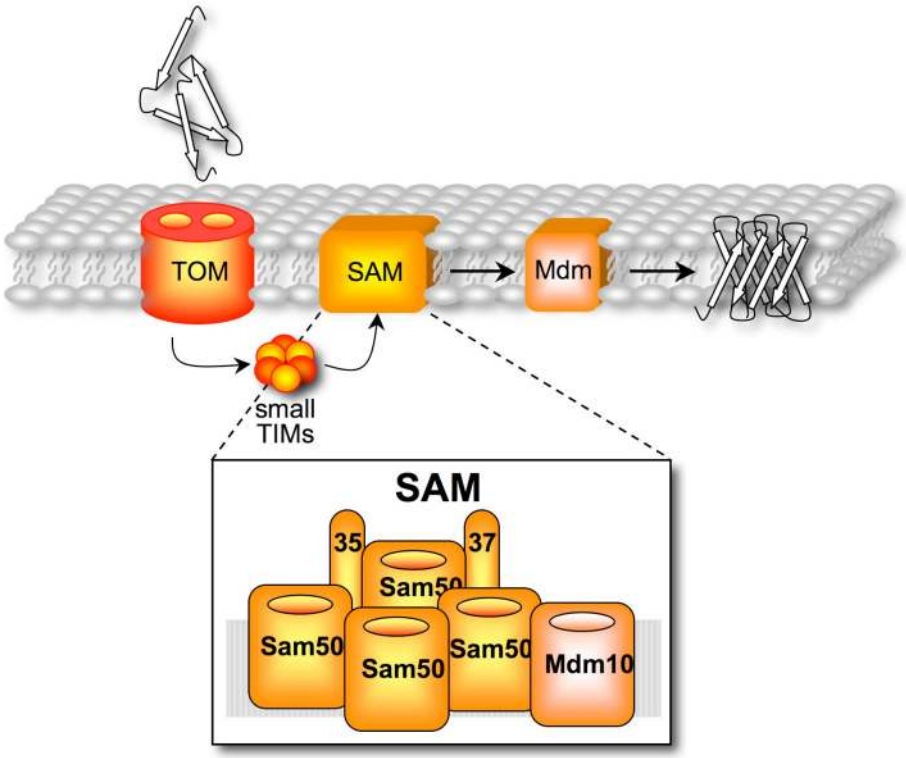

Fig. (3). Import and folding of β-barrel proteins in the outer membrane. The TOM complex translocates precursor proteins into the IMS, where they interact with small TIM proteins that pass the precursors to the SAM complex. Along with Mdm proteins, this second complex is involved in protein folding and insertion into the OMM. The SAM complex is built of four differing subunits: Sam50 and Mdm10 are β-barrel components, while Sam35 and Sam37 are hydrophobic subunits associated with the cytosolic side of the outer membrane.

identified and specified as a requisite for the biogenesis of Tom40; in addition, metaxin1 and metaxin2 have been identified as respective human homologues of Sam37 and Sam35 [27, 28].

2.2 The β-Signal for Protein Sorting

One of the most interesting and least resolved questions is how the SAM complex mediates insertion into the OMM and the folding of proteins into their native conformation. Recent studies have added significantly to our understanding of the given processes. It has been shown that the N-terminal portion of Sam50, the β-barrel component of the SAM complex, contributes to the translocation of β-barrel precursors across the TOM complex, as well as to their interaction with the SAM complex [8]. It has recently been demonstrated that deletion of the entire N-terminal domain of Sam50 inhibits neither yeast cell growth nor precursor binding to SAM, a finding which denies the notion that the POTRA domain is crucial for the recognition of β-barrel precursors [29]. In addition, the same authors identified a conserved sequence located in the last predicted transmembrane β-strand. The characteristic features of this consensus sequence, termed β-signal, are a large polar residue (Po), in which lysine or glutamine predominate, an invariant glycine and two large hydrophobic residues (Hy) (motif: xxx-Po-x-G-xx-Hy-x-Hy-x). Studies on the biogenesis of Tom40 β-barrel protein reveal that the four conserved residues of the β-signal are involved in distinct steps of β-barrel protein insertion into the membrane. The three large residues (the polar and the hydrophobic residues) are required for protein sorting to the SAM complex, while the subsequent membrane insertion depends not only on the β-signal but also on other regions of Tom40. The conserved glycine residues in β-strands of Tom40 are required for membrane insertion. This β-signal is necessary and sufficient for selective recognition by the SAM complex, and more specifically by the core complex formed by Sam50 and Sam35. In the presence of Sam35, the β-signal induces a change in the gating properties of the SAM channel and a significant increase in the channel conductance of the SAM complex. As a result of signal-induced enlargement, the SAM channel can accommodate several β-strands. This fits with the view that β-barrel precursors are not inserted into membranes as individual strands but contain a considerable amount of partially folded elements [17, 29].

These experimental findings indicate that Sam35 is essential for the recognition of the last β-strand of the precursor; interestingly, Sam35 is not integrated into the lipid phase of the OMM, but it is embedded into a proteinaceous membrane environment by its close association with Sam50 molecules. The hypothesis has accordingly been advanced that the translocation of β-barrel precursors into SAM is initiated by the binding of the last β-strand (containing the β-signal) to the hydrophilic, and predominantly α-helical Sam35, that is located in an oligomeric ring formed by the β-barrel domains of Sam50 molecules. The binding signal induces a conformational change that leads to the opening of the SAM channel, and thus several β-strands can be inserted into a hydrophilic, proteinaceous membrane environment. Subsequently, the precursor is laterally released from the SAM complex into the lipid phase of the OMM. Membrane integration strictly requires highly conserved glycine residues in the β-signal and additional β-strands. Since glycine residues provide the polypeptide chain with high flexibility, this step seems to involve conformational changes in the precursor to ensure correct integration into the lipid phase [29]. The role of Sam37 in β-barrel protein biogenesis is less defined, but recent data suggest that it is involved in the release of SAM-bound precursor proteins in the membrane and that it might play a stabilising role in the SAM complex [30].

2.3 Folding and Insertion of the β-Barrel Structure into the Outer Membrane

The forementioned findings begin to reveal the role of the SAM complex in β-barrel protein insertion into the membrane, but it does not clarify how Sam50 participates in the folding of β-barrel proteins.

Electron microscopy of purified Sam50 has revealed that it forms ring-shaped assemblies large enough to accommodate β-barrel substrate proteins [22]. This finding has led to the suggestion that Sam50 possibly aids polypeptide folding by the formation of an Anfinsen-type cage, analogously to the role played by the molecular chaperone GroEL (which in turn is the bacterial homologue of eukaryotic hsp60). However, since Sam50 is predicted to be a β-barrel membrane protein itself, the lateral release of folded substrates from the cage and into the membrane would require large structural rearrangements in the β-strands of Sam50, as well as the disruption of many hydrogen bonds. An alternative, hypothetical mechanism, involving the sequential insertion of β-strands into the membrane as the substrate folds, also poses problems, as the exposure of many open hydrogen bonds to the lipid bilayer would be energetically unfavourable. Arguably, this exposure could be plausible if evidence existed that open hydrogen bonds were transiently paired with polar groups in such surface patches within the SAM complex as are exposed upon substrate binding. Finally, the SAM complex might also be involved in the recruitment of certain lipids to assist in the protein folding of β-barrel substrates [31].

Studies *in vitro* have also shown that isolated and denatured β-barrel proteins can refold and insert into artificial lipid bilayers, with the probable involvement of several insertion intermediates, but without further assistance from other proteins. The spontaneous integration into hydrophobic lipid bilayers might be driven by the formation of the β-barrel structure upon membrane insertion, whereby all β-hairpins (the structures formed by two β-strands that are adjacent in primary structure and are oriented in an antiparallel arrangement) are inserted simultaneously, in a concerted manner. However, the kinetics of insertion into the artificial lipid bilayers is much slower than is insertion *in vivo,* and specific insertion into the OMM strengthens the case for some form of assistance. Accordingly, one possible function of Sam50 might be that of triggering an insertion-competent β-barrel precursor conformation, with Sam50 that would serve as a process scaffold. In this scenario, preformed β-hairpins would be inserted at the interface of the Sam50 β-barrel and the lipid phase of the OMM [18].

Additional experimental findings on membrane insertion and eukaryotic β-barrel membrane protein folding into lipid bilayer membranes have derived from studies on the human VDAC isoform 1 (hVDAC1) of the outer membrane of mitochondria [32].

This protein forms a transmembrane β-barrel with an additional N-terminal α-helix in the OMM. It has been demonstrated that protein folding and membrane insertion into lipid bilayers of phosphatidylcholines occurs spontaneously, and that native structure formation requires neither proteinaceous folding machinery nor an energy supply. Indeed, in aqueous solution hVDAC1 protein can organise its topology into a structure that comprises several β-sheets, while in lipid bilayers an α-helical structure can also form upon hVDAC1 folding. Moreover, a lipid bilayer is required for the formation of correctly inserted, folded, and stable hVDAC1 [32].

Although ATP is present in the IMS, mitochondrial hVDAC1 folding and insertion occur spontaneously and resemble the folding of bacterial outer membrane proteins. Eukaryotes inherit this mechanism from bacteria, and indeed there is no ATP in the periplasm of bacteria; accordingly bacterial β-barrel proteins have to fold and to be inserted into the membrane without additional energy supply. The gain in free energy derived from the formation of the native folded protein is sufficient to drive folding and membrane insertion. While phosphatidylcholine is a major lipid component (~50%) of the mitochondrial outer membrane, the physical properties of the OMM possibly differ, so as to prevent spontaneous insertion of proteins into the membrane and to provide a selection mechanism for specific membrane protein insertion. For this reason, proteinaceous machinery may be required for *in vivo* membrane insertion of mitochondrial outer membrane proteins.

On the basis of these findings, membrane-embedded or membrane-associated folding assistance can be said to provide a specific targeting mechanism for OMM proteins or to displace the chaperones needed to maintain OMM protein solubility in the IMS, possibly by altering the physical properties of the membrane in the direct vicinity of the given proteins so as to allow the latter's transmembrane insertion [32].

2.4 α-Helix Containing Proteins

Mitochondrial outer membrane hosts a class of proteins with β-barrel topology and proteins that cross the membrane because of the occurrence of an α-helix at the N- or C-terminal part of the polypeptide.

The membrane-anchored α-helix is localised either at the N-terminus (signal-anchored) or at the C-terminus (C-tail anchored) of proteins with a single transmembrane region. Although analysis of the targeting of signal-anchored and C-tail-anchored proteins to the mitochondria has revealed targeting information within their transmembrane regions and in flanking positive residues, no consensus sequence is apparent. This class comprises the proteins Tom70 and Tom20, which are anchored in the outer membrane with a transmembrane helix close to their N-terminus. In addition, there are proteins with a pair of internal transmembrane domains; the yeast fuzzy onions homolog protein, Fzo1, is an example [33, 34].

The import, folding and membrane insertion of this class of proteins, and the role of the TOM and SAM complexes in these processes, have been clarified by studies conducted on α-helix containing Tom proteins.

Precursors of α-helix containing proteins do not use the outer membrane Tom receptors and the protein-conducting channel of the TOM complex. Nevertheless, it has now been proposed that these proteins need the TOM translocase, possibly because the complex can facilitate protein insertion at its protein-lipid interface. The precursors can interact with the exposed domain of Tom40 and be inserted into the interface between the outer face of TOM complex and the lipid phase of the OMM [33, 34]. The portions of the Tom40 molecule that are presumed to mediate the insertion of α-helix containing transmembrane proteins within the lipid bilayer are distinct from the segment that forms the general import channel, and thus obviate the need for any lateral opening of the barrel structure or major rearrangements of the TOM complex [34]. According to this model, matrix-targeted preproteins with mitochondrial targeting signals are translocated through the Tom40 channel, while α-helix containing OMM proteins interact with the TOM complex *via* a different route. Interestingly, it has been shown that matrix-destined preproteins do not compete with α-helix transmembrane precursors [33].

After interaction with the TOM complex, the folding and membrane insertion of α-helix containing proteins also rely on SAM complexes. All of the three SAM subunits are required for the efficient membrane integration of Tom22, a component of the TOM complex with its membrane anchor in the C-terminal half of the protein. In contrast, the small Tom proteins Tom5, Tom6 and Tom7, which are C-terminal membrane-anchored proteins, need Sam37 for their assembly into the TOM complex [35]. The final two components of the TOM complex, Tom20 and Tom70, are anchored to the OMM by a single transmembrane α-helix located at the N-terminus.

It has recently been shown that membrane insertion and assembly into the TOM complex requires the Mitochondrial IMport 1 protein (Mim1), a protein that is also involved in the assembly pathway of Tom40 but not of porin. Mim1 associates with a fraction of SAM core complexes, and acts in the assembly of signal-anchored Tom receptors and of the small Tom proteins. Mim1 promotes membrane insertion of Tom6 and participates in the late steps that associate Tom5 and Tom7 with Tom40 [36].

2.5 The Assembly of TOM Complex: An Example of β-Barrel Multi-Protein Structure

The most thorough studies of the assembly pathways of OMM protein complexes are those that regard the TOM complex (Fig. **2**).

The assembly proceeds in a sequence of reactions that involve several intermediates. The Tom40 precursor first crosses the OMM *via* the pre-existing TOM complex and, subsequently, associates with the SAM complex. After SAM-mediated membrane insertion, the Tom40 precursor forms a further intermediate, which probably consists in a

Tom40 dimer and an associated Tom5 subunit. This assembly intermediate could then function as a scaffold for the formation of the TOM core complex by recruiting Tom6, Tom7 and Tom22. These late steps in the assembly of Tom40 with α-helical Tom proteins are promoted by Mdm proteins. In particular, Mdm10, itself a β-barrel protein, critically promotes the association of Tom22 and the small Tom proteins with Tom40. The last protein that participates in Tom complex assembly is the small, integral outer-membrane protein Mim1 [37]. Mim1 promotes the assembly of the signal-anchored receptor subunits Tom20 and Tom70 with the TOM core complex to form the mature holo-complex [36, 38].

Little is known about the assembly of the SAM complex. Sam50, the β-barrel protein, needs pre-assembled SAM complexes in the outer membrane for its own biogenesis. Possibly, the Sam50 precursor is initially released into the OMM and subsequently assembles with Sam35 and Sam37 into new SAM complexes. Newly synthesised Sam37 was observed to bind to SAM core complexes directly on the cytosolic side of the outer membrane without the TOM complex being involved. Sam35 probably associates with Sam50 in a similar manner, because it is also peripherally attached to the SAM complex [39].

3. PROTEIN FOLDING IN THE INTERMEMBRANE SPACE

The mitochondrial intermembrane space is the space enclosed by the outer and the inner mitochondrial membrane. The IMS includes the lumen between the outer and the inner membrane and the space enclosed by the cristae membrane, which is also called intracristal space. These two subcompartments are separated by cristae junctions, sites that are visible on electron micrographs at the neck of the cristae [40]. Many mitochondrial proteins are localised within this compartment, including components of the electron-transport chain, enzymes for metabolism processes, transporters for polypeptides and several apoptotic factors, of which the latter are sequestered in the IMS until they are released to trigger the events that lead to programmed cell death. IMS proteins are all imported *via* the TOM complex and then follow two differing routes, according to their molecular properties. Intermebrane space proteins with a mitochondrial presequence are directed to TIM23, which sorts them into the IMM. Proteins that do not carry a presequence are imported into the IMS and are trapped in the compartment once they achieve their folded conformation.

IMS-resident proteins can be classified into three different groups, on the basis of their energy requirement and of the sorting pathway they follow to reach their final destination (for reviews, see [6, 41]). These groups comprise: 1) "bipartite" sorting-signal-bearing proteins; 2) energy-independent IMS-transported proteins; 3) low-molecular-weight proteins that undergo oxidative folding. Members of the latter group are oxidised in the IMS and remain trapped within the intermembrane space, on account of their folded conformation. This oxidative folding pathway, which is peculiar to IMS proteins, will be described in detail in Section 3.3.

3.1 IMS Proteins with a "Bipartite" Sorting Signal

The first class of proteins comprises soluble IMS proteins that contain the canonical mitochondrial presequence followed by a hydrophobic sorting domain. These two features collectively constitute a "bipartite" sorting signal, and direct the N-terminal part of the protein firstly to the TOM complex and subsequently to the TIM23 complex for import and sorting. The import of these proteins is driven by $\Delta\psi_m$ and ATP, which enable even large and partially folded proteins to be translocated across the OMM. The presequence contacts numerous binding sites of increasing affinity, from which it sources specificity and its major pulling force, until it reaches TIM23 complex within the IMM. The hydrophobic sorting domain is localised shortly after the presequence and allows protein insertion into the lipid bilayer; the polypeptide is then locked in the inner membrane by the so called "stop-transfer mechanism" [42]. The matrix-targeted presequence is then generally removed into the matrix, in a processing event that is catalysed by the Mitochondrial Processing Peptidase (MPP). Depending on the protein, the whole bipartite signal can be removed by proteolytic pro-cessing, which accordingly releases the mature protein into the IMS.

Two examples of this class of proteins are: Mitochondrial Genome Maintenance protein 1 (Mgm1) and Cytochrome *C* Peroxidase 1 (Ccp1).

The first protein is a yeast dynamin-like GTPase that regulates the morphology of the mitochondrial network. Its biogenesis depends on two cleavage events. Mgm1 is synthesised with a mitochondrial presequence that is proteolytically removed in the matrix. Import then proceeds until the hydrophobic sorting domain is exposed to the Processing of Cytochrome *c* Peroxidase protein 1 (Pcp1), a serine protease that belongs to the rhomboid superfamily of intramembrane-cleaving peptidases. This protease catalyses protein cleavage within the IMM and thus generates an IMS soluble form of Mgm1. Mgm1 sorting is complicated by a second hydrophobic domain upstream of the Pcp1 processing sequence. This sequence can block the import of the Mgm1 precursor and thus result in a fraction of Mgm1 that is N-terminally anchored to the IMM, together with a fraction of IMS-soluble Mgm1 [43].

The mammalian homologue of yeast Mgm1 is OPtic Atrophy 1 protein (OPA1), a well-known regulator of mitochondrial morphology [44]. Upon import into the IMM, this protein is initially processed by MPP, which removes the mitochondrial targeting sequence, and then by two IMM metalloproteases of the AAA family (AAA, ATPases Associated with a variety of cellular Activities), the *i*ntermembrane space-facing AAA complex (*i*-AAA) and the *m*atrix-facing AAA complex (*m*-AAA). These proteases generate short OPA1 forms that are still anchored to the IMM [45-47]. Moreover, it has recently been demonstrated that the mammalian homologue of Pcp1, the rhomboid protease Presenilin-Associated Rhomboid-Like protein (PARL), is involved in OPA1 cleavage and IMS-soluble OPA1 generation, as shown in yeast. Soluble OPA1, together with membrane-bound OPA1, is required for cristae junction maintenance and apoptosis protection [48, 49].

The second example of an IMS protein with bipartite sequence is the yeast protein Ccp1. It is synthesised with a presequence that is cleaved by *m*-AAA protease in the MM. In addition, the *m*-AAA protease cooperates with Pcp1 in the maturation of Ccp1. The *m*-AAA protease mediates the ATP-dependent vectorial membrane dislocation of Ccp1 (*i.e.* the dislocation of the transmembrane segment of the protein from the lipid bilayer towards the MM), an activity that is independent of *m*-AAA proteolytic activity. Such mediation ensures the correct positioning of Ccp1 within the membrane bilayer, and enables intramembrane cleavage by the rhomboid protease Pcp1 [50]. The Pcp1-processed Ccp1 is then release from the IMM as a soluble mature protein.

3.2 Energy-Independent IMS Transported Proteins

The second class of proteins localises in the intermembrane space through interaction with binding sites on the outer or inner membrane, or with resident membrane proteins. These proteins do not contain N-terminal presequences, and their import does not require $\Delta\psi_m$ or ATP hydrolysis. The most thoroughly studied members of this class of proteins are the heme lyases. They facilitate the covalent attachment of heme to the apoforms of *c*-type cytochromes in the IMS. The precursors of heme lyases are synthesised in the cytosol without the typical N-terminal mitochondrial targeting signal.

Studies on import and folding of the two heme lyases of the yeast, namely cytochrome *c* (cyt *c*) heme lyase and cyt c_1 heme lyase, indicate that both proteins enter mitochondria *via* the TOM complex. Import occurred independently of membrane potential across the IMM and ATP in the matrix space, a feature that suggests the inner membrane is not required for transport along this direct sorting pathway [51]. The formation of a large folded domain in heme lyases occurred at the same rate as that of import, which indicates that these proteins fold either during or immediately after their transfer across the OMM [51]. Folding was not affected by depletion of ATP and $\Delta\psi_m$ or by inhibitors of peptidylprolyl *cis-trans* isomerases, nor did it involve homologues of known folding factors, such as Hsp60 and Hsp70; this implies that protein folding in the IMS follows different principles from those established for other subcellular compartments [51].

Interestingly, the targeting signal of mitochondrial heme lyases has been identified [52]. It is a hydrophilic sequence that includes a large fraction of both positively and negatively charged amino acid residues located in the third quarter of the protein. When inserted into a cytosolic protein, this targeting sequence directs the fusion protein into the IMS, even in the absence of a membrane potential or of ATP hydrolysis. The heme lyase targeting sequence represents the first topogenic signal for energy-independent transport into the IMS, and harbours two types of information. It assures recognition and translocation by the TOM complex, and it is responsible for driving the import reaction by undergoing high-affinity interactions with as yet unknown components of the IMS [52].

3.3 The Erv1-Mia40 Disulfide Relay System

The intermembrane space of mitochondria harbours a large number of proteins that contain disulfide bonds, undergo oxidative folding and remain trapped within the intermembrane space on account of their folded conformation. These proteins are efficiently oxidised in the Mitochondrial Intermembrane space Assembly (MIA) pathway (Fig. **4**) [53].

The components of MIA machinery are Mia40, a thioredoxin-unrelated minimal oxidoreductase, and the Essential for Respiration and Vegetative growth 1 protein (Erv1), a Flavin Adenine Dinucleotide (FAD)-linked sulfhydryl

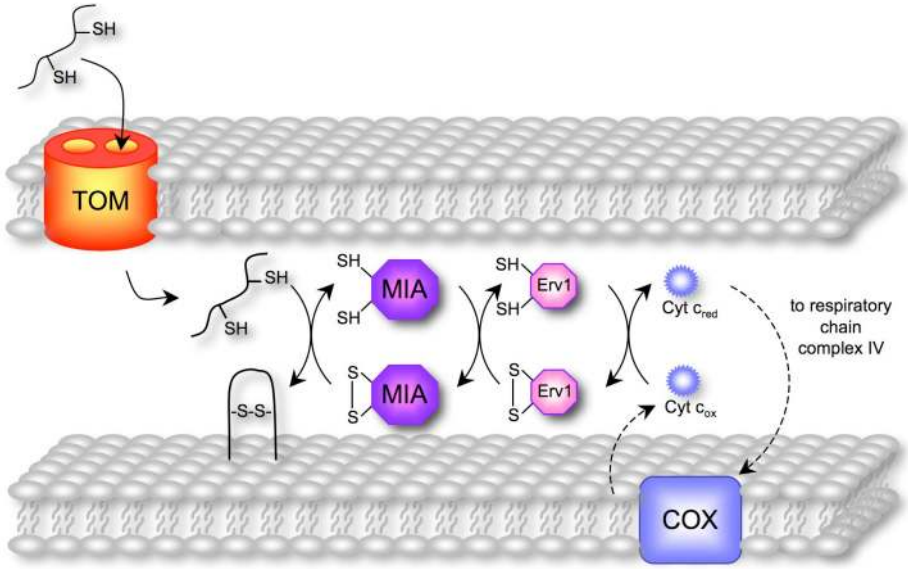

Fig. (4). Oxidative folding in the intermembrane space. Precursor proteins containing highly conserved cysteine residues are imported through the TOM complex in the IMS, where they undergo oxidative folding by Mia40. Mia40 is subsequently oxidised by Erv1, completing the relay system. Re-oxidation of Erv1 occurs by transfer of electrons to oxidised cytochrome *c*. The final product of these redox reactions is reduced cytochrome *c,* which in turn is re-oxidised by respiratory chain complex IV, cythocrome *c* oxidase (COX).

oxidase [54-56]. These proteins form a relay for disulfide formation in the precursor proteins.

Mia40 is the crucial oxidising component of this pathway, one that interacts early in import with the reduced unfolded precursor and indeed establishes a disulfide bond *via* a mixed disulfide intermediate. It functions as an import receptor that interacts transiently with newly imported polypeptides by converting them from a reduced to an oxidised and stably folded state. During this interaction, Mia40 is reduced but subsequently re-oxidised by Erv1, which participates in the oxidoreductase cascade without direct interaction with the precursor protein [53]. The electron acceptor of Erv1 is cyt *c*, which shuttles electrons onto cyt *c* oxidase and molecular oxygen, and thus provides a molecular link between the disulfide relay system and the respiratory chain [57-59]. It has been shown that Erv1 *in vitro* is able to pass its electrons directly onto molecular oxygen, thereby giving rise to the production of hydrogen peroxide [56]. The interaction of the disulfide relay system with the respiratory chain can thus prevent hydrogen peroxide production, which can be especially critical for higher eukaryotes.

Overall, the disulfide cascade in mitochondria involves a relay from Erv1 onto Mia40 and thence to the incoming precursor. In this oxidative folding pathway, the electrons pass from Mia40 to Erv1 and finally to *cyt c,* thus connecting oxidative folding with the respiratory chain as the final source of energy.

This pathway resembles the reaction cascade of the oxidative folding process in the Endoplasmic Reticulum (ER), and involves ER Oxidoreductin 1 (Ero1), Erv2 protein and Protein Disulfide Isomerase (PDI). In this system, PDI is the essential enzyme that catalyses both the oxidation of free thiols and the isomerisation of existing disulfides; Ero1 specifically oxidises PDI, which in turn passes the oxidising equivalent to folding substrates. Erv2 is an additional source of oxidising equivalents for PDI, independently of Ero1 [60].

The mitochondrial protein that relates functionally to PDI is Mia40, the catalyst of oxidative folding. Interestingly, yeast Mia40 is synthesised as a large protein of 45 kDa with an N-terminal presequence, it is imported through the TOM and the TIM23 complexes, and it localises within the IMM with the IMS-facing C-terminal domain ($Mia40_{CORE}$). In contrast, human Mia40 is a small protein of 16 kDa that lacks a presequence; it is a soluble IMS protein that consists exclusively of the C-terminal domain (homologous, or rather, orthologous with $Mia40_{CORE}$), and it is sorted to the MIA pathway [54, 61, 62].

The molecular mechanisms of Mia40-dependent oxidative folding have been clarified. Mia40 belongs to a protein family that contains six cysteine residues completely conserved among species, which constitute a CPC-Cx_9C-Cx_9C motif [63]. It introduces disulfide bonds into precursor substrates after the latter have crossed the OMM.

The N-terminal portion is the functional site of the molecule and the CPC motif forms the active center. This exclusive Mia40 motif is accessible to the solvent and its position enhances directness and ease of disulfide bond transfer to the substrate. The CPC motif functions as a redox-active site; since the second C (Cys55) hosts catalysis, the site shuttles between the oxidised and reduced states upon binding to the substrate, without structurally affecting the rest of the Mia40 molecule. The CPC segment mediates reactions with the substrate proteins and with the thiol oxidase Erv1. This motif protrudes into the solvent from a hydrophobic protein surface that is formed by numerous hydrophobic and aromatic residues, all of which are strictly conserved. This characteristic hydrophobic cleft acts as a substrate recognition and binding site; it stabilises the initial non-covalent interactions that so position the partially folded substrates as to enable the formation of the first mixed disulfide bond [64].

The cysteine residues of the twin Cx_9C motif play a structural role and stabilise Mia40. In particular, the disulfide bond formed by the third and the sixth cysteine residues appear to support a crucial functional conformation of the protein [65]. Since there is no evidence of a protein exerting disulfide-isomerase activity in the IMS, it seems possible that isomerase activity is dispensable in this oxidative pathway. Therefore, Mia40 activity *via* the CPC motif is deemed necessary and sufficient for the oxidation of its substrates [64].

Oxidative folding of the IMS proteins is also a crucial step for their import into this compartment. The import of precursor proteins is independent of both ATP and $\Delta\psi_m$. Interaction with the TOM complex (in particular, Tom5 and Tom40), and folding of the precursor in the IMS, are the bases for that this import is unidirectional. Studies on the import of small Tim proteins have demonstrated that both Mia40 and Erv1 are required for Tim protein oxidation. In particular, reduced Tim precursors bind to Mia40 during translocation into mitochondria, but they can diffuse back into cytosol, whereas the oxidised precursors are retained in the IMS and then assembled into oligomeric small Tim complexes. This indicates that protein oxidative folding determines vectorial transport of the precursors into the IMS [66].

3.4 IMS Proteins that Undergo Oxidative Folding

Although the literature has already identified a substantial set of disulfide relay substrates, the number of IMS proteins known to contain disulfide bonds is growing [67]. Typical substrates are characterised by one of two motifs of highly conserved cysteines: the twin Cx_3C motif, which occurs in the family of small Tim proteins, Tim8, Tim9, Tim10, Tim12 and Tim13 in yeast; and the twin Cx_9C motif that occurs in several IMS proteins, including the copper chaperone Cytochrome *C* Oxidase 17 (Cox17) and indeed Mia40 [63, 68, 69].

Oxidative folding of small Tim proteins has been well characterised. These proteins function as chaperones in the IMS during hydrophobic protein transit to the outer or inner membrane. The two Cx_3C motifs are juxtaposed in anti-parallel α-helices and linked by two disulfide bonds to form a hairpin-like structure [68, 70]. Studies on the biogenesis of the essential Tim9-Tim10 chaperone complex have shown that they form a hexameric complex with two intramolecular disulfide bonds in the Tim9 and Tim10 subunits. Each of the four cysteine residues is required for assembly of the subunit in the Tim9-Tim10 complex, but only the most N-terminal

cysteine of each precursor is critical for translocation across the OMM and for interaction with the Mia40 protein [71].

The twin Cx_3C is a zinc finger motif that can bind zinc in the cysteine-reduced form at a molar ratio of 1: 1. Interestingly, it has been shown that zinc plays a thiol-stabilising role, thus preventing oxidative folding of small Tim proteins in the cytoplasm, and maintaining the proteins in an import-competent state. It is likely that the zinc-linked protein cannot be imported directly into mitochondria, but it is currently unknown how the equilibrium between the apo- and the zinc- bound forms of small Tim proteins is mediated [72]. On the other hand, zinc is an inhibitor of Mia40 and Erv1 activities, and accordingly has to be removed during the process of oxidative folding. This inhibition could be overcome by the activity of a third participant in the biogenesis of small Tim proteins: the soluble IMS protein, Helper Of Tim of 13 kDa (Hot13). Hot13 is a conserved zinc-binding protein that interacts functionally and physically with Mia40, thus facilitating Mia40 demetallation and improving Erv1-dependent Mia40 oxidation [73].

There are additional substrates of the disulfide relay system that do not contain a twin $Cx_{3/9}C$ motif. A notable example is Erv1 itself, which contains six cysteine residues and two disulfide-bonded Cx_2C segments [74]. Another example is SuperOxide Dismutase 1 (Sod1), which protects the cell against oxidative damage inflicted by superoxide anions in two compartments of the cell, namely the cytosol and the IMS (the latter containing only a small percentage of total cell Sod1 content). Sod1 requires zinc and copper ions as cofactors, and contains a disulfide bond. The formation of this disulfide bond and the incorporation of the copper ion are mediated by the Copper Chaperone for Sod1 (Ccs1). Like Sod1, Ccs1 has a dual localisation in the cytosol and the IMS of mitochondria. Ccs1 promotes the import of Sod1, which has to be present in the reduced and unfolded apo-form to be able to cross the translocation pores of the OMM [75]. It has recently been demonstrated that the Mia40 and Erv1 system drives the transport of Ccs1 into the IMS. Mia40 forms disulfide intermediates with Ccs1, which is subsequently able to trigger the formation of disulfide bonds in Sod1. Moreover, over-expression of Mia40 increases the import of Ccs1 and Sod1, which in turn leads to changes in the distribution between the mitochondrial and the cytosolic fraction of these proteins. Therefore the disulfide relay system is required for oxidative folding of mitochondrial Ccs1 and Sod1, and has the ability to regulate the distribution of these two proteins between mitochondria and cytosol. Since the activity of the Mia40-Erv1 system is coupled to the respiratory chain, as previously described, regulation of the respiratory chain might arguably modulate the level of Ccs1 and Sod1 in mitochondria as an adaptation to the organelles' requirement [76].

4. INNER MEMBRANE PROTEIN BIOGENESIS

The inner membrane of mitochondria is the site where energy is produced. Energy accumulation is the result of the concerted action of the five respiratory chain complexes that couple the oxidation of substrate molecules with the phosphorylation of ADP to ATP. The activity of the respiratory chain generates a proton gradient across the IMM known as the mitochondrial membrane potential; this electrochemical gradient is used not only to produce ATP by the F_1F_o-ATPase, but also to transport proteins and metabolites across the membrane. To maintain the gradient, the inner membrane acts as a stringent permeability barrier that regulates the constant exchange of ions, molecules and polypeptides between the MM and the IMS. As a result, specialised metabolite carriers mediate the transport of metabolites and molecules through the membrane, and specific translocases are in charge of protein transport across the IMM or protein insertion into the membrane.

The proteins that localise in this compartment are inserted into the membrane on the basis of which of two differing complexes, TIM22 or TIM23 translocases, recognises their targeting signals. Multispanning inner membrane proteins contain internal targeting signals and are sorted from the entry gate, the TOM complex, to the carrier translocase TIM22, also known as the protein insertion complex [77]. Inner membrane proteins with a "bipartite" sorting signal, *i.e.* a presequence and a hydrophobic sorting domain, are directed to the TIM23 complex, which laterally inserts them into the IMM by a mechanism known as "stop-transfer mechanism" (as previously described for IMS proteins) [78]. Alternatively, proteins containing a presequence are imported in the MM and then sorted to the IMM through an export step [79]. Details of each of these sorting routes are presented in the following sections, 4.1 and 4.2.

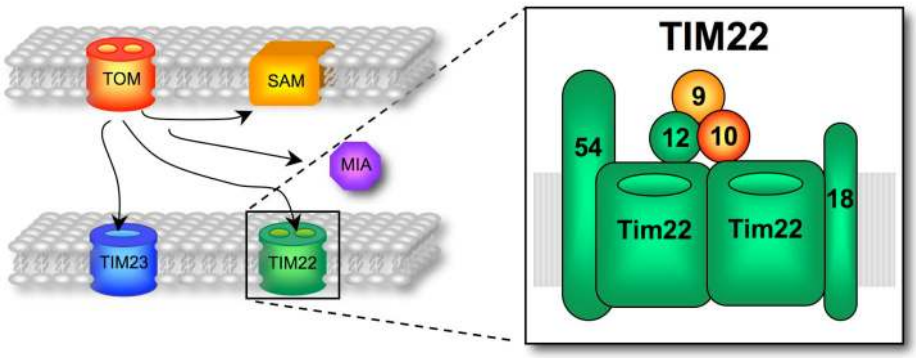

Fig. (5). Composition of the TIM22 complex. Two functionally-coupled Tim22 subunits form the hydrophilic channels. Tim54, and Tim18 are integral membrane proteins while Tim9, Tim10 and Tim12 are peripheral proteins.

4.1 The Mitochondrial Carrier Pathway

Most substrates of the TIM22 complex belong to the family of metabolite carriers, such as the ATP/ADP translocase, the dicarboxylate, the tricaboxylate, the pyruvate and the phosphate carriers. All these proteins are mainly hydrophobic proteins and share common structural features. They harbour three equal modules of approximately similar length, and each module consists of two transmembrane segments connected by a hydrophilic loop. Overall, the protein structure forms six transmembrane α-helices that span the IMM, with the N- and C-terminal portion of the protein exposed in the IMS. They then associate into dimers to generate the functional carrier [80]. Additional substrates of the TIM22 complex are Tim23 and Tim22 (respectively subunits of the TIM23 and TIM22 complexes), which are transported across and inserted into the membrane along the carrier transport pathway.

The TIM22 carrier translocase is a complex of approximately 300 kDa and contains six subunits, the integral membrane proteins Tim54, Tim22, Tim18 and the peripheral proteins Tim12, Tim10 and Tim9 (Fig. **5**).

The Tim22 protein, the central component of the translocase, forms a hydrophilic channel across the membrane, and provides two functionally coupled pores within the TIM22 complex. This twin-pore translocase mediates the insertion of the precursor proteins in accordance with internal targeting signals and $\Delta\psi_m$. This latter driving force acts on the precursor and promotes its docking to the translocase. The $\Delta\psi_m$ and the internal signal peptide subsequently induce gating transitions in one pore and closure in the second pore, and drive membrane insertion to completion [81]. The internal targeting signal, which has been shown to be critical for membrane insertion, is positioned in the third module of the metabolite carrier, in the N-terminal portion of the fifth transmembrane segment [82].

The route of sorting, folding and membrane insertion of mitochondrial carrier precursor proteins into the IMM *via* the TIM22 complex starts from the cytosol, where precursors are synthesised on cytosolic ribosomes (Fig. **6**).

In this scenario, the Hsp70 and Hsp90 chaperone proteins associate with unfolded precursor proteins to prevent protein aggregation for as long as they are associated with the receptor of the TOM complex, Tom70, and enter the mitochondrion [10]. Translocation across the outer membrane requires ATP hydrolysis for the detachment of the precursors from its chaperones and from the receptor. Carrier precursors traverse the TOM complex through the Tom40 channel in a hairpin loop-like conformation, and then interact in the IMS with the soluble Tim9-Tim10 chaperone complex. The hydrophobic transmembrane segments of the carrier precursors are recognised by the chaperone complex and are shielded during transit through the aqueous intermembrane space to the TIM22 complex [83-85]. In addition to Tim9 and Tim10, the small Tim protein Tim12, a peripheral membrane subunit of the TIM22 complex, participates in this process too. A soluble form of Tim12 assembles with Tim9 and Tim10 to form a Tim12-core complex that then docks onto the membrane-integrated subunits of the TIM22 complex to form the holo-translocase. Thus Tim12 links the soluble and membrane-bound subunits of the import pathway (Fig. **6**) [86]. Insertion of the precursors into the lipid bilayer is then driven by the TIM22 complex, with $\Delta\psi_m$ as the energy source. This driving force and the internal targeting signal within the precursor sequence activate the carrier translocase that mediates the insertion of the polypeptide chain into the membrane. The

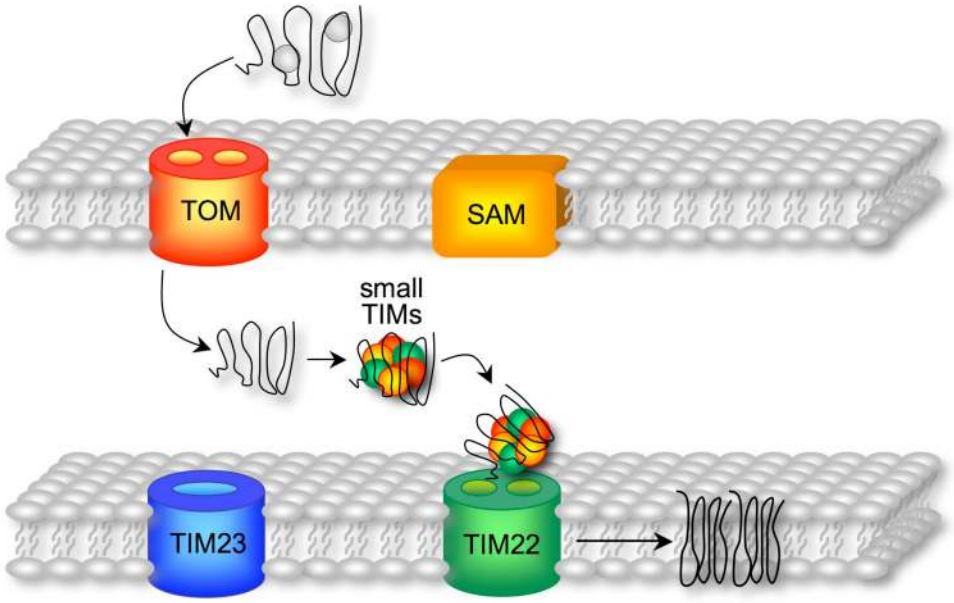

Fig. (6). The pathway of mitochondrial carrier proteins. Newly synthesised precursor proteins are protected from aggregation in the cytosol by chaperone proteins (in grey). After translocation through the TOM complex, precursors are recognised by the small Tim proteins Tim9, Tim10 and Tim12 (the latter in green) in the IMS. Small TIMs transport precursor proteins to the TIM22 complex, which mediates protein insertion into the IMM. The functional metabolite carrier is formed by protein dimers.

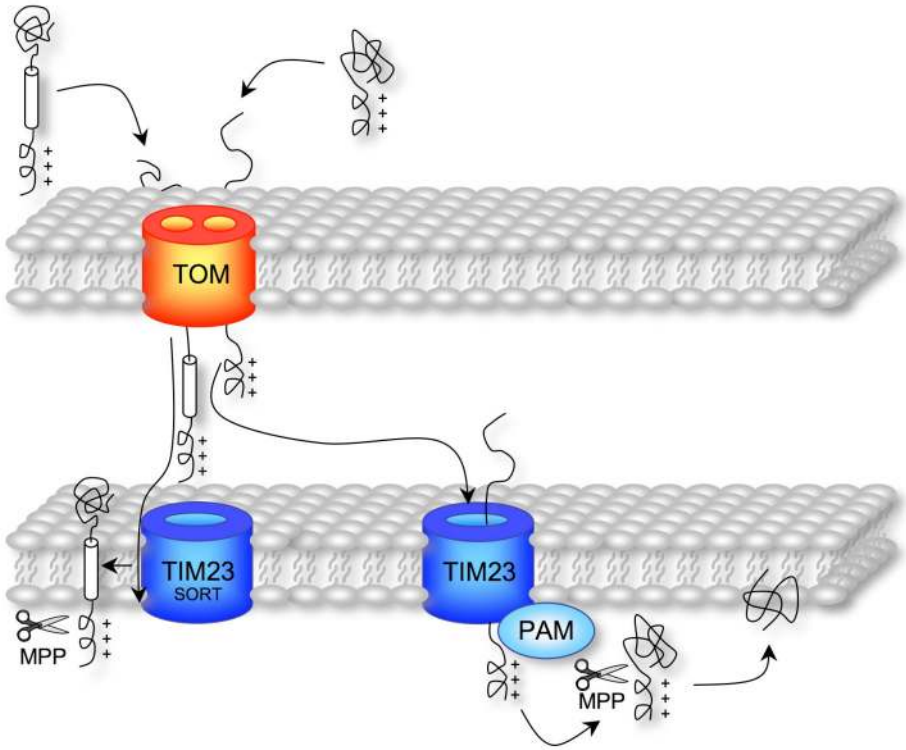

Fig. (7). Protein insertion into the inner membrane by the TIM23 complexes. Precursors of inner membrane proteins with a bipartite sorting signal are directly inserted into the lipid bilayer by the TIM23 inner membrane-sorting form, $TIM23^{SORT}$ (pathway on the left). Alternatively, the mitochondrial targeting sequence drives the translocation of precursors through the TIM23 complex and induces association of the TIM23 with the PAM complex. This interaction promotes the complete translocation of precursor proteins into the MM. Finally, proteins are sorted to the IMM through an export step (pathway on the right). The mitochondrial targeting presequence is cleaved off by the mitochondrial processing peptidase (MPP).

final step is the maturation of the metabolite carrier into its functional dimeric state [81].

4.2 Sorting Pathways *via* TIM23 Complex

Precursors of IMM proteins that contain a targeting presequence follow a differing route as they are sorted from TOM to the presequence translocase, the TIM23 complex (Fig. **7**). This complex delivers proteins to three different compartments: the intermembrane space, the inner mitochondrial membrane and the matrix.

The first energy source for these activities is $\Delta\psi_{m,}$ which drives presequence IMM translocations. This force is also sufficient for lateral protein insertion into the inner membrane. However, TIM23 additionally requires ATP hydrolysis to complete the translocation of precursors across the membrane into the MM. This is accomplished by interaction with the Presequence translocase-Associated Motor (PAM) complex (Fig. **7**).

Two different translocase forms of the TIM23 complex can be distinguished: a matrix transport form (termed $TIM23^{MOTOR}$) and an inner membrane-sorting form (termed $TIM23^{SORT}$). Their subunit composition is modulated in accordance with the two sorting pathways (Fig. **8**).

The core components of the TIM23 complex are the integral membrane proteins Tim23, the channel-forming subunit, Tim17, and Tim50 [78]. To enable the sorting of proteins with a bipartite sorting signal into the IMM, $TIM23^{SORT}$ contains the Tim21 subunit (Fig. **8**) [87]. Tim21 is an integral membrane protein that can interact with a subunit of the TOM complex, Tom22. The IMS domain of Tom22 binds to the presequence of precursor proteins that have passed through the TOM complex, and interacts with Tim21, which is exposed to the IMS. This interaction leads to the release from Tom22 of the precursor protein, which is then passed over to the TIM23 complex for membrane insertion [87]. It has been demonstrated that $TIM23^{SORT}$ interacts with the respiratory chain in a Tim21-dependent manner. Tim21 recruits proton-pumping respiratory chain complexes and stimulates preprotein insertion. Thus, the presequence translocase cooperates with the respiratory chain and promotes $\Delta\psi_m$-dependent protein sorting into the IMM [88].

In the absence of a hydrophobic sorting domain, the presequence enters the MM through the TIM23 complex (the core components without Tim21); this induces TIM23 to associate with the PAM complex to form the $TIM23^{MOTOR}$, which in turn translocates the precursor across the membrane. The central component of the PAM complex is the essential mitochondrial Heat Shock Protein 70 (mtHsp70) an-ATP dependent chaperone that binds to the incoming unfolded polypeptide chain and generates the driving force for the import process. Additional subunits of this complex are regulators of mtHsp70 activity: the co-chaperone Pam18, which stimulates mtHsp70 activity, Pam16, which regulates Pam18 activity, the MtHsp70 nucleotide exchange factor

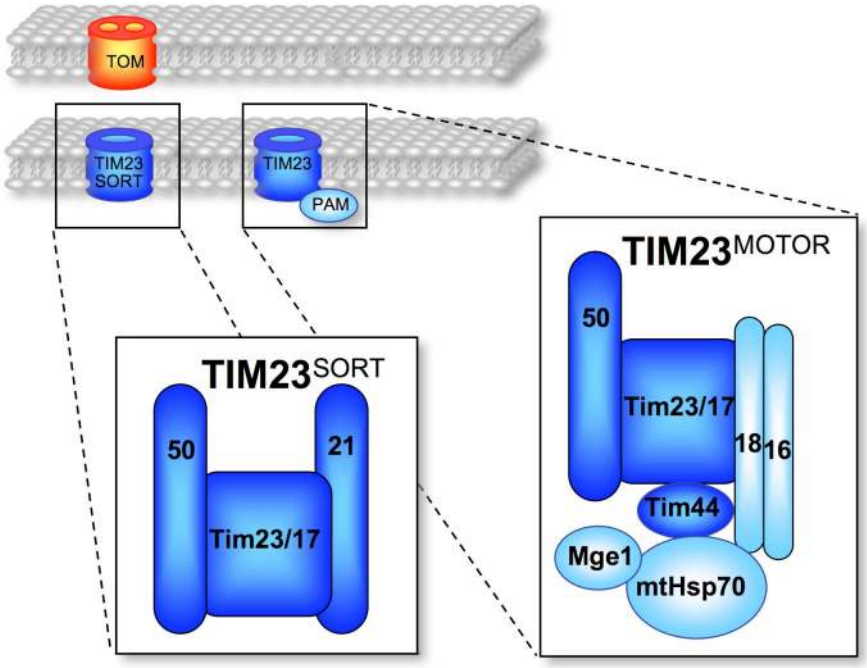

Fig. (8). Composition of the TIM23 complexes. The core components of the TIM23 complex are the channel-forming protein Tim23, and the Tim17 and Tim50 subunits. The addition of the Tim21 subunit results in the inner membrane-sorting form of TIM23, $TIM23^{SORT}$ (left square). Alternatively, core subunits interact with the PAM complex to form $TIM23^{MOTOR}$ (right square). MtHsp70 is the central component of the motor complex, Mge1 is the nucleotide exchange factor, and Pam18 and 16 are the co-chaperones. Tim44 connects TIM23 to the PAM complex.

Mitochondrial GrpE 1 (Mge1), and Tim44, which tethers mtHsp70 to the translocation channel (Fig. **8**) [89-91].

Interestingly, a recent report has shown that Pam16 and 18 interact with the respiratory chain too, independently of Tim21. Thus the association of respiratory chain complexes with presequence translocases occurs not only in preprotein sorting to the IMM by Tim21-containing $TIM23^{SORT}$ complexes, but also in an early stage of matrix translocation *via* $TIM23^{MOTOR}$ [92]. Regulated cycles of ATP-dependent binding and release of the precursor by mtHsp70 promote the forward movement of the polypeptide into the MM. The mitochondrial inner membrane proteins that are imported into the MM along the $TIM23^{MOTOR}$ pathway are then integrated with an export step into the IMM from the matrix side. The following section describes a notable player in this export step, namely a chaperone protein that belongs to the Alb3/Oxa1/YidC family.

4.3 The Role of Alb3/Oxa1/YidC Chaperone Protein Family

The inner membrane of mitochondria contains the entire respiratory chain, which provides most of the energy that cells are able to use. Four out of five respiratory chain complexes are composed of subunits encoded by the nuclear and the mitochondrial genomes; the single exception is respiratory complex II, which has no mitochondria-encoded subunit. Accordingly, the folding and assembly of these complexes rely initially on the coordinated activity of these two genomes, and subsequently on chaperones that assist the insertion of the subunits into a functional assembly.

Nuclear-encoded subunits are sorted and inserted into the IMM by the previously described protein translocases. Those subunits that undergo preliminary sorting into the MM, or that are encoded by mitochondrial DNA, require an additional sorting step for insertion into the IMM. The most exhaustively characterised pathway of membrane protein insertion from the MM involves proteins that belong to the Alb3/Oxa1/YidC family.

This family includes evolutionarily conserved membrane proteins that are able to catalyse membrane protein insertion and assembly processes in diverse biological systems. The YidC protein represents the prokaryotic member of this protein family and occurs both in Gram-negative and in Gram-positive bacteria. Alb3 is an integral membrane protein of the thylacoid membrane system of chloroplasts. Finally, the cytochrome OXidase Activity 1 protein (Oxa1) is located in the inner membrane of mitochondria, where it is required for the membrane insertion of several proteins with diverse membrane topologies [93].

The mature Oxa1 protein is a multispanning membrane protein with five transmembrane domains, an N-terminal domain that protrudes in the IMS, and a long C-terminal domain exposed to the MM. The monomer has a molecular weight of 36 kDa and forms homo-oligomeric complexes that facilitate integration of several mitochondrially encoded proteins into the inner membrane [94]. Oxa1 was first identified in yeast as a component required for the assembly of the cyt *c* oxidase complex; it was subsequently reported as participating additionally in the biogenesis of the cyto-chrome bc_1 and F_1F_o-ATP synthase complexes [95, 96]. In addition, Oxa1 mediates membrane insertion of nuclear

encoded proteins that reach the IMM in an export-like step from the MM [97].

Most mitochondrially encoded proteins are integrated within the lipid bilayer during their synthesis. This co-translational insertion is mediated by the C-terminal portion of Oxa1, which binds the large subunit of the mitochondrial ribosome through its matrix-exposed α-helical domain. Experimental evidence suggests that Oxa1 promotes co-translational insertion in the IMM by physical coupling protein synthesis with protein insertion machinery [98]. In addition, recent findings have shown that Oxa1 participates in the biogenesis of the F_1F_o-ATP synthase complex by supporting the assembly of the membrane-embedded F_o-sector in a post-translational manner. In particular, the matrix-localised domain of Oxa1 interacts with the newly synthesised mitochondrially encoded Atp9 protein, and maintains the assembly competence of this subunit for further assembly steps within the respiratory complex V [99].

Oxa1 is supported in its protein insertion activity by an additional mitochondrial protein, the Multi-copy Bypass of AFG3 protein 1 (Mba1), which associates with the matrix face of the IMM. As previously described for Oxa1, Mba1 too binds to the large subunit of mitochondrial ribosome, and acts as a membrane receptor for the ribosome, in concerted action with Oxa1, with the result that the ribosome is positioned in close proximity to the inner membrane [100].

5. PROTEIN FOLDING IN THE MATRIX

The majority of mitochondrial proteins reside in the matrix, where important mitochondrial metabolic functions take place, such as Krebs cycle, fatty acid metabolism and iron-sulfur cluster assembly. Resident MM proteins are imported by cooperation between the two main mitochondrial preprotein translocases, the TOM and TIM23 complexes (Fig. **9**).

Imported protein folding is undertaken by proteins belonging to three classes of molecular chaperones: Hsp70, Hsp60 and Hsp100. Proteins of the Hsp70 family, including the isoform mtHsp70, are monomeric proteins that are able to bind unfolded, hydrophobic segments of substrates in an ATP-dependent manner. Interaction of a substrate protein with Hsp70 stabilises and prevents its aggregation, and thus increases refolding efficiency. Hsp60 forms a large homo-oligomeric complex with an inner cavity that provides a protective environment for ATP-dependent folding of newly synthesised or unfolded proteins. Finally, proteins of the Hsp100 family, also named CaseinoLytic Peptidase (Clp) proteins, form homo-oligomeric complexes that are involved in protein aggregate re-solubilisation and in protein unfolding. These proteins participate in mitochondrial quality control systems, in concert with other factors that mediate protein turnover [101, 102]. The role of the mitochondrial components of the Hsp70 and Hsp60 families in matrix protein folding is discussed immediately below, while quality control systems are dealt with in Section 6.

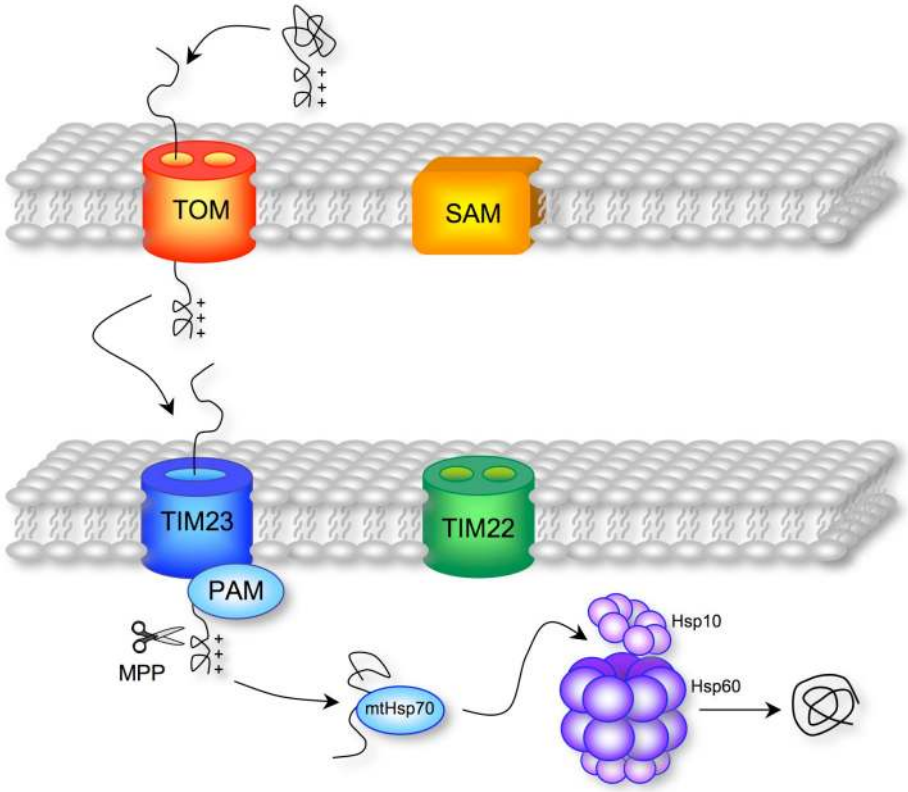

Fig. (9). Protein import and folding in the matrix. Precursor proteins are translocated into the MM by the TOM complex and then by the TIM23 and PAM complexes. Mitochondrial targeting presequence is cleaved off by MPP. Precursors are then subjected to folding attempts by mtHsp70 and Hsp60/Hsp10 chaperone proteins until the native conformation is achieved.

MtHsp70 and Hsp60 cooperate in the folding reaction of imported proteins in a sequential order. Preproteins first encounter mtHsp70 during the translocation process. After being released from mtHsp70, preproteins interact with the Hsp60 complex in order to reach the native folding state (Fig. **9**) [103].

5.1 The Role of mtHsp70 in Matrix Protein Folding

MtHsp70 (also called mortalin or, in yeast, Stress-Seventy subfamily C protein 1, Ssc1) plays a crucial role in preprotein translocation, because it is the core component of the PAM complex and the first chaperone that newly imported proteins meet, once these latter protrude into the matrix. In addition, MtHsp70 is actively involved in protein folding and assembly in the MM.

It consists of an N-terminal ATPase domain and a C-terminal peptide binding domain. The ATPase domain regulates the properties of the binding domain; specifically, ATP binding induces a conformational change in mtHsp70 that is correlated with a change in substrate binding affinity. In the ATP-bound state, the peptide binding domain is in an open conformation with low substrate affinity, while in the ADP-bound state the peptide binding domain is in a close conformation with high substrate binding affinity [104]. The activity of mtHsp70 is modulated through the regulation of its ATP/ADP-bound state by two co-chaperone proteins: Mge1 and Mitochondrial DnaJ 1 (Mdj1). Mge1 is a nucleotide exchange factor that induces recycling of mtHsp70 back into the ATP-bound state, thus permitting release of the substrate. The second co-chaperone, Mdj1, induces hydrolysis of the mtHsp70-bound ATP; the resulting ADP-bound mtHsp70 is thus allowed to interact with the substrate/unfolded protein [105]. Coordinated cycles of mtHsp70 reaction generate a productive interaction between the chaperone and the substrate.

MtHsp70 is the fundamental component of the PAM complex that cooperates with TIM23 to form the $TIM23^{MOTOR}$ complex, which in turn is responsible for protein import into the MM (Figs. **8** and **9**). The interaction between mtHsp70 and the incoming preprotein is essential for translocation. The $\Delta\psi_m$ drives the translocation of the positively charged N-terminal presequence into the matrix, but the bulk of the protein is such that ATP is required for translocation into the MM. This driving force is exerted by mtHsp70, the only ATPase that is involved in preprotein import [106, 107]. Co-chaperones that participate in the composition of the PAM complex regulate mtHsp70 activity. The co-chaperone Pam18 stimulates the ATPase activity of mtHsp70, and Pam16 regulates Pam18; Mge1 enables nucleotide exchange.

Despite its fundamental role as core component of the import machinery, debate persists on the molecular mechanism of mtHsp70-mediated translocation, and two differing hypothetical models stand out: the trapping model and the motor model. In the trapping model hypothesis, Brownian motion generates polypeptide movement in the translocation channel. The membrane potential drives the insertion of the preprotein N-terminal segment into the IMM. When this segment is exposed to the matrix, mtHsp70 binds transiting polypeptide and prevents backward movement, effectively acting as a molecular ratchet. Stimulated by Mge1 activity, several mtHsp70 binding steps lead to complete polypeptide translocation [108]. In contrast, the motor model hypothesis rests on the importance of mtHsp70-induced ATP hydrolysis and on mtHsp70's interaction with the Tim44 component of the $TIM23^{MOTOR}$ complex (see Section 4 for further information on Tim44). This model postulates that mtHsp70 exerts a direct force on the polypeptide during its translocation, and thus works as a translocation motor. The hypothesis holds that interaction with Tim44 acts as a lever for force generation [109]. Current evidence suggests that both mechanisms cooperate to obtain maximum import efficiency [102, 110].

In addition to its role in preprotein membrane translocation, mtHsp70 is also prominent in mitochondrial protein folding. Consequently, mtHsp70 can be found in association with Tim44 and the translocase complex or as a soluble folding complex together with its co-chaperones Mge1 and Mdj1 [111]. Furthermore, mtHsp70 participates in the biogenesis of mitochondrially encoded proteins. It interacts with newly synthesised polypeptides after translation by mitochondrial ribosomes, and facilitates their assembly into macromolecular complexes [112].

5.2 The Hsp60 Chaperone Complex in the MM

The second fundamental component of the matrix protein folding system is the co-chaperone Hsp60 (Fig. **9**). Once preproteins enter the mitochondrial matrix, they begin to refold and to assemble, and Hsp60 supports these processes [113].

Its function is a consequence of its singular molecular architecture. Our biophysical understanding of Hsp60 is primarily based on studies performed on the *E. coli* GroEL and GroES chaperonins, which are respectively homologous to eukaryotic Hsp60 and Hsp10. Hsp60 is a homo-oligomeric complex of 14 subunits arranged as two rings of 7 subunits each and forming a "double doughnut" structure. The double rings enclose a large inner cavity that harbours unfolded proteins and protects them from interactions with other components of the surrounding environment. Substrate proteins bind to hydrophobic residues that are exposed to the inner wall of the cavity. Hsp10 forms a lid at the top of the double ring system and closes the opening to the central cavity [114, 115].

The Hsp60 subunit can be divided into 3 domains: the apical domain, which binds to the substrate and to Hsp10; the equatorial domain, which contains binding sites for ATP and the other subunits of the ring; the intermediate domain, which acts as a mobile linker between the other two. The central cavity processes unfolded proteins in two sequential, transitional states. In the initial, peptide-accepting state, the cavity opens and exposes hydrophobic residues, which in turn capture non-native polypeptides and funnels them into the cavity; in the subsequent, peptide-folding state, Hsp10 association with the cavity opening and ATP binding induce large conformational changes in the Hsp60 subunits, and generate a fundamental change in the shape and hydrophobicity of the internal cavity. The cavity enlarges and its hydrophobic binding surface simultaneously twists away from the polypeptide and thus dispels the protein into the

cavity. The apical domains of the Hsp60 subunits of the ring switch from hydrophobic to hydrophilic, and vice versa. The coordinated movements of the 14 subunits swallow up the protein and the exposed hydrophobic residues, and reorient the latter until the polypeptide attains its native state and is released from the Hsp60 complex [116-118].

5.3 Iron-Sulfur Cluster Assembly Machinery

Mitochondrial preproteins require the chaperone protein mtHsp70 for translocation and folding in the MM. Two additional members of the Hsp70 family have been identified in yeast. The first is the ExtraCellular Mutant 1 protein (Ecm1), which shows very high sequence identity to that of mtHsp70, but whose functional relevance remains unclear [119]. The second is the Stress-Seventy subfamily Q protein 1 (Ssq1), which is involved in the assembly of iron-sulfur (Fe/S) clusters [120]. The basic steps that this singular pathway follows in the matrix will be described below as an example of additional folding and assembly mechanisms within the MM.

Fe/S clusters are small inorganic cofactors that act as catalysts in chemical reactions, as electron carriers in redox reactions and as stabilisers of protein structure. Proteins containing Fe/S clusters (Fe/S proteins) are localised within mitochondria, in the cytosol and in the nucleus of eukaryotic cells. Interestingly, the biogenesis of cytosolic and nuclear Fe/S proteins relies on the specialised Iron-Sulfur Clusters (ISC) assembly machinery that localises within the mitochondrial matrix; all cellular Fe/S proteins are generated by this machinery. Two additional systems are involved in the subsequent maturation of cytosolic and nuclear Fe/S proteins: the mitochondrial ISC export apparatus and the Cytosolic Iron-sulfur protein Assembly (CIA) machinery [121].

Most mitochondrial Fe/S proteins are localised within, or face, the matrix space. The first mitochondrial Fe/S proteins to be revealed as such include respiratory complexes I, II and III, Krebs cycle aconitase and ferrodoxin. Their assembly depends on the ISC assembly machinery. The central component of this system is the Iron-Sulfur cluster nifU-like 1 protein (Isu1), a scaffold protein on which Fe/S clusters are assembled before transfer to a recipient apo-protein. Isu1 contains three conserved cysteine residues that are critical for *de novo* Fe/S cluster assembly [122]. Additional factors cooperate with Isu1 in the first steps of ISC assembly. The cysteine desulfurase known as Nitrogen Fixation 1 (Nfs1) acts as a sulfur donor that catalyses the release of sulfur from cysteines. The iron-binding protein Yeast Frataxin Homolog 1 (Yfh1), the yeast homologue of human frataxin, binds iron and undergoes iron-stimulated interaction with Isu1-Nfs1; given these properties, it is held to be the iron donor of the reaction. Finally, the ferrodoxin reductase Adrenodoxin Reductase Homolog 1 (Arh1) and the ferrodoxin Yeast Adrenodoxin Homolog 1 (Yah1) provide electrons for the reduction of cysteine sulfur to sulfide [123-126].

The subsequent step is the transfer of the Fe/S cluster from the Isu1 scaffold protein to an apo-protein. This process involves 4 agents: Ssq1, a specialised molecular chaperone and a member of the Hsp70 protein family; its co-chaperone J-type Accessory Chaperone 1 (Jac1); the nucleotide exchange factor Mge1; and GlutaRedoXin 5 (Grx5p). Grx5 is a non-essential glutaredoxin that participates in Fe/S cluster transfer to the apo-protein, but the mechanism has yet to be elucidated. Jac1 binds to Isu1, targets it to Ssq1, and stabilises Isu1-Ssq1 interaction by stimulating Hsp70 ATPase activity. The mitochondrial chaperone is presumed to remodel the structure of Isu1 by facilitating transfer of the cluster to the recipient apo-protein. [127-129].

The iron-sulfur cluster assembly machinery in mitochondrial matrix was inherited from bacteria in the endosymbiotic event that led to the engulfed precursor of present-day mitochondria. Notably, bacteria continue to depend on ISC assembly machinery for the generation of the majority of cellular Fe/S proteins [130]. This feature is thus a further example of the high degree of continuity that folding and assembly mechanisms conserve in the evolution of prokaryotes to higher eukaryotes.

6. PROTEIN QUALITY CONTROL SYSTEMS

Biochemical processes and cellular protein homeostasis are controlled throughout the cell under normal and stress conditions. Defective or misfolded proteins can originate both during oxidative and/or environmental stress and as intermediates of folding processes, with deleterious effects on cellular metabolism. It is to combat such effects that cells possess quality control (QC) systems.

QC intervention begins with the recognition of damaged or misfolding in polypeptides and their binding by specific molecular chaperones for stabilisation and potentially refolding. When this cannot be accomplished, damaged proteins are transferred to ATP-dependent proteases for degradation. The most extensively characterised eukaryotic protein quality control machinery is the cytoplasmic ubiquitin-proteasome system, which is largely responsible for the degradation of soluble cytosolic proteins [131].

Additional QC systems are present in the endoplasmic reticulum and in mitochondria. Misfolded proteins in the lumen of the ER are bound to chaperones, retro-translocated through a multiprotein translocon complex across the ER membrane, ubiquitinated by ER membrane-associated or cytosolic ubiquitin ligases, depending on the substrate, and targeted for proteasomal degradation in the cytoplasm. This pathway is termed ER-Associated protein Degradation (ERAD) [132].

Organelles with an endosymbiotic origin such as mitochondria and chloroplasts have retained some of the original protein quality control enzymes, molecular chaperones and proteases that served their bacterial ancestors. The mitochondrial QC system comprises chaperones of the Hsp60, Hsp70 and Hsp100 family, which assist in protein import and folding and which prevent protein aggregation. In the mitochondrial matrix, mtHsp70 is involved in protein import as the major component of the PAM complex, but under stress conditions it protects proteins from aggregation or delivers them to the matrix protease for subsequent degradation [133]. The mitochondrial member of the Hsp100 family, Hsp78, maintains mtHsp70 in a soluble state, and cooperation between these two chaperones is required for the re-solubilisation of aggregated proteins [134].

6.1 Proteolytic Degradation in Mitochondrial Compartments

Apart from the stabilising action of molecular chaperones, the major mechanism of defence against the accumulation of damaged polypeptides is their specific removal by proteolysis. Defective proteins are degraded by proteases of the AAA family (ATPases Associated with a variety of cellular Activities). These proteases harbour a conserved ATPase domain of approximately 230 amino acid residues, which is characteristic of the AAA^+ superfamily of Walker-type ATPases. They form multimeric protein complexes, which constitute sequestered, proteolytic microcompartments. The energy derived from ATP hydrolysis is utilised to unfold specific substrate proteins and to transport them into this internal proteolytic cavity [135, 136].

All mitochondrial compartments possess a conserved proteolytic system, except for the outer membrane. Two protease complexes are involved in protein degradation in the matrix: the Lon and ClpXP proteases. The Lon protease (the yeast orthologous protease is called Proteolysis In Mitochondria 1, Pim1) is responsible for the degradation of most MM proteins. It forms a large, ring-like heptameric complex, and each subunit contains the ATPase and the proteolytic domain within the same polypeptide chain. The protease recognises misfolded or damaged proteins and mediates their complete proteolysis, thus preventing protein accumulation and harmful effects on mitochondrial activities [133].

The second ATP-dependent matrix protease, ClpXP, catalyses the unfolding and degradation of misfolded proteins. This protease is composed of two components, ClpX and ClpP, both highly conserved and present in the eubacteria, mitochondria and chloroplasts of higher eukaryotes. ClpX is the chaperone component of the complex, and ClpP is the compartmentalised protease that contains the proteolytic active site. The functional form of ClpP is a tetradecamer that is assembled from two heptameric rings stacked face-to-face to form a double ringed structure. The rings enclose a large aqueous chamber with the proteolytic active sites inside. ClpX forms hexameric rings axially aligned on each end of the double ring of ClpP, thus forming the ClpXP complex. ClpX rings are necessary for substrate binding and for the unfolding and translocation of the substrates into the proteolytic chamber formed by ClpP subunits [137, 138].

In the inner mitochondrial membrane, two IMM-integrated ATP-dependent proteolytic complexes termed AAA proteases selectively degrade non-assembled or damaged proteins: the *i*-AAA complex and the *m*-AAA complex [136]. These complexes can exert protein quality control on both sides of the membrane on the basis of their topology within the IMM.

The first is the homo-oligomeric complex that contains Yeast Mitochondrial Escape 1 protein (Yme1) subunits; this complex is called the *i*-AAA protease, because it is active in the *i*ntermembrane space. Each Yme1 subunit is an inner membrane protein that is inserted into the membrane with a "stop-transfer mechanism" through a single transmembrane domain; the catalytic C-terminal portion of the subunit contains the AAA domain and the proteolytic domain, and it is exposed into the IMS. The second complex is the *m*-AAA protease, a hetero-oligomeric complex that is composed of Yeast Tat-binding Analog 10 and 12 (Yta10 and Yta12) subunits and that degrades proteins on the *m*atrix side. The N-terminal region of each subunit contains two transmembrane helices that form the transmembrane segment of the protein. The C-terminal region consists of the AAA and the protease domains. Finally, a conserved coiled-coil region is located after the proteolytic domain; this region might contribute to AAA complex formation or to substrate binding [139-141].

The homologous proteins involved in human mitochondrial protein quality control in the IMM have been identified. Human *i*-AAA protease is formed by YME1-Like 1 protein (YME1L1), and is highly homologous to yeast Yme1 protein. YME1L1 localises in the inner mitochondrial membrane and is able to complement phenotypes observed in yeast *yme1* null mutant [142]. Human *m*-AAA protease is composed of two homologous subunits: paraplegin and ATPase Family Gene 3-Like 2 protein (AFG3L2). Complementation studies performed in a yeast strain that lacked both Yta10 and Yta12 demonstrated functional conservation of the human paraplegin and AFG3L2 complexes with the yeast *m*-AAA protease [143]. Interestingly, mutations in a subunit of the *m*-AAA protease, paraplegin, cause an autosomal recessive form of Hereditary Spastic Paraplegia (HSP), while mutations in *AFG3L2* have recently been associated with a dominant form of SpinoCerebellar Ataxia (SCA28) [144, 145]. We demonstrated that the absence of paraplegin affects the maintenance of respiratory chain complexes, in particular of respiratory chain complex I. The respiratory defect worsens in AFG3L2-depleted mouse models in which both the I and III assembled complexes are quantitatively reduced. An alteration in mitochondrial quality control within the inner membrane can therefore result in mitochondrial respiration defects [143].

Pathogenetic mutations that cause HSP have been identified in the gene encoding the human mitochondrial Hsp60 too. An autosomal dominant form of HSP is associated with a mutation that results in the V72I substitution in Hsp60 amino acid sequence [146]. Recent findings indicate that the V98I substitution affects the ATPase activity of Hsp60 and results in a dramatically decreased folding activity of complexes consisting of the mutant subunits only. The major effect of heterozygosity for this mutation is a moderately decreased activity of chaperonin complexes composed of mixed wild type and mutant Hsp60 subunits [147]. This is an additional example of the alteration of mitochondrial protein folding or of quality control that results in a neurodegeneration disease in humans.

Little is known about quality control within the IMS and the OMM. The *i*-AAA protease can exert surveillance on membrane proteins that are exposed within the IMS. In addition, it has been suggested that the serine protease High Temperature Requirement protein A2 (HtrA2/Omi) is involved in protein quality control of the compartment [148]. This protease offers protection from mitochondrial stress and may be part of an adaptive stress-signalling cascade. It has also been shown that HtrA2 is released from the intermembrane space during apoptosis. However its function has yet to be elucidated [148, 149].

In the outer mitochondrial membrane there is no evidence of QC chaperones or proteases. However, ubiquitin ligases, the enzymes that transfer activated ubiquitin onto the substrate, have been identified in the OMM [150]. The mitochondrial E3 ubiquitin ligase Membrane-Associated Ring-CH protein 5 (MARCH5) is a transmembrane protein of the outer membrane. It is responsible for the ubiquitination of the mitochondrial fission protein Dynamin-Related Protein 1 (Drp1) and is involved in the regulation of mitochondrial fission [151]. In addition, the mitofusin protein, an important regulator of mitochondrial fusion, is degraded through the ubiquitin-proteasome-dependent pathway [152]. Accordingly, the ubiquitin-conjugation system and the proteasomal degradation of mitochondrial proteins might contribute to the maintenance of mitochondrial homeostasis. Further studies are needed to clarify the role of this cytosolic degradation pathway in the mechanisms of mitochondrial quality control.

7. MITOCHONDRIAL UNFOLDED PROTEIN RESPONSE

Mitochondria contribute to several cellular metabolic pathways and generate energy for all the activities of the cell. The maintenance of the organelle in a productive state is based on the tightly coordinated expression of nuclear and mitochondrial genomes. In addition, the network of molecular pathways described in this review participates in the regulation of protein homeostasis through (i) translocases and chaperones that assist proteins during their sorting and folding routes and (ii) proteases that exert quality control surveillance.

Environmental stress can alter this balance by increasing the pool of unfolded or misfolded proteins and by activating signal transduction cascades to counteract these problems. These pathways are collectively called Unfolded Protein Responses (UPRs). In the cell, exposure to environmental stress results in protein unfolding and impairment of essential processes. This stress leads to the transcriptional activation of genes that harbour stress response elements within their promoters, which in turn enables cells to respond to global stress by increased synthesis of heat shock proteins and of other repair molecules [153].

This response can also occur in specific subcellular compartments; an imbalance between protein loading of the endoplasmic reticulum and protein folding inside the ER can indeed activate an analogous pathway termed the ER unfolded protein response (erUPR) [154]. Recent findings indicate the existence of a similar pathway in mitochondria, which has been called the mitochondrial UPR (mtUPR), though understanding of the primary molecular mechanisms is poor.

The first report on a mitochondrial-specific stress response in mammalian cells has shown that the accumulation of an unfolded protein within the MM triggers transcriptional upregulation of nuclear genes that encode such mitochondrial stress proteins as Hsp60, Hsp10, Mdj1 and ClpP. Moreover, analysis of the promoter regions of the above-mentioned genes has revealed that they contain an element which is recognised by the transcription factor C/EBP HOmology Protein (CHOP), a finding which suggests that CHOP is the activator of target genes in the mtUPR pathway [155].

As the reader will recall, CHOP is also involved in erUPR. Nonetheless, independent activation of mtUPR and erUPR has been demonstrated. This phenomenon can be explained by the presence of different regulatory features within the promoters of the genes of interest. In particular, the analysis of genes involved in mtUPR has revealed that additional elements within their promoter regions differentiate them from genes activated during erUPR. These genes contain an Activator Protein-1 (AP-1) element, and deletion of this element results in ablation of their mtUPR-responsiveness [156]. Moreover, bioinformatic analysis of mtUPR-responsive genes has shown that their promoters contain two additional promoter elements lying on either side of the CHOP element: Mitochondrial Unfolded Protein Response Element 1 and 2 (MURE1 and MURE2). Mutations in these elements have been found to reduce the activation of the promoters, which in turn suggests that these elements co-ordinate with CHOP in the regulation of mtUPR [157].

Parallel studies performed on mitochondrial stress response in *Caenorhabditis elegans* have shown that mtHsp70 and Hsp60 are selectively activated after perturbation of protein homeostasis, by impairment of multisubunit complex assembly or by RNA interference (RNAi) of genes encoding mitochondrial chaperones or proteases. Interestingly, inactivation of paraplegin or Oxa1 by RNAi resulted in Hsp70 and Hsp60 transcription activation; these data indicate that alterations in mitochondrial quality control mechanisms or in protein folding/membrane insertion specifically stimulate mtUPR [158].

Subsequent genome-wide screening performed in *C. elegans* with RNAi constructs directed to known genes has attempted to identify genes that activate mtUPR. A new protein has been discovered to be required for mtUPR induction: the mitochondrial matrix protease ClpP. This protein functions in bacterial heat shock response, a finding which suggests that eukaryotes utilise components from the protomitochondrial symbiont to signal mtUPR [159]. In addition, a signalling pathway from the mitochondrial matrix to the nucleus has been identified, as has been the involvement of a putative homeobox-like transcriptional factor termed Defective proVEntriculus 1 (DVE-1, homologous to *D. melanogaster* dve, Defective proVEntriculus) and the small UBiquitin-Like protein 5 (UBL-5). Protein misfolding in the mitochondrial matrix promotes the nuclear localisation of both UBL-5 and DVE-1, which cooperate in the transcriptional activation of *C. elegans* mtUPR targets [159]. Overall these experimental findings support the hypothesised existence of an mtUPR that activates gene expression in response to alterations in protein handling in the organelle.

8. CONCLUDING REMARKS

Other organelles populating the eukaryote cell depend substantially on central cellular establishment for concerted folding and degrading of misfit proteins. They generally send warning signals to a centralised security system that takes care of the element to be purged. In contrast, the mitochondrion preserves and defends its peptide-folding and

quality control autonomy from centralised management. Of course, as we have already said, crosstalk between the two genomes operates in close coordination, but mitochondria certainly behave as peripheral organelles that maintain distant though vital connections with the central systems.

This striking independence of action is further amplified by the multi-compartment structure of the mitochondrion, which specifically developed individual systems for the sorting and shaping of proteins in the four organellar partitions. The current review painstakingly describes the major known routes that mitochondrial proteins follow to reach appropriate localisation and native structural conformation within the organelle. Interestingly, all existing data highlight the close relationship between protein sorting and folding processes. Mitochondrial membrane translocases are committed to maintaining separate sorting pathways, while additional chaperones participate in protein folding and, when required, in membrane insertion. Furthermore, the compartmentalisation of the organelle facilitates local protein quality surveillance and cleaning, hence allowing the spatial coordination of the compartment-specific protein complexes devoted to the refolding and, if necessary, the degradation of proteins.

Our chosen focus for this review leaves other important phenomena in the background, particularly as regards organellar agony and cell poisoning, autophagy and apoptosis, which represent the mutually exclusive yin-yang pathways to self-degradation and cell suicide.

Although the puzzle of mitochondrial protein import, sorting and folding has begun to be delineated in recent years, important mechanistic aspects of this domain, suspended as it is between the prokaryote and eukaryote realms, deserve further investigation.

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