Available online at www.sciencedirect.com

ScienceDirect

Biochimica et Biophysica Acta 1783 (2008) 610–617

Biochimica et Biophysica Acta BBA

www.elsevier.com/locate/bbamcr

Review

The MIA system for protein import into the mitochondrial intermembrane space

Diana Stojanovski, Judith M. Müller, Dusanka Milenkovic, Bernard Guiard, Nikolaus Pfanner, Agnieszka Chacinska *

Institut für Biochemie und Molekularbiologie, Zentrum für Biochemie und Molekulare Zellforschung, Universität Freiburg, Hermann-Herder-Straße 7, D-79104 Freiburg, Germany

Received 21 September 2007; received in revised form 5 October 2007; accepted 15 October 2007
Available online 22 October 2007

Abstract

When thinking of the mitochondrial intermembrane space we envisage a small compartment that is bordered by the mitochondrial outer and inner membranes. Despite this somewhat simplified perception the intermembrane space has remained a central focus in mitochondrial biology. This compartment accommodates many proteinaceous factors that play critical roles in mitochondrial and cellular metabolism, including the regulation of programmed cell death and energy conversion. The mechanism by which intermembrane space proteins are transported into the organelle and folded remained largely unknown until recently. In pursuit of the answer to this question a novel machinery, the Mitochondrial Intermembrane Space Assembly machinery, exploiting a unique regulated thiol–disulfide exchange mechanism has been revealed. This exciting discovery has not only put in place novel concepts for the biogenesis of intermembrane space precursors but also raises important implications on the mechanisms involved in the generation and transfer of disulfide bonds.

Keywords: Mitochondria; Intermembrane space; Disulfide bonds; Protein import and assembly

1. Introduction

In basic terms a disulfide bond within a protein represents a covalent linkage between the sulfur atoms of two cysteine residues. This linkage plays a fundamental role in protein biochemistry through the regulation of protein structure and function. The formation of disulfide bonds is favored in the presence of an oxidative environment and thus disulfide bond containing proteins are predominately found in extra-cytoplasmic compartments. Within these compartments the introduction of disulfide bonds is required for the proper folding, enhanced stability or for activity of the protein. Systems developed to shuttle disulfide bonds are present in the periplasm of Gram-negative bacteria and the endoplasmic reticulum (ER) of eukaryotic cells (Fig. 1). Surprisingly, the mitochondrial intermembrane space has recently been revealed as a further eukaryotic compartment that accommodates thiol–disulfide exchange reactions.

Regardless of the compartment in which it is undertaken, reduction and oxidation of disulfide bonds is mediated by a variety of thiol-redox enzymes, which permit fast and reversible thiol–disulfide exchange (Fig. 1) [1–3]. As a general principle, disulfide bonds are not formed spontaneously in living cells but are rather created by specialized enzymes in order to be donated to another set of thiols within the same or a different polypeptide chain. As a consequence of this the formation of mixed disulfide species or reaction intermediates is a distinctive feature of systems incorporating disulfide bonds. We will first briefly summarize the principles of oxidative protein folding established for bacterial and ER proteins as a basis to discuss the components and mechanisms of the mitochondrial machinery.

Abbreviations: ERV1, essential for respiration and vegetative growth; MIA, mitochondrial intermembrane space assembly; PDI, protein disulfide isomerase; SAM, sorting and assembly machinery; TIM, translocase of the inner membrane; $\Delta\psi$, membrane potential; TOM, translocase of the outer membrane

* Corresponding author. Tel.: +49 761 203 5245; fax: +49 761 203 5261.
E-mail address: Agnieszka.Chacinska@biochemie.uni-freiburg.de (A. Chacinska).

0167-4889/$ - see front matter © 2007 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamcr.2007.10.004

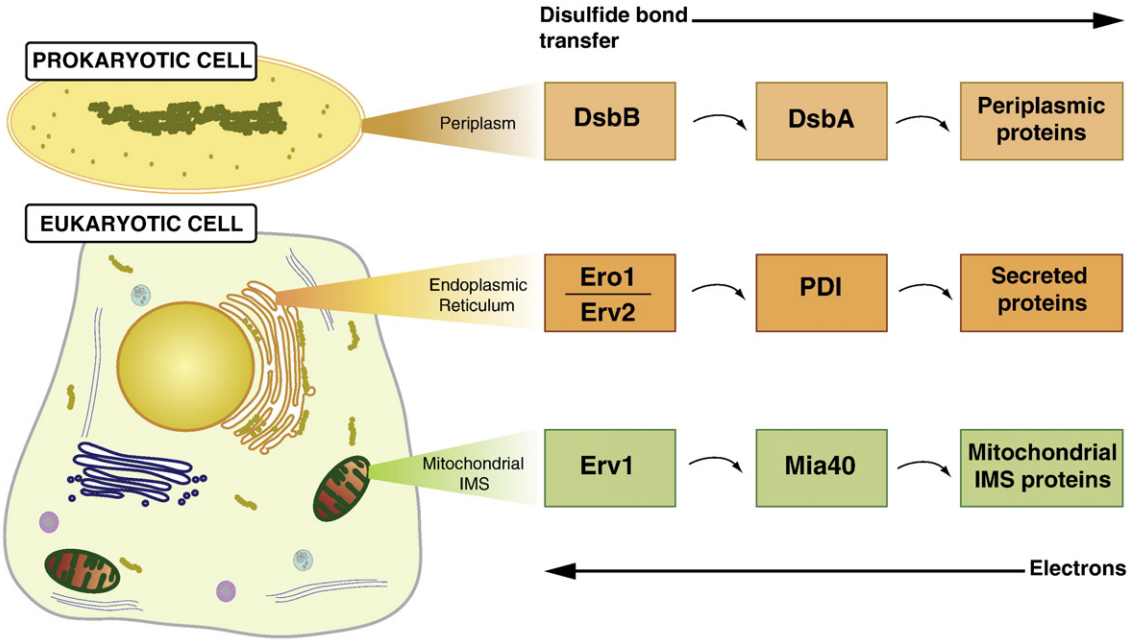

Fig. 1. Pathways for disulfide bond formation. The acquisition of disulfide bonds within proteins is mediated by dedicated oxidoreductase systems and a chain reaction of events permitting the transfer of oxidizing equivalents. In prokaryotic cells disulfide bond formation is catalyzed in the oxidative environment of the periplasm. Here, the catalyst DsbA donates its disulfide bonds to target periplasmic proteins and as a consequence is released in a reduced form. To start a new oxidation cycle DsbA must be subsequently reoxidized and its partner protein DsbB accomplishes this reoxidation step. In eukaryotic cells catalysts necessary to promote disulfide bond formation are found within the ER and mitochondrial intermembrane space. Oxidizing equivalents flow from Ero1 or Erv2 to PDI to secretory proteins in the ER, or from Erv1 to Mia40 to intermembrane space proteins in the mitochondria. The direction of disulfide bond transfer and electron flow is shown.

The disulfide bond (Dsb) machinery of prokaryotes has been extensively studied in *Escherichia coli* (Fig. 1). The regulation of disulfide bonds in the bacterial periplasm by the Dsb family of oxidoreductases occurs through two distinct electron transfer pathways: the oxidation and the isomerization pathways [2,4–6]. The oxidation pathway encompasses the donation of disulfide bonds from the catalyst DsbA, which possesses a thioredoxin-like domain, to the newly translocated protein (Fig. 1) [2,7,8]. As a consequence of this transfer event two cysteines in the active site of DsbA acquire a reduced state and must be restored to an oxidized state [2]. The cytoplasmic membrane protein DsbB, which uses the oxidizing power of the electron transport system, executes this oxidation step [1,8]. The pathway catalyzed by DsbA is rapid and can therefore pose problems for proteins with multiple thiols resulting in the formation of non-native bonds. However, in *E. coli* the isomerase or DsbC–DsbD pathway catalyses the shuffling of incorrectly arranged disulfide bonds [2,4–6]. A combined effort between the periplasmic disulfide bond isomerase, DsbC and a membrane bound enzyme, DsbD, that is responsible for maintaining DsbC in a reduced state, permits the rescue and proper maturation of such proteins.

In addition to protein modification and quality control the ER accommodates successful protein folding through the acquisition of disulfide bonds for many eukaryotic proteins. There are a number of oxidoreductases with thioredoxin-like domains in the ER, but the most prominent and most well characterized of this group is protein disulfide isomerase (PDI) (Fig. 1). PDI is an essential enzyme that has the ability to catalyze both the oxidation of free thiols and the isomerization of existing disulfides [9–11]. Oxidation reactions involve the transfer of an active site disulfide from PDI to substrate proteins, while isomerization requires that the active site cysteines of PDI be in a reduced state. The redox state of PDI *in vivo* is determined by its interaction with Ero1, an essential ER membrane-associated protein that generates disulfides in a flavin-dependent reaction that consumes molecular oxygen [3,12,13]. Ero1 does not transfer oxidizing equivalents to substrate proteins but rather specifically oxidizes PDI, which in turn passes the oxidizing equivalent to folding substrates. Erv2, a small FAD-binding protein was shown to participate in a bypass pathway for disulfide bond formation within the ER [14]. A combination of genetic and biochemical techniques revealed that Erv2 could consume molecular oxygen as a source of oxidizing equivalents for PDI, independently of Ero1. Erv2 belongs to the family of Erv1p/ALR proteins that are found in both higher and lower eukaryotes. Members of this family are present in different cellular compartments including mitochondria [15–17]. Furthermore, a complete pathway for disulfide bond formation and transfer encoded by the vaccinia virus has been shown to oxidize structural virion protein in the eukaryotic cytoplasm during the life cycle of poxviruses [18]. The diverse distribution of these proteins had raised the possibility that the formation of disulfide bonds and the mechanisms governing this process may not be restricted to the environment provided by the ER in eukaryotes.

Mitochondria had not been presumed to harbor processes involved in the formation and exchange of disulfide bonds.

The regulated transfer of disulfides within the mitochondrial intermembrane space was indeed an unexpected finding given that it was believed that the intermembrane space was continuous with the reducing environment of the cytosol via outer membrane porins. Mia40 together with Erv1, a sulfhydryl oxidase homologous to the above mentioned Erv2 from the ER, form a novel MIA (Mitochondrial Intermembrane Space Assembly) pathway. This pathway plays a pivotal role in post-translational events of intermembrane space-directed proteins from their translocation across the mitochondrial outer membrane through to their subsequent folding and maturation (Fig. 1) [19–22].

2. MIA machinery in the intermembrane space of mitochondria

Many mitochondrial intermembrane space proteins are small (<20 kDa) and contain conserved cysteine residues that are involved in binding of cofactors, metals and the formation of disulfide bonds [19,23]. Typical examples of proteins that have served as model substrates in analyzing protein biogenesis in the mitochondrial intermembrane space include the family of small Tim proteins, which act as chaperone complexes within the intermembrane space for the transport of hydrophobic precursors [21,23,24]. There is now ample data that demonstrates that essential cysteine residues of the mature small Tim proteins are engaged in intramolecular disulfide bonds [25–27]. In spite of these observations it remained a mystery for many years where and how the acquisition of disulfide bonds in mitochondrial proteins was taking place. The discovery of the MIA pathway has given a novel perspective on how the biogenesis of essential small Tim proteins and other components of the intermembrane space is accomplished.

2.1. Components of the MIA machinery

Upon availability of the yeast mitochondrial proteome a subset of previously uncharacterized and essential mitochondrial proteins were revealed [28]. Amongst these was the product encoded by the *YKL195w* open reading frame, now referred to as Mia40, which was shown to be located within the mitochondrial intermembrane space [29]. Mia40 is ubiquitously expressed in fungi, plants and animals and contains 6 essential cysteine residues, four of which are arranged in two Cys–X_9–Cys motifs [29–32]. Although conserved through evolution, differences between higher and lower eukaryotes exist with respect to the size of Mia40 and its membrane anchoring [29–32]. Depletion of Mia40 or disruption of its function through mutation specifically inhibited the accumulation of small cysteine rich intermembrane space members, while other protein import pathways into mitochondria remained unaffected [29,30,32]. Mia40 was shown to directly interact with newly imported intermembrane space proteins, and this interaction involves the formation of transient intermolecular disulfide bonds (Fig. 2) [29,33].

The identification of Mia40 represented a breakthrough in understanding the biogenesis pathway undertaken by intermembrane space precursors. The formation of transient mixed disulfides between Mia40 and the translocating protein raised the view that Mia40 was behaving as an oxidizing component that donated disulfides to incoming precursors. Given that disulfide bond formation is a process dependent on a dedicated oxidoreductase system the search for additional players involved in the intermembrane space pathway commenced. This work revealed the FAD-dependent sulfhydryl oxidase Erv1 as an additional contributor to the disulfide exchange pathway within the intermembrane space in cooperation with Mia40 [21,22]. Lisowsky [34] disclosed Erv1 as a factor that was essential for respiration and indispensable for vegetative growth and Erv1 was later shown to function as a FAD-linked sulfhydryl oxidase in the intermembrane space [35], however, its physiological substrates were unknown. Erv1 was also implicated in the biogenesis of the iron–sulfur clusters [36]. Recent efforts have revealed that Erv1 transiently interacts with Mia40 via disulfide bonds and moreover is also required for precursors to productively reach their final destination in the intermembrane space (Fig. 2) [33,37,38].

2.2. Transfer of disulfide bonds driven by the MIA machinery

The detection of transient disulfide conjugates between Mia40 and substrates, as well as between Mia40 and Erv1 raised a possibility that the MIA machinery resembles other systems that serve in the transfer of disulfide bonds. Analogous to the more well-characterized systems detailed above (DsbB–DsbA; PDI-Ero1/Erv2), Erv1 would represent a primary redox centre involved in disulfide bond formation similar to its homolog Erv2 and the functionally related Ero1 from the ER or DsbB from the bacterial periplasm. Mia40 would then function in the transfer of oxidizing equivalents from the primary oxidoreductase to substrate proteins initiating their oxidative folding (Figs. 1 and 2).

Although not yet demonstrated, it is conceivable to suggest that intermembrane space proteins such as small Tim proteins acquire their disulfide bonds while bound to Mia40 and this permits their release in an oxidized state. This turn of events would result in the release of Mia40 in a reduced state. In support of such a scenario only the oxidized form of Mia40 is capable of forming disulfide intermediates with incoming precursors and Erv1 maintains the oxidative state of Mia40 [33,38]. Accordingly, post precursor release it is believed that Mia40 is re-oxidized by Erv1 favoring successive rounds of interaction with newly incoming precursors via mixed disulfide intermediates (Fig. 2) [33]. However, the function of Erv1 may not be limited to the oxidation of Mia40 but, in the case of small Tim substrates, Erv1 appears also to be required in subsequent steps involving their maturation into oligomeric complexes [38]. The reality of such an additional function was realized through the analysis of a conditional *erv1* mutant where intermembrane space substrates were arrested at the Mia40-bound stage and blocked in subsequent assembly [38]. The capacity for Mia40–substrate conjugates to form in this mutant infers that Mia40 is in an oxidized state and that the assembly defects observed are due to an additional and post-Mia function of Erv1. It remains open as to how the interplay between Mia40 and Erv1 is achieved and regulated.

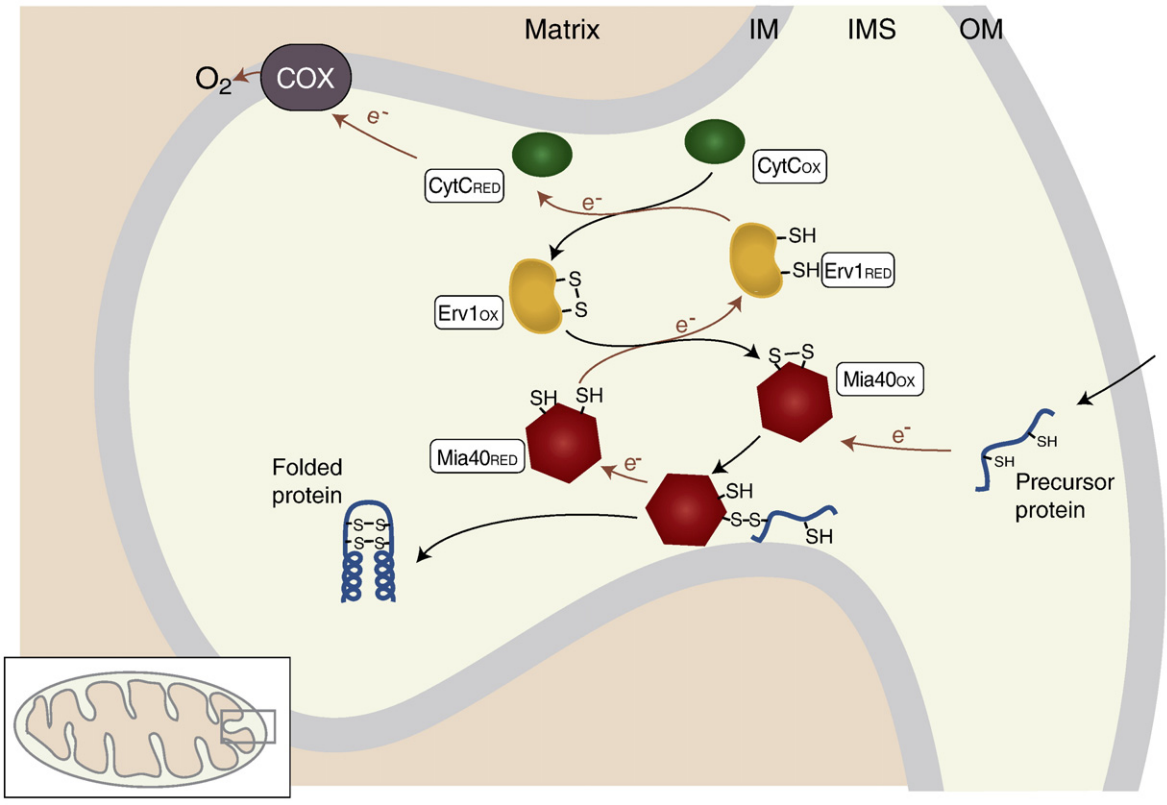

Fig. 2. Disulfide bond formation in the intermembrane space — The MIA pathway. The reactions that mediate redox-regulated protein import into the mitochondrial intermembrane space commence with an association of incoming precursors with the receptor Mia40 via a transient disulfide intermediate. The donation of disulfides by Mia40 to substrates permits their folding and retention within the intermembrane space and results in the release of Mia40 in a reduced form. The sulfhydryl oxidase Erv1 maintains Mia40 in an oxidized state and thus competent for subsequent rounds of precursor import through the donation of electrons to cytochrome *c*. Electron flow from cytochrome *c* is coupled to respiratory chain complexes within the inner membrane to ultimately reduce molecular oxygen to water.

Another interesting problem is what serves as a final electron acceptor for Erv1 in the intermembrane space. Biochemical studies with purified components by Farrell and Thorpe [39] have demonstrated that cytochrome *c* is an efficient electron acceptor for Erv1 (Fig. 2). It seems plausible to pass electrons to respiratory chain complexes via cytochrome *c* to ultimately reduce molecular oxygen to water. Indeed, genetic studies support such a scenario [37]. However, it should be noted that cytochrome *c* could not be the sole electron acceptor for Erv1, since both cytochrome *c* and cytochrome *c* oxidase are dispensable for life under fermentative conditions, whereas the MIA machinery and the biogenesis of intermembrane space proteins are strictly essential.

3. Protein import into mitochondria

The MIA pathway couples thiol–disulfide exchange to protein translocation into the mitochondrial intermembrane space. To emphasize the novel features of this import pathway we will first describe the principles of protein translocation into mitochondria.

Protein sorting and translocation across biological membranes is characterized by a number of common features. Receptor-like proteins that are capable of recognizing targeting elements within precursor proteins serve as a first point of contact. Precursor proteins pass the membrane via water-filled channels formed by membrane-embedded protein complexes. Increasing binding affinities towards consecutive components and/ or force generated by nucleotide hydrolysis drives such translocation events. These general considerations hold true for protein import into mitochondria [40].

The prokaryotic ancestry of mitochondria has rendered the organelle one of the more structurally complex organelles within the eukaryotic cell defined by the presence of four sub-compartments, the mitochondrial outer and inner membranes that serve as barriers for the two aqueous compartments, the mitochondrial intermembrane space and matrix. Given that the majority of mitochondrial proteins are nuclear encoded the task of precursor import and sorting into one of the mitochondrial sub-compartments is essential for maintaining organelle function and ultimately cellular survival. These complex structural features of the organelle have led to the evolution of alternative import pathways and mechanisms for the successful delivery and integration of different precursors into mitochondria (Fig. 3) [21,41–46].

3.1. Protein import across and into mitochondrial membranes

The Translocase of the Outer mitochondrial Membrane (TOM complex) represents the first point of contact for nuclear encoded precursors with the organelle, providing not only a recognition site but also an entry gate through which precursors

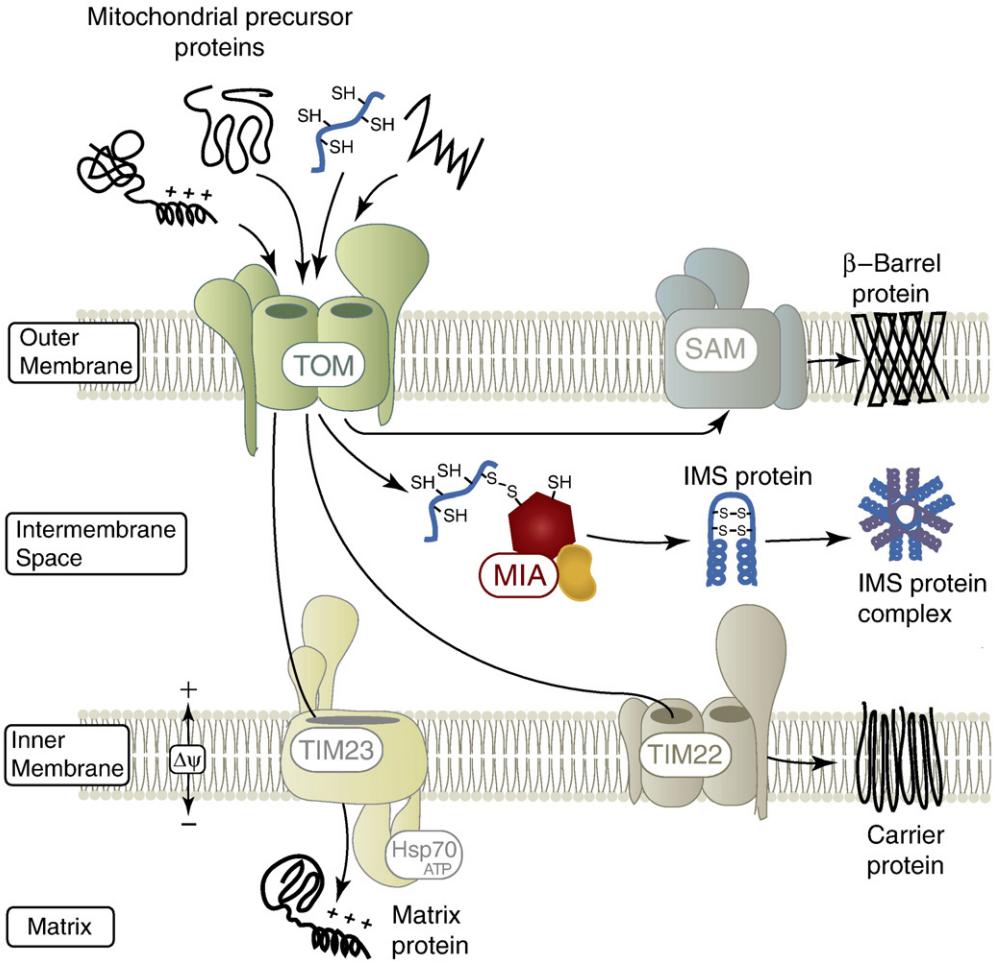

Fig. 3. Biogenesis of mitochondrial proteins. The greater part of mitochondrial precursors are synthesized on cytosolic ribosomes and are subsequently imported into the organelle in a post-translational manner. Common to all precursors is their delivery to the translocase of the outer membrane (TOM complex), which serves as a recognition point and entry gate through which precursors can enter the organelle. Following translocation across the outer membrane import pathways diverge depending on individual or multiple targeting elements contained within precursors. Precursors possessing an N-terminal presequence are sorted to the presequence translocase of the inner membrane (TIM23 complex) in a membrane potential ($\Delta\psi$)-dependent manner. Complete translocation of precursors into the mitochondrial matrix is an ATP-driven process and upon entry into the matrix proteolytic removal of the presequence permits folding of matrix residents into their functional state. Hydrophobic inner membrane proteins (carriers) possessing non-cleavable targeting elements are delivered to and inserted into the inner membrane by the $\Delta\psi$-driven carrier translocase (TIM22 complex). Small cysteine rich intermembrane space precursors are translocated through the TOM complex and their import is facilitated by the intermembrane space specific and redox regulated import machinery, consisting of Mia40 and Erv1. Precursors of outer membrane β-barrel proteins are translocated through the TOM complex into the intermembrane space and directed to the sorting and assembly machinery (SAM complex) for their integration into the outer membrane.

can traverse the outer membrane (Fig. 3) [41–46]. After translocation through the TOM complex there is a specific segregation of precursor import pathways, and successive involvement of further machineries permitting precursor translocation across or integration into the mitochondrial membranes. The "classical" import pathway into mitochondria is defined by the presence of an N-terminal positively-charged extension termed a presequence, which is typically found in precursors destined for the mitochondrial matrix or inner membrane. Such precursors are directed to the presequence Translocase of the Inner Membrane (TIM23 complex) for their subsequent translocation across the inner mitochondrial membrane upon which proteolytic removal of the presequence by the matrix processing peptidase results in release of the mature protein [21,42,47,48]. Some proteins of the inner membrane and intermembrane space are also synthesized with N-terminal presequences. These proteins contain a hydrophobic sorting signal behind the positively charged signal and are arrested in the inner membrane. In case of intermembrane space proteins, a second proteolytic cleavage leads to release of the mature protein into the intermembrane space [21,47,48].

All other classes of mitochondrial precursors do not possess any cleavable signal including proteins localized to the outer membrane and the majority of intermembrane space and inner membrane proteins. Precursors of β-barrel outer membrane proteins, utilize the TOM complex for their translocation into mitochondria, upon which they require the further action of the Sorting and Assembly Machinery (SAM complex) of the outer membrane for their successful integration and assembly into functional complexes [45,49]. Polytopic inner membrane proteins employ an alternative translocase of the inner membrane, the TIM22 or Carrier Translocase [21,42,45,47]. Upon translocation through the

TOM complex these largely hydrophobic precursors are guided through the intermembrane space by chaperone complexes of the small Tim proteins, permitting their delivery to the TIM22 translocon for insertion into the inner membrane [23].

Integration into and import across the mitochondrial inner membrane relies on the membrane potential across the inner membrane both for cleavable and non-cleavable precursor proteins. Complete translocation of cleavable preproteins into the mitochondrial matrix requires an additional driving force gained through ATP hydrolysis by the mitochondrial chaperone, Hsp70 (Fig. 3) [41–45,50].

3.2. Exchange of disulfide bonds as a basis for protein import into the mitochondrial intermembrane space

Like essentially all mitochondrial proteins intermembrane space residents are also nuclear encoded and are synthesized as mature sized proteins within the cytosol. Initial studies indicated that protein import into the intermembrane space requires the TOM complex for translocation across the outer membrane. However, these studies revealed no dependence on an inner membrane potential, ATP or translocation machinery within the inner membrane [51,52]. Referring to the general principles of protein translocation, a striking feature of the intermembrane space pathway was the absence of any known component that would serve as a specific receptor and thus stimulate translocation across the outer membrane. Additionally, cysteine residues within precursors seemed to play an essential role in the accumulation of the proteins in the intermembrane space [26,52]. These observations led to the proposals of the "folding trap" model favoring the passive diffusion of proteins through the TOM complex and the retention of precursors via their rapid and spontaneous folding involving cysteine residues and thus inability to slide back through the TOM complex [24,52]. However, this model lacked the potential existence of a specific import machinery for intermembrane space proteins. The identification of the MIA pathway has put into perspective this biogenesis pathway and it can now be defined as a chain reaction of events that permits translocation and oxidative folding of precursors resulting in their retention within the intermembrane space.

A major question surrounding this topic of study was if the intermembrane space system encompasses general redundancy permitting random cysteine-rich substrates to interact with Mia40. Detailed *in vivo* and *in vitro* analysis revealed that Mia40 is able to specifically recognize its native substrates among a number of other cysteine rich-proteins including those located in other cellular compartments [53]. Moreover, in the case of the model substrates from the small Tim protein family, Mia40 is able to distinguish between single cysteine residues within precursors in a site-specific manner [53,54]. Thus, Mia40 functions as a receptor-like protein dictating the entry of precursors into the intermembrane space-specific biogenesis pathway. In this respect Mia40 possesses some unique features as a receptor as it is capable of tightly coupling the processes of precursor recognition and translocation to the transfer of disulfide bonds [53]. To the best of our knowledge such a mechanism for the translocation of proteins across biological membranes has not been reported so far in any other system, including those of the ER and bacterial periplasm. Enzymes transferring disulfide bonds may begin their mode of action as soon as cysteine residues of substrates emerge from the protein translocating channels. However, in contrast to the MIA machinery, neither their activity nor cysteine residues affect ongoing translocation of precursor proteins in the lumen of the ER or periplasm.

Assigning a specificity and selectivity function to the MIA machinery is a step forward in the understanding of mechanisms that govern biogenesis of intermembrane space proteins. However, the full complement of determinants within the MIA machinery leading to productive import and protein biogenesis in the intermembrane space remains to be determined. A further candidate that likely participates in this pathway is Hot13, which has been identified by its interaction with intermembrane space precursors. It is not yet clear at which stage Hot13 participates in intermembrane space precursor biogenesis, but the available data favour the scenario that Hot13 is involved in late steps of assembly or recycling of small Tim complexes [55].

4. Substrates and substrate specificity of the MIA pathway

The initial characterization of Mia40 and Erv1 employed a subset of substrate proteins including the before mentioned members of the small Tim family, the copper chaperone Cox17 and the related protein Cox19 [29]. These substrates differ in their characteristic cysteine motif with the small Tim proteins belonging to the Cys–X_3–Cys family, while Cox17 and Cox19 belong to the Cys–X_9–Cys family [20,23,29,33]. Recently, the number of substrates exploiting the MIA pathway was increased, with a further three members belonging to the Cys–X_9–Cys family, Mdm35, Mic17 and Mic14, shown to have a dependence on functional Mia40 and Erv1 for import into mitochondria [56]. Additionally, Erv1 itself was also shown to import into the intermembrane space in a MIA-dependent manner [56,57]. These findings not only increased the number of Cys–X_9–Cys substrates exploiting the MIA pathway but also increased the substrate size limit of the MIA pathway. The two largest proteins that had been previously shown to require MIA were Tim13 (11 kDa) and Cox19 (11 kDa) and this additional analysis revealed that precursors up to 22 kDa, such as Erv1, could also exploit this pathway [56,57]. Furthermore, the import of Erv1 in a MIA-dependent manner reveals the capacity of this pathway to import precursors with alternative signature motifs than those belonging to the Cys–X_3–Cys and Cys–X_9–Cys families.

The role of individual cysteine residues in diverse MIA substrates remains an open question. Do intermembrane space proteins require an isomerization or reduction activity for their biogenesis? These and many more questions will undoubtedly be the source for future investigation.

5. Concluding remarks and future perspectives

Although some parallels may be drawn between the intermembrane space oxidative folding pathway to that of the

bacterial periplasm or ER systems, based on several observations the mitochondrial intermembrane space likely utilizes unique mechanisms for the generation of disulfide bonds. Firstly, the formation of mixed disulfide intermediates within the intermembrane space is tightly coupled to precursor translocation across the outer membrane. This represents a novel concept where thiol–disulfide exchange reactions dictate vectorial translocation events, which has not been shown for any other system. Secondly, Mia40 does not share any obvious homology to DsbA or PDI that are its functional equivalents within the periplasm and ER, respectively. Furthermore, Mia40 does not possess any detectable thioredoxin-like motif that is a characteristic feature for PDI and DsbA, and thus its true mode of action remains unknown. Mixed disulfide intermediates between PDI or DsbA with substrates are difficult to detect, likely due to the short-lived nature of these intermediates. However, the mitochondrial system permits the efficient detection of Mia40-bound substrate conjugates. Could this simply be due the different features of the Mia40 protein or alternatively could the coupling of intermediate formation to translocation enhance their stability, lifetime and ultimately detection? Third, the function of Erv1 in mitochondria appears to include a higher level of complexity than its functional equivalents Erv2 and Ero1 from the ER due to its dual role in the biogenesis of small Tim precursors. At which stage of the MIA pathway does Erv1 come into action? How is its more active role in the import of small Tim proteins facilitated? Are there alternative substrates for which Erv1 behaves as a direct donor of oxidizing equivalents?

The novel features defined above for the MIA system are not disheartening by any means as they strongly imply unique mechanisms governing oxidative folding within the mitochondrial intermembrane space. The identification of additional players and the capacity to reconstitute the mitochondrial system *in vitro* will provide a powerful means to address the true mechanisms that permit the acquisition of disulfide bonds in a range of mitochondrial intermembrane space precursors. In the short time since its discovery this field has made significant strides and as further discoveries are brought to light many parallels to more established systems, or conversely unique features and mechanisms may be realized.

Acknowledgments

This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 746, Gottfried Wilhelm Leibniz Program, Max Planck Research Award, Bundesministerium für Bildung und Forschung, and the Fonds der Chemischen Industrie. D.S. is recipient of an Alexander von Humboldt Research Fellowship.

References

[1] J.-F. Collet, J.C. Bardwell, Oxidative protein folding in bacteria, Mol. Microbiol. 44 (2002) 1–8.
[2] H. Kadokura, F. Katzen, J. Beckwith, Protein disulfide bond formation in prokaryotes, Ann. Rev. Biochem. 72 (2003) 111–135.
[3] C.S. Sevier, C.A. Kaiser, Conservation and diversity of the cellular disulfide bond formation pathways, Antioxid. Redox Signal. 8 (2006) 797–811.
[4] R. Glockshuber, Where do the electrons go? Nature 401 (1999) 30–31.
[5] A. Rietsch, J. Beckwith, The genetics of disulfide bond metabolism, Annu. Rev. Genet. 32 (1998) 163–184.
[6] J. Messens, J.-F. Collet, Pathways of disulfide bond formation in *Escherichia coli*, Int. J. Biochem. Cell Biol. 38 (2006) 1050–1062.
[7] J.L. Martin, J.C. Bardwell, J. Kuriyan, Crystal structure of the DsbA protein required for disulphide bond formation *in vivo*, Nature 365 (1993) 464–468.
[8] K. Inaba, S. Murakami, M. Suzuki, A. Nakagawa, E. Yamashita, K. Okada, K. Ito, Crystal structure of the DsbB–DsbA complex reveals a mechanism of disulfide bond generation, Cell 127 (2006) 789–801.
[9] R.B. Freedman, T.R. Hirst, M.F. Tuite, Protein disulfide isomerase: building bridges in protein folding, Trends Biochem. Sci. 19 (1994) 331–336.
[10] B. Wilkinson, H.F. Gilbert, Protein disulfide isomerase, Biochim. Biophys. Acta 1699 (2004) 35–44.
[11] A. Benham, R. Sitia, The diversity of oxidative protein folding, Antioxid. Redox Signal. 8 (2006) 271–273.
[12] B.P. Tu, J.S. Weissman, Oxidative protein folding in eukaryotes: mechanisms and consequences, J. Cell Biol. 164 (2004) 341–346.
[13] E. Gross, D.B. Kastner, C.A. Kaiser, D. Fass, Structure of Ero1p, source of disulfide bonds for oxidative protein folding in the cell, Cell 117 (2004) 601–610.
[14] C.S. Sevier, J.W. Cuozzo, A. Vala, F. Aslund, C.A. Kaiser, A flavoprotein oxidase defines a new endoplasmic reticulum pathway for biosynthetic disulphide bond formation, Nat. Cell Biol. 3 (2001) 874–882.
[15] G. Hofhaus, G. Stein, L. Polimeno, A. Francavilla, T. Lisowsky, Highly divergent amino termini of the homologous human ARL and yeast scERV1 gene products define species specific differences in cellular localization, Eur. J. Cell Biol. 78 (1999) 349–356.
[16] J. Gerber, U. Mühlenhoff, G. Hofhaus, R. Lill, T. Lisowsky, Yeast Erv2p is the first microsomal FAD-linked sulfhydryl oxidase of the Erv1/Alrp protein family, J. Biol. Chem. 276 (2001) 23486–23491.
[17] D.L. Coppock, C. Thorpe, Multidomain flavin-dependent sylfhydryl oxidases, Antioxid. Redox Signal. 8 (2006) 300–311.
[18] T.G. Senkevich, C.L. White, E.V. Koonin, B. Moss, Complete pathway for protein disulfide bond formation encoded by poxviruses, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 6667–6672.
[19] K. Tokatlidis, A disulfide relay system in mitochondria, Cell 121 (2005) 965–967.
[20] C.M. Koehler, K.N. Beverly, E.P. Leverich, Redox pathways of the mitochondrion, Antioxid. Redox Signal. 8 (2006) 731–733.
[21] D. Milenkovic, J. Müller, D. Stojanovski, N. Pfanner, A. Chacinska, Diverse mechanisms and machineries for import of mitochondrial precursors, Biol. Chem. 388 (2007) 891–897.
[22] J.M. Herrmann, R. Köhl, Catch me if you can! Oxidative protein trapping in the intermembrane space of mitochondria, J. Cell Biol. 176 (2007) 559–563.
[23] C.M. Koehler, The small Tim proteins and the twin Cx_3C motif, Trends Biochem. Sci. 29 (2004) 1–4.
[24] J.M. Herrmann, K. Hell, Chopped, trapped or tacked — protein translocation into the IMS of mitochondria, Trends Biochem. Sci. 30 (2005) 205–211.
[25] S.P. Curran, D. Leuenberger, W. Oppliger, C.M. Koehler, The Tim9p–Tim10p complex binds to the transmembrane domains of the ADP/ATP carrier, EMBO J. 21 (2002) 942–953.
[26] H. Lu, S. Allen, L. Wardleworth, P. Savory, K. Tokatlidis, Functional TIM10 chaperone assembly is redox-regulated *in vivo*, J. Biol. Chem. 279 (2004) 18952–18958.
[27] C.T. Webb, M.A. Gorman, M. Lazarou, M.T. Ryan, J.M. Gulbis, Crystal structure of the mitochondrial chaperone TIM9–10 reveals a six-bladed α-propeller, Mol. Cell 21 (2006) 123–133.
[28] A. Sickmann, J. Reinders, Y. Wagner, C. Joppich, R. Zahedi, H.E. Meyer, B. Schönfisch, I. Perschil, A. Chacinska, B. Guiard, P. Rehling, N. Pfanner, C. Meisinger, The proteome of *Saccharomyces cerevisiae* mitochondria, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13207–13212.

[29] A. Chacinska, S. Pfannschmidt, N. Wiedemann, V. Kozjak, L.K. Sanjuán-Szklarz, A. Schulze-Specking, K.N. Truscott, B. Guiard, C. Meisinger, N. Pfanner, Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins, EMBO J. 23 (2004) 3735–3746.
[30] M. Naoé, Y. Ohwa, D. Ishikawa, C. Ohshima, S. Ishikawa, H. Yamamoto, T. Endo, Identification of Tim40 that mediates protein sorting to the mitochondrial intermembrane space, J. Biol. Chem. 279 (2004) 47815–47821.
[31] S. Hofmann, U. Rothbauer, N. Mühlenbein, K. Baiker, K. Hell, M.F. Bauer, Functional and mutational characterization of human MIA40 acting during import into the mitochondrial intermembrane space, J. Mol. Biol. 353 (2005) 517–528.
[32] N. Terziyska, T. Lutz, C. Kozany, D. Mokranjac, N. Mesecke, W. Neupert, M. Herrmann, K. Hell, Mia40, a novel factor for protein import into the intermembrane space of mitochondria is able to bind metal ions, FEBS Lett. 579 (2005) 179–184.
[33] N. Mesecke, N. Terziyska, C. Kozany, F. Baumann, W. Neupert, K. Hell, J.M. Herrmann, A disulfide relay system in the intermembrane space of mitochondria that mediates protein import, Cell 121 (2005) 1059–1069.
[34] T. Lisowsky, Dual function of a new nuclear gene for oxidative phosphorylation and vegetative growth in yeast, Mol. Gen. Genet. 232 (1992) 58–64.
[35] J. Lee, G. Hofhaus, T. Lisowsky, Erv1p from *Saccharomyces cerevisiae* is a FAD-linked sulfhydryl oxidase, FEBS Lett. 477 (2000) 62–66.
[36] H. Lange, T. Lisowsky, J. Gerber, U. Mühlenhoff, G. Kispal, R. Lill, An essential function of the mitochondrial sulfhydryl oxidase Erv1p/ALR in the maturation of cytosolic Fe/S proteins, EMBO Rep. 2 (2001) 715–720.
[37] S. Allen, V. Balabanidou, D.P. Sideris, T. Lisowsky, K. Tokatlidis, Erv1 mediates the Mia40-dependent protein import pathway and provides a functional link to the respiratory chain by shuttling electrons to cytochrome *c*, J. Mol. Biol. 353 (2005) 937–344.
[38] M. Rissler, N. Wiedemann, S. Pfannschmidt, K. Gabriel, B. Guiard, N. Pfanner, A. Chacinska, The essential mitochondrial protein Erv1 cooperates with Mia40 in biogenesis of intermembrane space proteins, J. Mol. Biol. 353 (2005) 485–492.
[39] S.R. Farrell, C. Thorpe, Augmenter of liver regeneration: a flavin-dependent sulfhydryl oxidase with cytochrome *c* reductase activity, Biochemistry 44 (2005) 1532–1541.
[40] G. Schatz, B. Dobberstein, Common principles of protein translocation across membranes, Science 271 (1996) 1519–1526.
[41] N. Pfanner, A. Geissler, Versatility of the mitochondrial protein import machinery, Nat. Rev., Mol. Cell Biol. 2 (2001) 339–349.
[42] R.E. Jensen, A.E. Johnson, Opening the door to mitochondrial protein import, Nat. Struct. Biol. 8 (2001) 1008–1010.
[43] T. Endo, H. Yamamoto, M. Esaki, Functional cooperation and separation of translocators in protein import into mitochondria, the double-membrane bounded organelles, J. Cell Sci. 116 (2003) 3259–3267.
[44] P. Dolezal, V. Likic, J. Tachezy, T. Lithgow, Evolution of the molecular machines for protein import into mitochondria, Science 313 (2006) 314–318.
[45] W. Neupert, J.M. Herrmann, Translocation of proteins into mitochondria, Ann. Rev. Biochem. 76 (2007) 723–749.
[46] M.J. Baker, A.E. Frazier, J.M. Gulbis, MT. Ryan, Mitochondrial protein-import machinery: correlating structure with function, Trends Cell Biol. 17 (2007) 456–464.
[47] M. van der Laan, M. Rissler, P. Rehling, Mitochondrial preprotein translocases as dynamic molecular machines, FEMS Yeast Res. 6 (2006) 849–861.
[48] D. Mokranjac, W. Neupert, Protein import into mitochondria, Biochem. Soc. Trans. 33 (2005) 1019–1123.
[49] D. Stojanovski, M. Rissler, N. Pfanner, C. Meisinger, Mitochondrial morphology and protein import — a tight connection? Biochim. Biophys. Acta 1763 (2006) 414–421.
[50] N.J. Hoogenraad, L.A. Ward, M.T. Ryan, Import and assembly of proteins into mitochondria of mammalian cells, Biochim. Biophys. Acta 1592 (2002) 97–105.
[51] M. Kurz, H. Martin, J. Rassow, N. Pfanner, M.T. Ryan, Biogenesis of Tim proteins of the mitochondrial carrier import pathway: differential targeting mechanisms and crossing over with the main import pathway, Mol. Biol. Cell 10 (1999) 2461–2474.
[52] T. Lutz, W. Neupert, J.M. Herrmann, Import of small Tim proteins into the mitochondrial intermembrane space, EMBO J. 22 (2003) 4400–4408.
[53] D. Milenkovic, K. Gabriel, B. Guiard, A. Schulze-Specking, N. Pfanner, A. Chacinska, Biogenesis of the essential Tim9–Tim10 chaperone complex of mitochondria: site-specific recognition of cysteine residues by the intermembrane space receptor Mia40, J. Biol. Chem. 282 (2007) 22472–22480.
[54] D.P. Sideris, K. Tokatlidis, Oxidative folding of small Tims is mediated by site-specific docking onto Mia40 in the mitochondrial intermembrane space, Mol. Microbiol. 65 (2007) 1360–1373.
[55] S.P. Curran, D. Leuenberger, E.P. Leverich, D.K. Hwang, K.N. Beverly, C.M. Koehler, The role of Hot13p and redox chemistry in the mitochondrial TIM22 import pathway, J. Biol. Chem. 279 (2004) 43744–43751.
[56] K. Gabriel, D. Milenkovic, A. Chacinska, J. Müller, B. Guiard, N. Pfanner, C. Meisinger, Novel mitochondrial intermembrane space proteins as substrates of the MIA import pathway, J. Mol. Biol. 365 (2007) 612–620.
[57] N. Terziyska, B. Grumbt, M. Bien, W. Neupert, J.M. Hermann, K. Hell, The sulfhydryl oxidase Erv1 is a substrate of the Mia40-dependent protein translocation pathway, FEBS Lett. 581 (2007) 1098–1102.