Sorting of Preproteins into Mitochondria

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1. Introduction

Mitochondria are the center of the oxidative metabolism of all known eukaryotes. Several observations, including sequence comparisons of the mitochondrial 16S RNA, indicate that mitochondria evolved from early α-proteobacteria by endosymbiosis.[1] Out of the some 1000 different proteins that are present in mitochondria, only 1–2%, depending on the organism, are encoded by the small mitochondrial genome.[2–4] The majority of them is nuclear-encoded, synthesized on cytosolic polysomes, and posttranslationally imported.[5, 6] Due to the compartmentalization of an eukaryotic cell, an effective sorting of proteins synthesized in the cytosol to their target organelle is required.[7] Proteins that have to leave the cytosol after their synthesis exhibit signal sequences that enable the cell to transport these proteins to their respective target compartment and to translocate them into the organelles.[8] Due to their double membrane, mitochondria contain four subcompartments: the outer mitochondrial membrane, the intermembrane space, the inner mitochondrial membrane, and the matrix. Mitochondrial preproteins contain sorting sequences that determine the final localization within these compartments.[9, 10]

The recent sequencing of several eukaryotic genomes has confirmed that the machineries involved in intracellular transport are highly conserved. Nearly all studies on the mitochondrial protein import machinery were carried out using the yeast *Saccharomyces cerevisiae* and the filamentous fungus *Neurospora crassa* as model organisms. However, the mitochondria of higher eukaryotes, including those of mammals, appear to contain a homologous set of Tom and Tim proteins and to apply the same mechanistic principles.

2. Protein targeting to mitochondria

To be sorted, most preproteins destined for the mitochondrial matrix, the intermembrane space, or the inner membrane carry an amino-terminal presequence. Mitochondrial presequences are able to form amphipathic α helices.[11] A conserved primary sequence is not observed, but positively charged, hydroxylated, and hydrophobic amino acids are characteristic, while negatively charged residues are completely absent. Structural elements and the positive net charge seem to be more important for the recognition by mitochondrial receptors than single residues.[12, 13] Perhaps it is this variability in sequence that makes it possible to import virtually all mitochondrial proteins with the help of the same protein complex in the outer membrane. In the mitochondrial matrix, the presequences (20 to 80 amino acids in length) are cleaved off by the heterodimeric mitochondrial processing peptidase (MPP).[14–16]

For targeting into the intermembrane space, several preproteins contain a specific sorting sequence directly following the amino-terminal presequence. These sorting sequences contain a central hydrophobic segment and resemble the signal sequences of bacterial secretory proteins. In the intermembrane space these sequences are cleaved off by the inner-membrane peptidase (IMP).[17–20] While the function of amino-terminal presequences in targeting is well established,[21, 22] less is known about the functions of sorting sequences. Some mitochondrial proteins, especially those of the outer and inner mitochondrial membranes, do not have a presequence. Therefore the mature protein itself must carry the necessary targeting information.[23] No consensus sequences or more general structural properties could be identified, which could be responsible for the specific recognition of this type of proteins. For many proteins the targeting signal is still unknown.

In contrast to secretory proteins, which are mostly translocated cotranslationally into the endoplasmic reticulum,[24] mitochondrial proteins are generally synthesized on free ribosomes and imported posttranslationally.[25] Already during their synthesis, these proteins are bound to cytosolic chaperones of the Hsp70 family (heat shock proteins of a molecular mass of ca. 70 kDa) and thus kept in an unfolded state, a prerequisite for their subsequent translocation (Figure 1).[26] Preproteins that contain folded domains prior to their translocation have to be unfolded during import into the mitochondria.[27–29]

Binding of cytosolic Hsp70, however, is not specific for mitochondrial proteins, since Hsp70 also binds to proteins that are posttranslationally transported into the endoplasmic reticulum.[29] Certain proteins that guide the transport to the receptor

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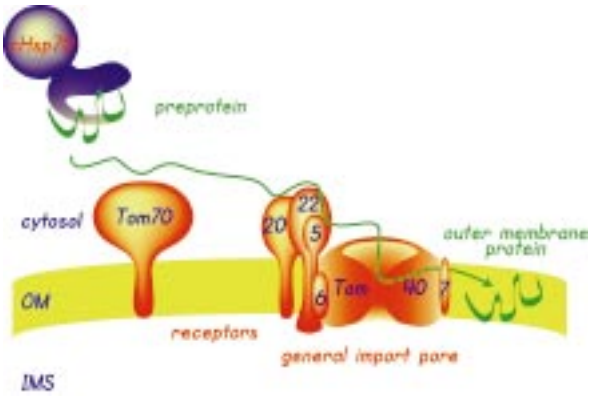

Figure 1. *Membrane insertion of mitochondrial outer-membrane proteins. Mitochondrial preproteins destined for the outer mitochondrial membrane (OM) are synthesized on cytosolic polysomes, bound by cytosolic chaperones (cHsp70), and recognized by receptor proteins (Tom70 or Tom20) on the outer membrane. With the help of Tom22 and Tom5, these proteins are transferred to the channel component Tom40. They are subsequently inserted into the outer mitochondrial membrane by diffusing laterally out of the general import pore. IMS = inter-membrane space.*

proteins on the surface of the outer mitochondrial membrane recognize specifically the signal sequences of mitochondrial proteins. The mitochondrial import-stimulating factor (MSF) may serve as an example. In the mammalian cytosol, MSF prevents the aggregation of some preproteins and supports their binding to mitochondria.[30–32]

Although most preproteins are thought to follow the post-translational import mechanism described above, some proteins may be imported in a cotranslational manner. In this process, translating ribosomes bind directly to the translocation site, thereby coupling the translocation directly to the translation of the proteins. When mitochondria are isolated from yeast cells arrested in translation, ribosomes are bound on the surface of mitochondria, suggesting that cotranslational transport is a possible mechanism in yeast.[33] In vitro and in vivo data confirm this observation.[34, 35]

3. Binding to the import receptors of the outer mitochondrial membrane

At the mitochondrial surface, most preproteins are recognized and transiently bound by specific import receptor proteins and finally transferred to the general protein import channel of the outer membrane, the general import pore (GIP).[36] Proteins of the outer membrane which are involved in translocation are termed Tom proteins (*t*ranslocase of the *o*uter *m*itochondrial membrane) and are further specified by a number corresponding to their approximate molecular mass in kDa.[37]

The two receptor proteins Tom20 and Tom70 show different, but partially overlapping specificities for preproteins.[38] Both receptors contain an amino-terminal membrane anchor and a carboxy-terminal cytosolic domain (Figure 1).[39, 40] Tom20 recognizes mainly proteins that carry a presequence by direct, mostly hydrophobic interactions with the α helix of the presequence.[21, 41, 42] Recently, some proteins were found that bind Tom20 although they lack a mitochondrial presequence. For example, the outer-membrane proteins porin[43] and Tom40,[44] the intermembrane-space protein cytochrome *c* heme lyase[45] as well as the inner-membrane protein Tim22 (*t*ranslocase of the *i*nner *m*itochondrial membrane).[46] Proteins bound to MSF prefer binding to Tom70. In this case, the preprotein is thought to bind with different parts to several molecules of Tom70 at the same time. Thus, Tom70 could fulfil the tasks of a chaperone and prevent irreversible aggregation of preproteins in transit.[42, 47–50]

Both receptors transfer the bound preproteins to the general import pore (GIP).[49] In this context Tom22 plays an important role. First, Tom22 interacts with presequences and serves as an additional import receptor. Second, Tom22 is an integral part of the GIP and serves as a central organizer of this complex.[50] Tom22 mediates the interaction of Tom20 with the GIP, and it appears to interact with the hydrophilic side of presequences.[41] Therefore it is assumed that presequences sequentially bind to Tom20 with their hydrophobic part and to Tom22 with their hydrophilic part.[49, 51, 52] The complex of Tom70 with the TOM complex is less stable. The interactions are mediated by tetratrico peptide repeat (TPR) motives.[53]

Mitochondrial protein import is not entirely dependent on the import receptors. It was recently shown that the precursors of some components of the intermembrane space do not require receptor proteins during import. They can directly enter the mitochondria through the GIP.[46]

4. Transport into and across the mitochondrial outer membrane

Further translocation of receptor-bound preproteins is mediated by the GIP complex. The components of the GIP complex are Tom22, Tom40, and the small Tom proteins Tom5, Tom6, and Tom7.[54] The intact complex has a molecular mass of about 400 kDa; in the absence of Tom22, this complex disassembles into subcomplexes of ca. 100 kDa, demonstrating the central role of Tom22.[50, 55]

Besides Tom22, only Tom5 possesses an amino-terminal cytosolic domain, whereas the other components of the TOM complex are deeply buried in the membrane.[56] Tom5 was shown to facilitate the transfer of preproteins from the import receptors to the GIP. The pathway of preproteins is thus formed by a series of Tom proteins: Preproteins are picked up by Tom20 and then transferred to Tom22, Tom5, and finally to Tom40 that forms the pore.[56]

Tom40 spans the membrane presumably in a porin-like manner with several β strands that form a β barrel with a pore of ca. 2 nm in width. The channel was characterized electrophysiologically after reconstitution of purified Tom40 into liposomes. Reconstituted Tom40 specifically reacted with synthetic presequence peptides, demonstrating that the channel contains a binding site for presequences.[57] Interestingly, electron micrographs of the complete TOM complex that was isolated from mitochondria show up to three pores, each of

which displays an inner diameter of about 2 nm.[58] Tom22 modulates the channel properties by facilitating the closing of the channel.[50] Tom6 and Tom7 modulate the stability of the TOM complex in an antagonistic fashion, providing both sufficient stability to allow rapid transfer of preproteins from the import receptors to the Tom40 channel and sufficient flexibility for the opening of the translocon. Tom6 promotes assembly of Tom22 with Tom40 and facilitates the transfer of preproteins.[59] In contrast, Tom7 supports a dissociation of the translocase and thus is involved in the import of outer-membrane proteins that first insert into the Tom40 pore, but then diffuse laterally into the membrane.[60, 61]

The driving force for the transport of preproteins across the general import pore is not known to date. In contrast to the transport of preproteins across the inner mitochondrial membrane, neither membrane potential nor ATP hydrolysis are required.[55] Preproteins with a positively charged presequence could make use of the patches of negative charges that are found in some Tom proteins.[49] According to the acid-chain hypothesis,[62] these negative patches should permit a binding to Tom20, Tom70, and Tom22. Although the initial recognition of the preproteins by Tom20 occurs through a hydrophobic interaction, the negatively charged residues probably function as the first binding site in a sequential binding and release chain. Negative charges in the amino terminus of Tom5 would direct the presequences to Tom40. The intermembrane-space domain of Tom22 also contains a patch with a negative net charge, which could function as a trans binding site. In this scenario, preproteins would be guided across the outer membrane by a chain of binding sites, including ionic interactions of increasing strength, and then handed over to the translocation machinery of the inner membrane.[63] Accordingly, the amphipathic character of cleavable presequences might reflect a bifunctionality. While the hydrophobic side facilitates the initial recognition by Tom20, the charged side is responsible for the import driving by the mechanism described above.

Some proteins lacking a cleavable presequence contain internal segments with presequence-like properties that may use an acid chain. However, additional mechanisms may be involved. The soluble Tim proteins of the intermembrane space were shown to bind to some hydrophobic proteins as soon as they emerge out of the GIP. By this mechanism, at least a backsliding could be prevented. The mechanism that allows the release from the GIP and the transport into the intermembane space remains to be established.[64]

The mechanisms by which outer-membrane proteins are imported have not been investigated in detail yet. The involvement of components of the GIP complex in the import of some outer-membrane proteins suggested the active participation of the GIP in their biogenesis.[50, 56, 61] Using liposomes and purified Tom20, it was proposed, however, that porin, the most abundant outer-membrane protein, can be inserted into a membrane in the absence of Tom5, Tom40, or other components o the GIP.[43, 65] A detailed study on the effects of several Tom mutants has now clarified that with intact mitochondria, receptors and the GIP are required for efficient import and assembly of porin in the outer membrane.[66]

5. Transport into the intermembrane space

For targeting into the intermembrane space, preproteins can follow different pathways. Some proteins of this compartment are imported independently of the outer-membrane import receptors. The precursors of the small Tim proteins, Tim9, Tim10, and Tim12, apparently do not require Tom20 or Tom70 for efficient import.[46] These soluble proteins of the intermembrane space are all synthesized without a presequence. Their import is independent of the membrane potential $\Delta\psi$ across the inner membrane, and the proteins do not require the involvement of the inner-membrane TIM complexes[46, 67] (Figure 2, pathway I).

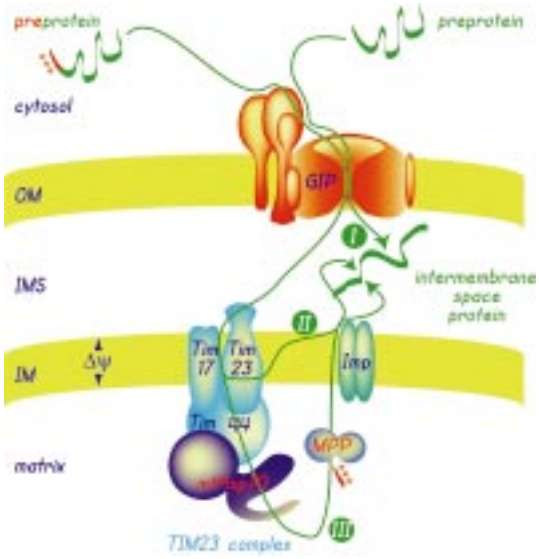

Figure 2. *Translocation of preproteins into the intermembrane space (IMS). Several preproteins of the intermembrane space are synthesized without an N-terminal presequence. Following insertion into the general import pore (GIP) complex, the preproteins are translocated across the outer membrane (OM) and arrive in the IMS (pathway I). Other IMS-located proteins are synthesized with a typical matrix-targeting sequence (red segment; positive charges are indicated). These proteins cross the outer membrane and insert into the TIM23 complex of the inner membrane (IM). This complex mediates translocation at least of the matrix-targeting sequence of the preproteins into the mitochondrial matrix. Here it is cleaved off by the action of the mitochondrial processing peptidase (MPP). The sorting sequence, located behind the matrix-targeting sequence, typically arrests the protein in the inner membrane (stop-transfer). The inner-membrane peptidase (Imp) cleaves at the intermembrane-space side to release the mature protein (pathway II). Some proteins may first be imported into the matrix with the mature part and then be exported across the IM (conservative sorting, pathway III). Translocation of proteins into the intermembrane space via the TIM23 complex requires the presence of a membrane potential ($\Delta\psi$).*

Other proteins destined for the intermembrane space (or the intermembrane-space side of the inner membrane) are synthesized with a bipartite presequence, including a positively charged matrix-targeting sequence and an additional sorting sequence,[20] and make use of the TIM23 complex of the inner membrane. To address the possible mechanisms that are involved, the import of cytochrome b_2 as a model protein was investigated in several studies. Unlike direct import into the intermembrane space by transport through the TOM complex, import pathways that involve the TIM components depend on

the membrane potential $\Delta\psi$.[70, 71] Two possible pathways were discussed to explain the participation of the TIM complex:

- In the stop-transfer mechanism (Figure 2, pathway II), the positively charged first half of the presequence of cytochrome b_2 is translocated across the inner membrane and is cleaved off in the matrix by MPP, while the sorting sequence gets stuck in the TIM23 complex and is laterally released into the inner membrane.[9, 69, 70] The second sequence is subsequently cleaved at the intermembrane-space side by Imp1.[19] The mature protein finally is a soluble component in the intermembrane space. The sorting sequence includes a hydrophobic segment and positively charged amino acid residues. The sorting sequence is not simply arrested in the inner membrane due to its hydrophobic nature, but more complex structural properties seem to be required for the recognition of a "stop-transfer" signal by the inner-membrane import machinery.[71] It has been proposed that the sorting sequence assumes loop-like structure when inserting into the inner membrane.[69, 72]
- Other proteins, for example the Rieske iron – sulfur protein, are first translocated completely into the matrix.[10] The presequence is cleaved and the protein is exported to the intermembrane-space side (conservative sorting, Figure 2, pathway III). Like all proteins that are translocated through the Tim23/Tim17 channel, proteins sorted to the intermembrane space by this mechanism need the membrane potential $\Delta\psi$ to reach their functional destination.[73, 74]

An attractive aspect of the conservative sorting hypothesis is the relation to the evolutionary origin of mitochondria. Preproteins are first completely imported into the matrix to reach the situation of proteins that are exported by bacteria. In fact, the sorting sequences of several mitochondrial preproteins resemble the signal sequences of bacterial proteins. However, the sequencing of the yeast genome has revealed that yeast does not contain homologues of the bacterial Sec complex, demonstrating that this part of the bacterial protein transport machinery was lost during evolution.[75] It is now clear that there is no general pathway for protein sorting in mitochondria since different preproteins show significant differences in their sorting mechanisms.

6. Transport into the inner mitochondrial membrane

Many proteins of the inner mitochondrial membrane are synthesized without a cleavable presequence, for example, the metabolite transporters (carriers). These proteins bind on the trans side of the GIP to a complex of soluble Tim proteins of the intermembrane space.[76, 77] Tim9 and Tim10 are thought to form a 70-kDa heterohexamer or -octamer.[78, 79] In this ternary complex the hydrophobic preprotein is shuttled through the aqueous intermembrane space to a translocase of the inner membrane that consists of Tim12, Tim18, Tim22, and Tim54 and is termed the TIM22 complex (Figure 3).[80–82] Tim12 is a peripheral membrane protein,[83] but Tim18, Tim22, and Tim54

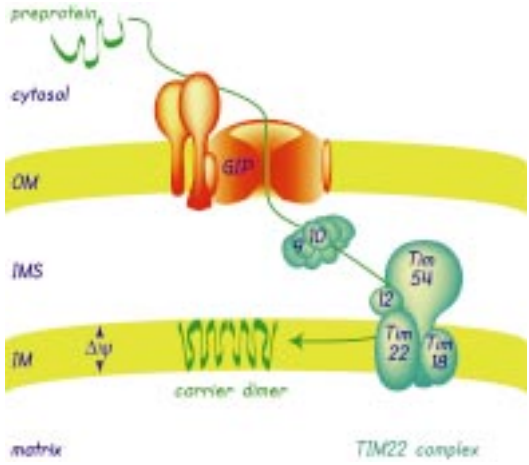

Figure 3. *Membrane insertion of carrier proteins into the inner membrane (IM). The carrier preproteins traverse the general import pore (GIP complex) and bind to a complex consisting of Tim9 and Tim10 in the intermembrane space (IMS), which transfers these preproteins to the TIM22 complex in the inner membrane. This complex mediates the insertion of the carrier preproteins with the help of the membrane potential $\Delta\psi$ into the inner membrane. Here the carrier proteins dimerize and adopt their functional state.*

are integral membrane proteins.[82] The preprotein gets in contact with the TIM22 complex, possibly by exchanging one subunit in the Tim9/Tim10 complex against membrane-associated Tim12.[76, 78, 84] The membrane potential $\Delta\psi$ across the inner membrane is required for insertion of the preproteins into the TIM22 complex.[64] The carrier protein is then released into the inner membrane and assembles into the functional dimer (Figure 3). The exact mechanism of this newly discovered transport pathway as well as the specific tasks of the proteins involved are still under investigation. Meanwhile, two additional small Tim proteins have been identified, Tim8 and Tim13, that are highly homologous to Tim9 and Tim10.[85]

Until now, the Tim22/Tim54 pathway was shown to be used by members of the carrier family.[81] Moreover, all components of the mitochondrial import machinery are themselves encoded by nuclear genes and must be imported from the cytosol. The preproteins of Tim17, Tim23,[86] and Tim22[46] are integrated via the TOM apparatus and the TIM22 complex, whereas the preproteins of Tim54 use the TOM apparatus and the TIM23 complex like presequence-containing preproteins.[46]

Besides the Tim machinery, the inner membrane contains two additional components that are involved in the insertion of proteins into this membrane by an export mechanism from the matrix. The Oxa1 protein was shown to be crucial for the export of N-terminal tails of nuclear-encoded proteins from the matrix to the intermembrane space, as well as for the insertion of inner-membrane proteins encoded by the mitochondrial genome.[87–91] Interestingly, Oxa1 is highly homologous to YidC and Albino3, proteins that are essential for the import of a class of proteins into the *Escherichia coli* plasma membrane and into the thylakoid membrane of chloroplasts.[92–94] Recently Pnt1 was identified as a possible component involved in the export of mitochondrially encoded proteins.[91] A possible interaction of the two components is not known to date.

7. Transport into the mitochondrial matrix

Proteins containing positively charged mitochondrial presequences do not need soluble proteins in the intermembrane space for their translocation. After leaving the TOM complex, the presequences bind to the integral membrane protein Tim23.[92, 93] Tim23 exposes numerous negative charges in its amino-terminal intermembrane-space domain that are discussed to provide a binding site for presequences. Preproteins spanning both the outer-membrane TOM complex and the inner-membrane TIM complex form TIM – TOM supercomplexes of about 600 kDa.[79, 94–96] More recently, evidence was provided that the amino terminus of Tim23 inserts into the outer membrane, thus bridging both membranes in the absence of translocating preproteins.[97] The function of this unique topology of a membrane protein remains to be established.

Tim17 is a homologue of Tim23.[98, 99] Tim23 and Tim17 together form a 90-kDa complex in the inner membrane (Figure 4);[96, 100] additional components may be involved. Tim17 and Tim23 both span the inner membrane with four

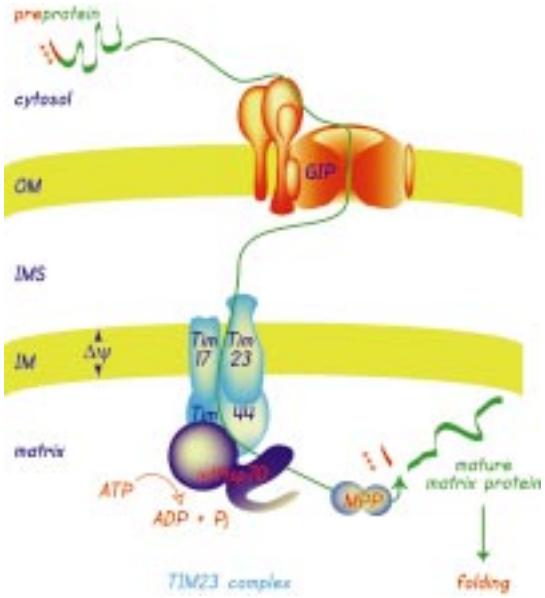

Figure 4. *Translocation of proteins of the mitochondrial matrix. These proteins are typically synthesized with an N-terminal presequence (red segment; positive charges are indicated) in the cytosol. After passing the mitochondrial outer membrane (OM) by using of the general import pore (GIP complex), these proteins bind to Tim23 of the inner membrane (IM). They are then transported through the Tim23 – Tim17 channel across the inner membrane into the matrix, driven by the membrane potential $\Delta\psi$. The mitochondrial heat shock protein 70 (mtHsp70), which is attached to the inner membrane via Tim44, drives the import in an ATP-dependent manner. The matrix-processing peptidase (MPP) cleaves off the presequence and the mature proteins are folded into their native states.*

putative α helices, and both proteins show a sequence homology of 46% in the membrane-spanning region.[98] By the help of a leucin zipper in its amino terminusm, Tim23 is capable of forming homodimers.[93] Disassembly of these dimers due to the arrival of a presequence is thought to be the first step in translocating a protein across the inner membrane. In addition, the membrane potential $\Delta\psi$ (negative inside) exerts an electrophoretic force on the positively charged presequences and therefore is an essential prerequisite for translocation into the matrix.[72, 74, 101]

The translocation of the complete protein, however, requires the hydrolysis of ATP by mtHsp70, a soluble heat shock protein of 70 kDa in the mitochondrial matrix.[68, 102, 103] Most ATP that is hydrolyzed during the translocation of a preprotein is probably required for the interaction of preproteins with mtHsp70. However, additional ATP is required for productive interactions with the chaperone proteins in the cytosol.[103, 104] Preproteins that are transported through the narrow import channels of the mitochondrial membrane are completely unfolded, and it is very likely that they arrive in the matrix in an extended conformation. Therefore they are excellent substrates for binding to the mtHsp70 of the mitochondrial matrix.[28] A mechanism for rapid and efficient transfer of mtHsp70 to translocating preproteins is dependent on Tim44, a peripheral membrane protein that is bound to the inner surface of the TIM23 complex (Figure 4).[96, 105–109] Tim44 is an overall hydrophilic protein that is apparently not part of the import pore and not required for the formation of the pore.[110] Tim44 and mtHsp70 form a 1:1 complex.[106] The molecular details of the transfer of mtHsp70 from Tim44 to the translocating polypeptide are still unclear. Two models are discussed for the mechanism of Tim44/mtHsp70-mediated protein translocation:

- The "Brownian ratchet model" postulates an oscillation of the preprotein in the import channel due to Brownian motion.[111, 112] Binding of mtHsp70 to matrix-exposed parts of the unfolded peptide chain could hinder the backsliding of this part of the peptide once it protrudes into the matrix. Additional mtHsp70 molecules would bind newly exposed segments and thus give a direction to this random movement.[113, 114] It is proposed that the unfolding of all preproteins during translocation is solely due to a spontaneous process. Tim44 would only be responsible for a high local concentration of mtHsp70 at the import site. An extension of this model is the "hand-over-hand model" that takes into account the possible dimerization not only of Tim23, but also of entire TIM23 complexes, including Tim44.[115] mtHsp70, bound to Tim44, would catch a matrix-exposed segment of the preprotein and would then dissociate from Tim44. A second mtHsp70 molecule, already bound to the second Tim44 molecule, would immediately bind to the next segment of the preprotein, while a third mtHsp70 molecule could bind to the first Tim44 molecule, which is no longer occupied. Repeating this cycle, a preprotein could be efficiently loaded with mtHsp70 and translocated into the matrix, provided that unfolding of the protein in the cytosol is fast and spontaneous and thus not rate-limiting.[115]
- Observations from studies of the import of preproteins that contain stably folded domains led to the "pulling motor model" for the action of mtHsp70.[116–118] As in the other two models, in this scenario mtHsp70 also binds to Tim44 and to the prepotein in transit to form a ternary complex.[105, 106, 119, 120] The hydrolysis of ATP would now lead to a conformational change in mtHsp70, initiating a power stroke

that pulls an additional segment of the preprotein across the membrane.[110, 120] As protein folding is highly cooperative, a limited power stroke could facilitate the unfolding of a protein domain and thus promote further translocation.

In fact, several studies have indicated that both mechanisms, active pulling and passive trapping, contribute to the mechanism of mitochondrial protein import.[110, 118, 121, 122] Less stably folded preproteins seem to be translocated even if the binding of mtHsp70 to Tim44 is impaired. Preproteins that have tightly folded domains, however, are strongly dependent on the interaction between Tim44 and mtHsp70. The pulling motor model is supported first by the finding that mtHsp70 undergoes a measurable conformational change upon hydrolysis of ATP,[123] which could execute an active pulling force. Secondly, recent results obtained with yeast mutant strains indicate that hindering a backsliding alone is not sufficient for an efficient import of preproteins into the mitochondrial matrix.[118] Additionally, it could be demonstrated that mtHsp70 binds to Tim44 with its ATPase domain.[124] Consequently, movements of the peptide-binding domain that are triggered by changes in the state of the bound nucleotide could easily be transferred to the bound substrate polypeptide. The exact contributions of both mechanisms to the translocation of preproteins can probably only be determined by a more detailed elucidation of the interactions between the Tim44/mtHsp70 complex and the preprotein.

While the structure of Tim44 is still unknown, a model of the structure of mtHsp70 already exists which is based on the high evolutionary conservation of heat shock proteins of the 70-kDa family in nearly all organisms.[125] It is reasonable to assume that the structure of mtHsp70 corresponds to the already solved ones of bovine Hsc70[126] and bacterial DnaK.[127] Single domains of these Hsp70 proteins were crystallized, including complexes with different binding partners.

Another interesting feature of the interaction of mtHsp70 and Tim44 is the fact that the deletion of a segment of 18 amino acids in Tim44 weakened this interaction considerably, while binding of Tim44 to Tim23 was not affected.[122] The deleted segment shows a significant similarity to helix II of the J domain, which is highly conserved among the co-chaperones of the J protein family.[106, 116] The J domain of these proteins is responsible for the interaction of J proteins with their partner proteins of the Hsp70 family.[128, 129] In this way, the bacterial DnaK interacts with DnaJ,[130–132] and the Hsp70 of the endoplasmic reticulum, Bip, interacts with its J protein Sec63.[133, 134] At least in some respect, Tim44 seems to play the role of a J protein for mtHsp70 in mitochondrial protein import.

8. Folding of imported proteins

Besides its essential role in protein import, mtHsp70 is involved in the first steps of folding of newly imported preproteins in the mitochondrial matrix.[135–137] mtHsp70 interacts with the co-chaperones Mdj1 and Mge1 in a fashion similar to the bacterial DnaK/DnaJ/GrpE system.[138, 139] Some preproteins, however, reach their native conformation even if mtHsp70 is not fully functional. Other mitochondrial members of the Hsp70 family, identified by the complete sequencing of the yeast genome,[140] may substitute for mtHsp70 in this process. The biogenesis of Yfh1, the yeast homologue of human frataxin, was found to depend on Ssq1, the second Hsp70 protein of the mitochondrial matrix.[141] Some newly imported proteins bind to Hsp60, which is a homologue of bacterial GroEL and representative of an additional class of chaperone proteins.[142, 143] Other components of the mitochondrial protein folding machinery do not belong to the chaperone proteins, but exhibit an enzymatic protein folding activity. The peptidyl-prolyl *cis – trans* isomerase cyclophilin was shown to support folding of a newly imported protein.[144, 145] However, it should be noted that it is still unclear how many proteins require prolyl isomerases for rapid folding. It is likely that only a small fraction of mitochondrial proteins interact with cyclophilin.

While the pathways and the components of the mitochondrial protein folding machinery are only partly understood yet, it is clear that proteins can only enter the matrix compartment by interactions with mtHsp70. In this respect the role of mtHsp70 is essential. Consequently, a better understanding of the mechanism of mtHsp70-driven protein transport will continue to be a central issue in future work on the biogenesis of mitochondria.

9. Tom and Tim as potential targets in drug development

The power of yeast genetics has allowed the testing of virtually all components of the TOM and TIM machinery if they are essential for growth. These studies have revealed that nearly all essential genes of *Saccharomyces cerevisiae* that encode mitochondrial proteins are Tom proteins, Tim proteins, or chaperones involved in the biogenesis of the mitochondria. Most other mitochondrial proteins are not essential in this organism. Interestingly, *S. cerevisiae* can live without respiration but not without mitochondrial protein import. The Tom and Tim proteins should therefore be of potential interest in designing drugs to fight human-pathogenic fungi or parasites that require their mitochondria for growth, or to find inhibitors of plant growth for agricultural means. Inhibitors of the mitochondrial respiratory chain are already applied as important pesticides: Strobilurin A is an inhibitor of respiratory chain complex III specifically in fungi, derivatives of oxathiin are potent inhibitors of respiratory chain complex II of basidiomycota. A remarkable specificity is achieved although respiratory chain complexes are conserved structures in all higher eukaryotes. Inhibitors of the TIM and TOM complexes should therefore be similarly suitable. So far this approach has not been used, since our knowledge of the components is only recent: The first component of the Tom protein was identified in 1989, the first Tim protein was found in 1992. The only high-resolution structure that has been solved so far is the solution structure (determined by NMR spectroscopy) of the hydrophilic presequence-binding domain of Tom20.[21] The purified TOM complex was visualized by electron microscopy and reconstituted in liposomes.[58] In our own laboratory, we recently reconstituted Tom40,[57] Tim22, and Tim23 for characterization by electrophysiological techniques.[146] Recent progress in the determination of structures of membrane proteins, including the components of the mitochondrial

respiratory chain, is encouraging. The next decade should witness additional structures in the field of the mitochondrial protein import machinery, and the current genome projects will facilitate the accessibility of homologous structures in different organisms.

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