Biochimica et Biophysica Acta 1808 (2011) 990–1001

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Review

Understanding the molecular mechanism of protein translocation across the mitochondrial inner membrane: Still a long way to go☆

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ARTICLE INFO

Article history:
Received 11 May 2010
Received in revised form 8 July 2010
Accepted 10 July 2010
Available online 18 July 2010

Keywords:
TOM
TIM23
Tim44
mtHsp70
Translocation motor

ABSTRACT

In order to reach the final place of their function, approximately half of the proteins in any eukaryotic cell have to be transported across or into one of the membranes in the cell. In this article, we present an overview of our current knowledge concerning the structural properties of the TIM23 complex and their relationship with the molecular mechanism of protein transport across the mitochondrial inner membrane. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

Contents

1. Introduction

The vast majority of proteins in any eukaryotic cell are synthesized on cytosolic ribosomes. However, only about half of these proteins function in the cytosol; the rest function in one of the cellular organelles or in the plasma membrane. In order to reach their final destination, they must be translocated across or into one of the various membranes in the cell. Intracellular sorting of proteins relies on the presence of a specific targeting signal on the one hand and on complicated proteinaceous machineries called protein translocases on the other. Translocases recognize the targeting signals, and subsequently mediate the transport of proteins encompassing those signals across or into the specific organellar membranes [1,2].

☆ This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

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doi:10.1016/j.bbamem.2010.07.011

Mitochondria contain a double layer of membranes, the outer and inner mitochondrial membranes, which define two aqueous compartments, the intermembrane space (IMS) and the innermost mitochondrial matrix. The transport of proteins into mitochondria is a particularly demanding task as it not only requires targeting to the organelle but it also necessitates the proper sorting of the proteins to the correct intramitochondrial compartment. With the exception of a few proteins encoded by the mitochondrial DNA, all mitochondrial proteins are synthesized in the cytosol in the form of precursor proteins [3–6]. Mitochondrial proteins carry a large number of different targeting signals. However, they are all recognized at the mitochondrial surface by receptors of the TOM (translocase of the outer mitochondrial membrane) complex. The TOM complex, covered in more detail in another article of this special issue (Rapaport D) is composed of the receptor proteins Tom20, Tom70 and Tom22, the ß-barrel protein Tom40 which forms a translocation channel, and three small Tom proteins, Tom5, Tom6 and Tom7, which most likely contribute to the dynamics of the TOM complex but may also have a more direct role in translocation of proteins (Fig. 1). Essentially all mitochondrial proteins are transported across the outer membrane through the translocation channel of the TOM complex. However, the various mitochondrial import pathways diverge at the intermembrane space (IMS) side of the TOM complex. So far, three proteins, Tom40, Tom22 and Tom7, have been implicated in the formation of this so called trans binding site of the TOM complex. It is likely that the trans site has binding sites for translocation machineries present in the IMS and/or inner membrane so that proteins are more efficiently transported into the organelle. The various import pathways present in mitochondria are thoroughly covered in several articles in this special issue. Here we will concentrate on the translocation of proteins across the mitochondrial inner membrane mediated by the TIM23 complex.

2. The translocase of the inner membrane of mitochondria — the TIM23 complex

Essentially all matrix proteins and a large number of inner membrane proteins are targeted to mitochondria by a positively charged N-terminal segment called a presequence or matrix targeting signal (MTS). The transport of these precursor proteins into the organelle depends on the cooperative action of the TOM complex in the outer membrane and the TIM23 complex in the inner membrane. Using the energy of the membrane potential across the mitochondrial inner membrane and ATP in the matrix, the TIM23 complex mediates translocation of proteins across and their insertion into the mitochondrial inner membrane. The large number of components involved, the diversity of tasks it performs and the energy sources it requires, make the TIM23 complex the most complicated of all mitochondrial translocases. The precursor proteins are recognized at the outlet of the TOM channel by the IMS-exposed receptor proteins Tim50 and Tim23, which guide them to the translocation channel formed by Tim23 and, possibly, Tim17 [7–13]. This part of the complex is sufficient for membrane-potential dependent transport of the presequence into the matrix. However, the translocation of the complete polypeptide chain into the matrix requires the ATP-dependent action of the import motor of the TIM23 complex [14]. The ATP-consuming subunit of the complex is mtHsp70. It binds and releases segments of the translocating chain in an ATP hydrolysis regulated manner [15–17]. This process is regulated by the various co-chaperones of mtHsp70: Tim44 which recruits mtHsp70 to the translocation channel in the inner membrane [18–22], Tim14 (Pam18) which stimulates ATP hydrolysis by mtHsp70 [23–25], Tim16(Pam16) which controls the activity of Tim14 [26–28] and Mge1 which stimulates the release of ADP [29–31]. Repeated cycles of binding to and release from mtHsp70 lead to the vectorial transport of the polypeptide chain into the matrix [32]. This pathway is followed by all precursor proteins in which the N-terminal presequence is their only targeting signal. However, if the translocating precursor protein contains an additional signal which is recognized by the TIM23 complex as the lateral sorting signal [33], the complex undergoes a conformational change which leads to the lateral opening of the translocation channel and insertion of the precursor protein into the inner membrane [10,34]. Thus, the TIM23 complex is actively remodeled during sorting of proteins into two different compartments of mitochondria. The subunits of the TIM23 complex involved in recognition of the lateral sorting signal are largely unclear as are the molecular mechanisms underlying the lateral opening of the TIM23 complex and subsequent insertion of the membrane spanning

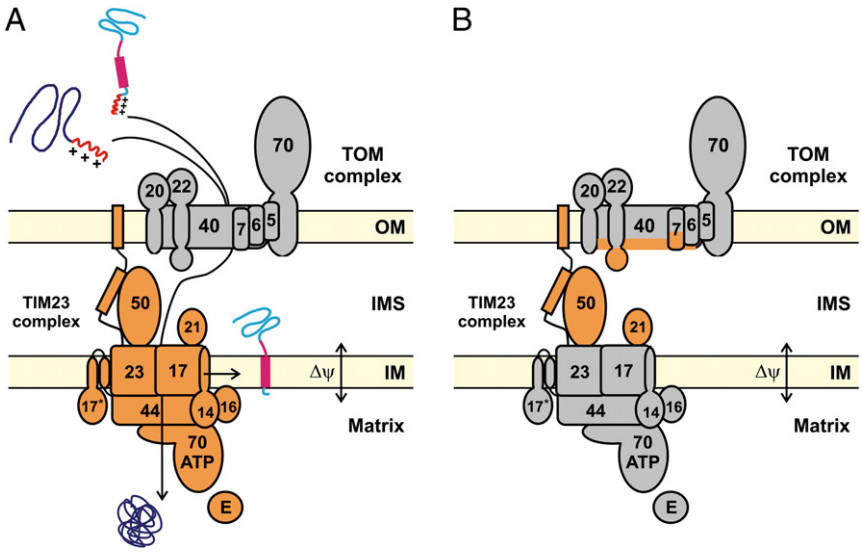

Fig. 1. Import pathway of precursor proteins with N-terminal presequences. (A) Import of precursor proteins with N-terminal presequences (in red) requires a cooperative action of the TOM complex (in grey) in the outer membrane and the TIM23 complex (in orange) in the inner membrane. If the presequence is the only targeting signal present, the precursor protein will be translocated completely into the matrix. If, however, an additional lateral sorting signal (in magenta) is present, it will be recognized by the TIM23 complex and the precursor protein will be laterally released into the inner membrane. (B) Several components of the TOM and TIM23 complexes were implicated to contribute to the cooperation of the complexes in the intermembrane space. They are indicated in orange, and the rest are shown in grey. OM, outer mitochondrial membrane; IMS, intermembrane space; IM, inner mitochondrial membrane; numbers indicated the molecular weights of the respective TOM and TIM components. 17* indicates Pam17.

segments into the inner membrane. The only two components of the TIM23 complex that are not essential for viability of yeast cells, Tim21 and Pam17 [35–37], modulate the activity of the complex in an antagonistic manner [34]. Below we address the various aspects of the function of the TIM23 complex in greater detail.

3. Cooperation of the TIM23 complex with the TOM complex

It was recognized early on that upon removal of the outer mitochondrial membrane by hypotonic swelling, so called mitoplasting, matrix targeted precursor proteins can be recognized and transported by the translocation machinery of the inner membrane [38]. This demonstrated that the TIM23 complex is able to recognize and transport proteins on its own, even though, under normal conditions, translocation of precursor proteins through the TIM23 complex is preceded by the translocation through the TOM complex. In contrast, the TOM complex alone is not able to mediate complete translocation across the outer membrane. When a precursor protein was added to isolated outer membrane vesicles, the MTS was transported across the membrane and was accessible to the MPP enclosed in the vesicle, however, the majority of the polypeptide chain remained outside [39,40]. Moreover, when precursor proteins were incubated with intact mitochondria in the absence of a membrane potential (to prevent transport across the inner membrane), only the extreme N-terminal segments were transported through the TOM complex, while the majority of the polypeptide chain remained outside of the organelle [41]. Thus, in intact mitochondria, transport across the outer membrane and transport across the inner membrane are interdependent processes in which the TOM and TIM23 complexes cooperate to transport proteins simultaneously across both membranes. Such crosstalk is mandated by the fact that the N-terminal segment of a translocating chain reaches the matrix while its more C-terminal segments are still in the cytosol. Indeed, when translocation of the C-terminal segments of precursor proteins is artificially blocked by for example methotrexate-stabilized dihydrofolate reductase, a two-membrane spanning intermediate can be generated [42,43]. Thus, a supercomplex consisting of a translocating precursor protein together with both TOM and TIM23 complexes can be isolated [34,44]. We are only beginning to understand the molecular mechanisms which underlie the cooperation of TOM and TIM23 complexes. Several proteins from both of the TOM and TIM23 complexes have domains that project into the IMS which could, in principle, contribute to the cooperation of the two translocases (Fig. 1). However, the relative contributions of the individual components to the overall process are still largely unclear.

The first hint of a TOM–TIM23 cooperation at the molecular level came ten years ago, when an unexpected finding concerning the topology of Tim23 was reported. Tim23 is anchored to the inner membrane via probably four transmembrane helices at its C-terminus. The N-terminal part of Tim23 is hydrophilic and resides in the IMS. The rather surprising observation was that the N-terminal segment of the IMS domain of Tim23 is accessible to proteases in intact mitochondria, demonstrating that the protein spans two membranes [45]. Hence, it could have the ability to physically bring the two membranes together, and thus draw the two translocases into proximity of one another. Although this finding was questioned by others [44], subsequent work showed that the exposure of Tim23 at the mitochondrial surface is dependent upon the association of Tim23 with Tim50. In Tim50-depleted mitochondria [9], or in mitochondria which harbor mutations that impair the interaction of Tim23 and Tim50 [10,46], the susceptibility of Tim23 to protease added externally to intact mitochondria was reduced. On the other hand, the translocation load imposed on the TIM23 complex increases the exposure of Tim23 on the mitochondrial surface [34]. Therefore, this segment of Tim23 plays an active role during transport of precursor proteins even though it is not absolutely essential for the activity of the TIM23 complex. The actual interaction of the N-terminal segment of Tim23 with the TOM complex, or more precisely with the IMS-exposed domain of Tom22, was demonstrated only very recently, using an *in vivo* site specific crosslinking approach [10]. It will be interesting to see the effects of precursor proteins on this interaction.

The second important clue to understanding the cooperation of the two translocases was the finding that Tim50 interacts with precursor proteins which are only partly translocated through the TOM complex [7,9]. Precursor proteins that were incubated with mitochondria in the absence of membrane potential were found to be crosslinked not only to the components of the TOM complex but, intriguingly, also to Tim50. None of the other TIM23 components was observed to be crosslinked to precursor proteins at this translocation stage [7], suggesting that Tim50 is the first component of the translocase to bind precursor proteins. Tim50 recognizes all known types of TIM23 substrates [8] supporting the notion that it is the presequence itself which is recognized by Tim50. Interestingly, this receptor function of Tim50 was shown to be dependent upon its association with the translocase. Specifically, in mitochondria which either lack Tim23 or carry mutations in Tim23 that impair its interaction with Tim50, no crosslinking of precursor proteins to Tim50 was observed [8,10]. This raises the possibility that the two proteins contribute to a hypothetical presequence recognition site, although crosslinking was observed only to Tim50. Alternatively, it is possible that Tim50 adopts a conformation conducive to recognition of presequences only upon binding to Tim23. Recognition of precursor proteins by Tim50 is also required for stable binding of precursor proteins to the TOM complex [35]. An early translocation intermediate of the Oxa1 precursor in the TOM complex which is visible on Blue Native PAGE (BN-PAGE) with wild type mitochondria, is not observed in mitochondria carrying a mutant Tim50. This further supports a role of Tim50 in very early stages of transport of precursor proteins into mitochondria. Approximately 60 residues of a translocating chain have to be inserted into mitochondria before the precursor protein is recognized by Tim50 [8]. Intriguingly, the same length of precursor protein is required for binding to the trans site of the TOM complex [47]. This suggests that Tim50 is found in close proximity to the TOM complex. Indeed, the interaction of the IMS-exposed domain of Tom22 with Tim50 has been demonstrated using site specific crosslinking *in vivo* [10]. The residues of Tim50 involved in interaction with the TOM complex are not currently known, nor have the effects of translocating chains on the interaction of Tim50 with the TOM complex been reported. It also remains to be elucidated whether the IMS domain of Tom22 is the only binding site for Tim50 on the TOM complex. Interestingly, Tim50 appears to be required for generation, but not for stabilization, of the two-membrane spanning intermediates [44] suggesting that its major role is in the early steps of protein translocation across the inner membrane.

A third component of the TIM23 complex that has been implicated in the cooperation with the TOM complex is Tim21. Recombinantly expressed, IMS-exposed domain of Tim21 was found to be efficiently and specifically bound to the TOM complex when incubated with mitochondrial lysates [35,36]. This interaction was shown to be mediated by the IMS-exposed domain of Tom22, upon deletion of which no binding was observed [35]. Two negatively charged residues of Tom22, D131 and D135, were reported to be especially important for the interaction between $Tim21_{IMS}$ and $TOM22_{IMS}$, which is electrostatic in nature [48]. The recent crystal structure of $Tim21_{IMS}$ demonstrated the presence of conserved positively charged residues on the protein surface [48]; however, their role in binding to the TOM complex has not been investigated. In view of the above data, it is rather surprising that Tim21 is not essential for cell viability [35,36] and that its importance is manifested only when the energetic activity of mitochondria is limiting [49]. This may, however, lend credence to the notion that the system is somewhat redundant and that several contacts contribute to the cooperation of the two translocases.

4. The translocation channel of the TIM23 complex

The TIM23 complex recognizes precursor proteins already at the outlet of the TOM channel. From thereon, the translocating chain is guided through the translocation channel of the TIM23 complex. The composition of this translocation channel is still not clear. Both Tim17 and Tim23 have multiple transmembrane spanning helices which could contribute to the formation of the channel [50–53]. Predicted transmembrane helix II of Tim23 was recently shown to be amphipathic in nature [54]. Its water-exposed face is in contact with translocating chains making it a likely candidate for channel formation. Furthermore, Tim23 recombinantly expressed in *Escherichia coli*, formed voltage-dependent and presequence-sensitive channels after refolding from inclusion bodies and insertion into black lipid bilayers [12]. The C-terminal domain, which is predicted to form four transmembrane helices, was sufficient to produce channels with similar characteristics to the full length protein, with the exception of presequence sensitivity. The latter was largely absent when only the C-terminal domain of Tim23 was reconstituted. This is in agreement with the receptor function attributed to the N-terminal domain of Tim23 [55]. Similar experiments with recombinantly expressed Tim17 have not been reported so far. Electrophysiological investigations of mitoplasts prepared from mitochondria depleted of Tim23 failed to reveal any channels corresponding to the TIM23 complex [56]. However, the analysis of mitochondria depleted of Tim17 revealed that the twin pore structures, observed in wild type mitochondria, collapsed to a single pore [56]. It is thus possible that Tim23 is the major component of the translocation channel whereas Tim17 contributes to its formation and/or stabilization. The crystal structure of the channel will likely be required to resolve this issue.

The inner membrane of mitochondria must be tightly sealed so that the proton gradient, generated by the respiratory chain complexes, can be used by the FoF1-ATPase to produce ATP. Furthermore, the translocation of precursor proteins by the TIM23 complex is strictly dependent upon the membrane potential. Thus a constantly open, presumably large protein-conducting channel of the TIM23 complex would be deleterious for the cell. Therefore, opening of the TIM23 complex must necessarily be tightly regulated. Closure of the channel has been attributed to several components of the TIM23 complex. The dimerization of Tim23, mediated by the second half of its IMS domain, is promoted by membrane potential. On the other hand, precursor proteins lead to dimer dissociation. It was therefore suggested that the Tim23 dimerization is responsible for the regulated opening of the translocation channel [55]. The second component implicated in closure of the channel is Tim50. The permanently open channel formed by recombinant Tim23 in black lipid bilayers was closed upon addition of the IMS domain of Tim50 [57]. However, intact mitochondria depleted of Tim50 [7,9,11] and those harboring mutations that impair the Tim23–Tim50 interaction [10,46] are still able to generate and maintain membrane potential, raising the possibility that the reconstituted system may differ in some way from that of intact mitochondria. The last component whose mutations were reported to affect membrane potential is Tim17. Removal of the short N-terminal segment of Tim17 led to a severe reduction of the membrane potential across the inner membrane [58] suggesting the involvement of this segment in the maintenance of the permeability barrier of the inner membrane. Again, it is likely that several components contribute to the regulated opening of the translocation channel of the TIM23 complex.

The voltage sensing component of the TIM23 complex remains a mystery. None of the TIM23 components contains segments which resemble voltage sensors related to those of the known voltage-gated ion channels, suggesting that the nature of the TIM23-complex voltage sensor may be completely different from anything known today. The membrane-potential dependent dimerization of the IMS domain of Tim23 led to the proposal that this segment is the voltage sensor of the complex [55]. Although the latter possibility cannot be excluded, it is more likely that the actual sensor resides somewhere within the membrane-embedded section of the complex, where the effects of the membrane potential are expected to be most pronounced. The recently reported membrane-potential dependent crosslinking between Tim17 and Tim23 may shed some light on this issue [59].

5. The mitochondrial protein import motor

Once the presequence has reached the matrix, the membrane-potential requirement no longer exists. The translocation of the polypeptide chain into the matrix is then mediated by the import motor of the TIM23 complex. This fascinating translocation motor is characterized not only by its complexity but also by the tight regulation of its functional cycle. At the heart of the motor lies the only ATP-hydrolyzing component in the system, the mitochondrial 70 kDa heat shock protein (mtHsp70), which is a soluble protein that resides in the mitochondrial matrix. The universal and most characterized function of this group of chaperones is to fold stress denatured and newly synthesized proteins, as amply demonstrated in the past for the homologous bacterial protein, DnaK. This bacterial chaperone carries out its protein-folding activity in cooperation with two co-chaperones, DnaJ and GrpE. A similar, protein-folding task is also facilitated by mtHsp70 in the mitochondrial matrix, with the assistance of the respective soluble co-chaperones Mdj1 and Mge1 [60]. A completely different role is played by mtHsp70 in the process of mitochondrial protein import, where it helps to promote the unfolding of preproteins to allow for their accommodation in the translocation channel. The functional interaction of mtHsp70 with the TIM23 translocase imposes several intriguing difficulties. i) The import function of mtHsp70 entails its intimate interaction with the import channel. How is the soluble chaperone anchored to the inner membrane? ii) The interaction of mtHsp70 with the import channel is dynamic and transient; hence, it should be accurately coordinated with the action of the TIM23 channel. e.g. a correct positioning of mtHsp70 next to the import channel when a matrix targeting signal emerges in the matrix. iii) The association/dissociation of the preprotein must be precisely coordinated, since premature release of a precursor protein may lead to its sliding back from the import channel. Tim44, the first component of the TIM23 complex to be discovered [61], was shown to play a key role in anchoring mtHsp70 to the import channel. However, Tim44 is not only a passive anchor but rather plays an active role in the regulation of the motor operation. When a polypeptide chain emerges into the matrix, it binds mtHsp70, and through repeated cycles of binding and release, mtHsp70 promotes the unfolding and entry of the polypeptide into the matrix. The efficiency of this process is guaranteed by the various motor components, mtHsp70 co-chaperones that regulate and modulate its function. Tim14 is a J-domain protein that stimulates the ATPase activity of mtHsp70. Tim14 facilitates the tight locking of mtHsp70 onto a polypeptide chain when it emerges in the matrix. Tim16 inhibits the ATPase stimulatory effect of Tim14 and may regulate its function in the absence of a translocating precursor. Since Tim14 and Tim16 are present in a stable complex, conformational changes, rather than dissociation, are suggested to control Tim14 inhibition by Tim16 [62,63]. Finally, the nucleotide exchange factor Mge1 was shown to be essential for the import process [31,64,65]. Mge1 interacts with the ADP-bound form of mtHsp70 and helps to exchange bound ADP with ATP.

6. The assembly of the import motor

The theoretical separation of the TIM23 complex into two distinct subcomplexes, the core complex and the import motor, is based mainly on the structural and functional properties of the individual

proteins, as well as on strength of interaction between the various proteins. For example, in BN-PAGE the TIM23 complex dissociates, whereas the core, Tim23–Tim17 interaction, is stable and the two proteins are detected in a 90K assembly [43]. Several studies examined the stability of the Tim23–Tim17 interaction in the absence of the other translocase components. Since most of the constituents of the TIM23 complex are essential in yeast, depletion of individual proteins was carried out, rather than knock-out studies. The observation that depletion of any of the other complex components, except for Tim23 and Tim17, did not affect the Tim23–Tim17 association, suggested that this couple represents the core assembly of the translocase [13]. Consequently, it is reasonable to conclude that the assembly of other motor components is hierarchal. The question remains as to which component serves as the primary anchor and assembly site for the translocation motor?

As mentioned before, Tim23 and Tim17 form a stable complex that does not dissociate in the absence of other components. However, further depletion experiments showed that if Tim23 and Tim17 do not assemble properly, the import motor detaches from the core translocase [23,26]. Does recruitment of Tim50 also require the presence of an existing Tim23–Tim17 core? In the absence of Tim23, Tim50 dissociates from the translocase. When Tim17 is absent, Tim50 can still partially associate with Tim23 [23]. This indicates that Tim50 is recruited to the channel through interactions with Tim23.

Several lines of evidence suggest that Tim44 is the primary component that anchors the import motor to the TIM23 complex. First, upon depletion of Tim44, the Tim23–Tim17–Tim50 core complex assembles correctly, while Tim14, Tim16, mtHsp70 and Mge1 were not able to form a complex with the translocase. Second, when other components of the motor (Tim14/Tim16) were depleted, the association of Tim44 with the core complex was not affected [26]. The interaction between Tim14 and Tim16 is very stable and is not dependent on the presence or absence of other proteins. It is not clear whether the formation of this stable complex is required for association of these two proteins with the channel. One report showed that depletion of Tim14 results in the release of Tim16 from the translocase, whereas the assembly of the other components is not affected. Similarly, upon depletion of Tim16, Tim14 does not associate with the TIM23 complex [26]. This data indicates that only in the context of a stable complex these two proteins are recruited to the translocase, probably through interactions with Tim44. On the other hand it was also shown that when the Tim14–Tim16 interaction is destabilized, Tim14 is released from the translocase while Tim16 can still be detected in the TIM23 complex [66]. This suggests that Tim16 interacts with the translocase independently of Tim14, and is responsible for the recruitment of Tim14 to the complex.

Combining the above-mentioned results, a model for the assembly of the TIM23 translocase can be proposed (Fig. 2). First, Tim23 and Tim17 interact to form the core of the translocase. If Tim23 and Tim17 fail to assemble correctly, the entire translocase disintegrates. On the IMS side, Tim50 joins the Tim23–Tim17 core complex through interactions with Tim23. Tim44 associates with the core translocase and constitutes the vital link between the membrane-embedded core complex of the translocase, and the import motor. When Tim44 is correctly positioned, it recruits mtHsp70 in addition to the Tim14–Tim16 complex. Mge1 joins the complex through its association with mtHsp70, and together with Tim14–Tim16, regulates the dynamics of the import motor by modulating the progression through the ATP hydrolysis-driven cycle.

7. Structural properties of the individual motor proteins

Unraveling the mechanism of function of the mitochondrial import motor will be greatly facilitated by high resolution structural information of the individual proteins and their complexes. Here, important aspects of structural properties of the translocation motor are summarized.

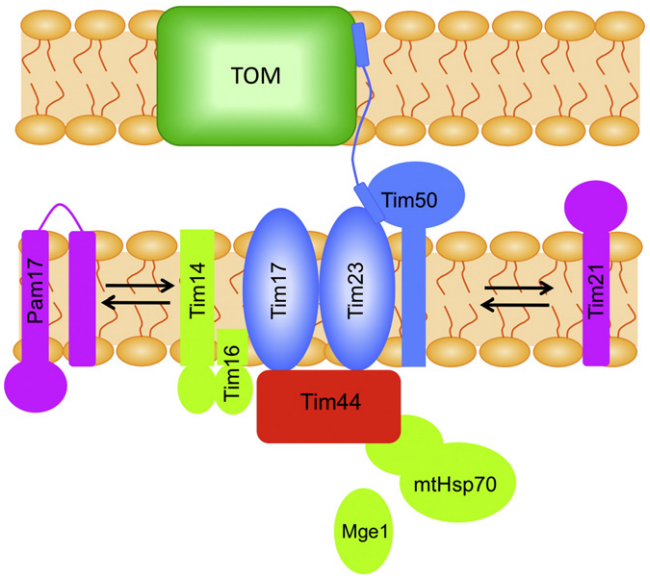

Fig. 2. The assembly of the TIM23 complex. Schematic illustration of the components of the TIM23 complex. Different colors indicate differences in the order of recruitment to the translocase. First, the core complex, which forms a platform for the assembly of the other components, is colored blue. Then in red, Tim44, serves as the main anchor for the import motor. Finally, the components of the motor that are recruited last to the complex are depicted in light green. Tim21 and Pam17 are not permanently attached to the complex, and their dynamic association is illustrated by the double arrows.

7.1. Tim44

Tim44 is a unique, essential and matrix-localized mitochondrial protein of 44 kDa that has no known homologues in other eukaryotic cellular compartments. As such, it has been the subject of many studies attempting to elucidate its structural and functional properties. Tim44 was discovered in two independent studies. The first was a genetic screen for mutants exhibiting defective protein import [67], and the second, was a crosslinking analysis of proteins bound to a precursor stuck in the import channel [61,68]. Results from the *in organello* crosslinking experiments raised the possibility that Tim44 binds directly to precursor proteins in transit. However, a second option – that they are only found in close proximity – is also conceivable.

One of the first issues to be addressed was the nature of the Tim44 interaction with the membrane. Although the sequence of Tim44 does not contain any predicted transmembrane segments, the protein is detected in the membrane fraction when mitochondria are disrupted in the absence of detergent [67,69]. However, in contrast to integral membrane proteins, Tim44 is released from the membrane upon treatment with alkaline pH [68]. It was therefore concluded that, Tim44 is peripherally attached to the mitochondrial inner membrane. The strong association of this protein with the membrane can be explained by the observation that, in addition to its direct interaction with the TIM23 core complex, Tim44 is able to associate with negatively charged phospholipids [70,71].

Structural analysis of purified Tim44 showed that the protein is elongated and composed of two domains, a C-terminal and an N-terminal. The C-terminal domain of yeast Tim44 starts at amino acid 210 and extends to the end of the protein. This domain is globular and very stable. However, the N-terminal domain is much less stable, and is highly prone to proteolytic degradation. Tim44 was shown to bind specifically to cardiolipin-containing liposomes. In the absence of cardiolipin, the interaction of Tim44 with the liposomes was significantly weaker [70,71]. This issue has taken on a new significance with

the discovery of the central role of cardiolipin in biogenesis of mitochondrial proteins. Indeed, it was demonstrated that cardiolipin is involved in the assembly and function of mitochondrial carriers [72,73], in respiratory chain integrity [74–76] and in the assembly of the TIM translocases [77,78]. Similar to the full length Tim44, the C-terminal domain associates specifically with cardiolipin-containing liposomes [70,71].

The crystal structure of the yeast Tim44 C-terminal domain (starting at amino acid 234) provided further insight regarding the structural properties of the protein [79]. The structure revealed a large hydrophobic pocket that may also constitute a lipid binding site. Moreover, a uniquely positioned α-helix (named A1) was observed, which protrudes out vertically from the main body of the protein, and has a positively charged surface. The positively charged residues on this helix are highly conserved [80]. In the high resolution structure of human Tim44, a hydrophobic pocket is also observed. However, in this structure, the protruding helix A1, together with helix A2, is replaced by one long helix that is folded towards the hydrophobic pocket.

A recent study provided a more detailed understanding of the lipid binding properties of the C-terminal domain. It was shown that the two N-terminal positively charged α-helices of the C-terminal domain (A1 and A2) are responsible for its lipid binding properties (Fig. 3). Deletion of those two helices completely abolished the interaction with cardiolipin-containing liposomes. Thus, it is reasonable to suggest that the interaction of Tim44 with cardiolipin is governed mainly by electrostatic forces and it involves the negatively charged head groups of the phospholipid. A truncation construct of the Tim44 C-terminal domain that contains the hydrophobic pocket, cannot bind cardiolipin-containing liposomes, therefore excluding the suggestion that this is the major binding site. However, one cannot rule out the possibility that the hydrophobic pocket may participate in stabilizing the interaction between Tim44 and the phospholipid, once it is initiated by helices A1 and A2 [70,71].

Interestingly, when the ability of the N-terminal domain to interact with acidic phospholipids was analyzed, this domain was also able to associate with cardiolipin-containing liposomes (M. Marom, unpublished results). When a short β-strand and an α-helix (as predicted from the secondary structure analysis) (Fig. 3) were removed from the extreme C-terminus of this domain, its interaction with the liposomes was abolished (M. Marom, unpublished results). This observation suggests that a long central stretch of full length Tim44, which extends into both the N and C-terminal domains (ca. amino acids 165–263), constitutes the lipid binding site of Tim44 (Fig. 3).

The functional significance of the direct interaction of Tim44 with cadiolipin, in addition to its direct interaction with the core of TIM23 complex is unclear. Since the lipid binding site of Tim44 appears to be a long stretch in the middle of the protein, analyzing its functional significance *in vivo* is a difficult task. It is tempting to speculate that a direct binding of Tim44 to the inner membrane, via association with cardiolipin, may help to increase the local concentration of the protein next to the import channel which may facilitate its binding to the Tim23–Tim17 complex. Thus, association with cardiolipin might enhance the efficiency of Tim44 function.

Tim44 is a highly conserved protein that is found in animals, fungi, plants and diverse protists. In mice, Tim44 was found to be one of the proteins that is up-regulated under hyperglycemic conditions and was suggested as a therapeutic target for diabetes [81]. Interestingly, despite the presence of a limited number of components of the protein import machinery in mitosomes (i.e. Tom40 and SAM50) of *Entamoeba histolytica*, a Tim44 homologue has not been identified so far, pointing to an overall mitochondrial reduction in this organism. Notably, a protein containing a C-terminal like domain of Tim44 was identified in α-proteobacteria. The function of this protein in bacteria is unknown [82].

7.2. MtHsp70

MtHsp70 belongs to the ubiquitous family of 70 kDa heat shock proteins. The members of this family are molecular chaperones present in various compartments in the cell where they perform diverse functions including protein folding, protein degradation, translocation across membranes and involvement in the heat shock response [83]. Three members of this family reside in the mitochondrial matrix of *S. cerevisiae*: Ssc1 (mtHsp70), Ssq1 and Ecm10 (Ssc3) [84]. Ssc1, which is also known as mtHsp70, is the most abundant of the three, and its deletion is lethal for yeast cells [85]. MtHsp70 is highly homologous to the *E. coli* chaperone DnaK, which is the most characterized member of the family [86]. The high degree of homology may be derived from the prokaryotic origin of mitochondria. In the matrix, mtHsp70 mediates protein folding and stands at the heart of the mitochondrial protein import motor. For its folding function, similar to the bacterial DnaK system, mtHsp70 cooperates with a J-protein, named Mdj1 in mitochondria, and with the nucleotide exchange factor, Mge1. Since Mdj1 is essential only at high temperatures, the lethal phenotype observed upon deleting mtHsp70 is probably due to its vital function in the import process [85,87]. Hsp70s are composed of two domains, an N-terminal nucleotide binding domain (NBD) and a C-terminal peptide binding domain (PBD), which are connected by a short linker. The peptide binding domain is composed of two sub domains: a conserved β-stranded core and an α-helical lid [88]. The β-stranded core forms the substrate binding pocket with eight anti-parallel β-strands, and the lid is composed of five α-helices. The closure of the peptide binding pocket is achieved by the folding of the lid over the β-stranded core [89]. Despite the high degree of sequence conservation and the implied structural conservation, Hsp70s are rarely interchangeable.

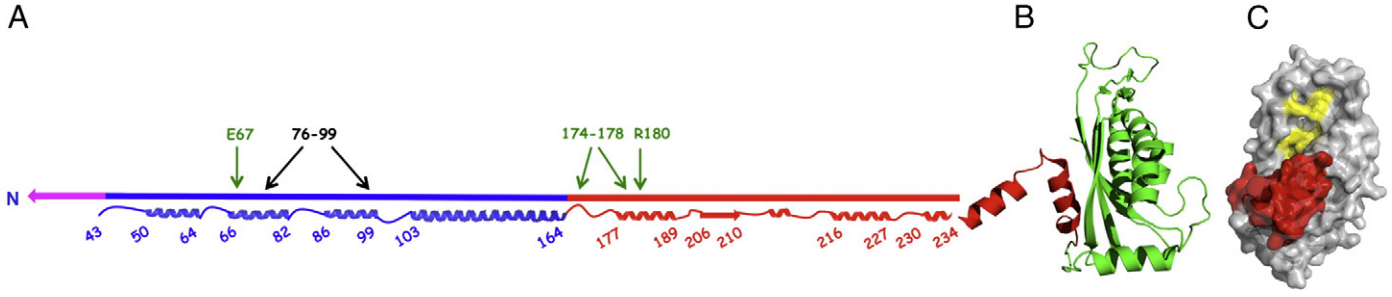

Fig. 3. The structure of Tim44. (A) Cartoon illustration of a secondary structure prediction for the Tim44 N-terminal domain. The presequence is colored purple and the region predicted to bind phospholipids is shown in red. Green arrows point out amino acids suggested to participate in mtHsp70 binding. Black arrows indicate the region that was suggested to participate in Tim16 binding based on a suppressor analysis. (B) Cartoon illustration of the Tim44 C-terminal domain: red signifies the region that was shown to bind phospholipids. (C) Surface representation of the Tim44 C-terminal domain [79]. The phospholipid binding region is shown in red, and hydrophobic amino acids that were suggested to form the hydrophobic pocket are colored yellow.

For example, DnaK targeted to the yeast mitochondrial matrix, could not rescue the temperature sensitive phenotype of an mtHsp70 mutant, which showed a defect in protein import. [90]. Subsequent experiments carried out in *E. coli* showed that mtHsp70 cannot complement the function of DnaK [91]. Thus, during evolution Hsp70 chaperones underwent fine-tuning to carry specific functions in different organisms and cellular compartments [92].

Conformational dynamics of mtHsp70 was recently analyzed in an extensive FRET study. It was shown that the ATP state of the chaperone is well defined with an open PBD and the two domains docked onto each other. In contrast, the ADP state was heterogeneous, both with respect to PBD–NBD interactions and the conformation of the SBD. Moreover, it was demonstrated that substrates modulate the structure of mtHsp70 in that they are required for closure of the PBD and undocking of the two domains. Similar experiments were conducted with DnaK revealing differences between DnaK and mtHsp70, which probably reflect the tuning of Hsp70 chaperones to meet specific functions [92].

Interestingly, human mitochondria harbor only one mtHsp70 homologue, mortalin. Mortalin presumably takes on all functions that are performed by the three yeast mtHsp70s (Ssc1, Ssq1 and Ecm10). Several studies have shown that mortalin is implicated in diverse pathological conditions. As such, it was found to be overexpressed, or to change its subcellular location, from mitochondria, in normal cells, to the cytosol, in cancerous cells. Altered expression of mortalin was also observed in neurodegenerative diseases such as Parkinson and Alzheimer. Finally, several lines of evidence showed that mortalin performs additional, diverse extra-mitochondrial functions. The most apparent evidence for this is the fact that mortalin has been found to associate with a large number of unrelated cytosolic proteins. The list includes Mps1 kinase, fibroblast growth factor-1 (FGF-1), cytosolic J-proteins, p53 and others [93]. Further studies are required to determine the precise functional significance of these cytosolic interactions of mortalin and how this matrix-localized protein ends up in the cytosol. Possible mechanisms are discussed in another review in this issue (Yogev and Pines).

7.3. Mge1

Mge1, the mitochondrial homolog of bacterial GrpE [30,64,94], resides exclusively in the mitochondrial matrix and has no homologues in other cellular compartments [95]. Similarly to bacterial GrpE, Mge1 functions as a nucleotide exchange factor for mitochondrial Hsp70s. As such, it serves as a key modulator of mtHsp70 function in mediating protein import and folding [29,31,60,65,96]. In the import process, Mge1 controls the nucleotide-regulated interactions of mtHsp70 with Tim44 and a translocating precursor, which are essential for an effective import process [64,97,98]. Similarly to its bacterial homolog, Mge1 forms homodimers [99–101] and is able to replace the function of GrpE in *E. coli* [94,102]. This indicates a high functional and structural conservation of this co-chaperone across biological kingdoms. The dimeric structure of Mge1 is essential for proper interaction with and modulation of mtHsp70. Indeed, it was shown that the reversible dimer–monomer transition of Mge1 serves as a thermosensor for the mitochondrial Hsp70 system, regulating the nucleotide exchange rates under heat shock [103,104].

7.4. Tim14–Tim16 complex

Following the discovery that mtHsp70 is a key component of the yeast import motor [15,105], it became clear that its efficient functioning entails cooperation with specific co-chaperones, a nucleotide exchange factor and a J-domain protein. For a long time, the identity of its J-domain partner in import remained obscure. J-domain proteins are divided into three types. The classical homologues of the bacterial DnaJ are known as type I J-domain proteins. These contain three typical domains, a compact helical J-domain which is connected by a glycine-rich region to a zinc finger domain, and ends in a C-terminal domain. Type II co-chaperones contain the J-domain which is connected by the glycine-rich region to a C-terminal domain. Type III members encompass only the J-domain [106]. Five J-domain containing proteins are currently known to reside in the yeast mitochondrial matrix namely, Mdj1, Mdj2, Tim14, Jac1 and Jid1 [23,87,107–109]. However, only Tim14 was shown to be essential for protein import into mitochondria. Tim14 belongs to the family of type III J-domain proteins. In contrast to Type II and Type I, members of the Type III sub-class cannot bind substrate proteins. Tim14 is anchored to the inner mitochondrial membrane via its single transmembrane helix and exposes its C-terminal conserved J-domain to the matrix. Interestingly, Tim14 that lacks its transmembrane anchor and contains only the matrix domain (which includes the J-domain) is still functional *in vivo* [63]. In yeast, a non-conserved N-terminal hydrophilic domain, which is absent in some of the mammalian homologues, protrudes into the intermembrane space [23]. Tim14 exists in a stable complex with the J-like protein Tim16. [26]. When in complex with Tim16, Tim14 is inhibited from exerting its ATPase-stimulation function on mtHsp70 [63]. Tim16 is highly homologous to Tim14 but instead of an HPD motif, which is essential for stimulation of the ATPase activity of mtHsp70, it contains a DKE sequence. Intriguingly, adding an HPD to Tim16 does not convert it to a functional J-protein [63]. Tim16 does not contain a predicted transmembrane segment but rather a conserved hydrophobic N-terminal domain. The N-terminal domain is connected through a short linker to the conserved C-terminal J-like domain. Tim16 is strongly attached to the inner membrane, most likely via peripheral interactions of the hydrophobic N-terminus with phospholipids, together with its interactions with Tim14. The J-like C-terminal domain of Tim16 was shown to be the only essential part of the protein [63].

The interaction between Tim14 and Tim16 is defined by several interesting characteristics. It is a very strong interaction which does not dissociate even in the presence of 1% Triton X-100, a condition under which even the TIM23 complex is not stable [26]. Furthermore, it was shown *in vitro* that on their own, the soluble domains of the two proteins are much less stable (T_m values are 16.5 °C for Tim14 and 29 °C for Tim16) than when they are in complex (T_m value of 41 °C). This indicates that at 30 °C, the physiological growth temperature of yeast, the individual proteins will begin to denature, while their complex will remain intact [62].

The crystal structure of the Tim14–Tim16 hetero-dimer reveals that the J-domain of Tim14 and the J-like domain of Tim16 form a stable complex with a 1:1 stoichiometry. Tim14 and Tim16 share a high degree of structural similarity that is also shared by DnaJ and includes a typical J-domain fold. The major difference between the proteins lies in their surface. The surface of Tim14 is mainly positively charged, a common feature of J-proteins. However, the surface of Tim16 is neutral or negatively charged. In the complex, three helices of Tim14 (helices I, II and III) are tightly packed, which renders a constraint on the conformation that is necessary for its ATPase enhancement function. This explains why Tim14 is not active when in complex with Tim16 [63]. Based on the above-mentioned results, it is reasonable to suggest that *in vivo* the Tim14–Tim16 complex does not dissociate, rather, the regulation of its ATPase-stimulation function, occurs through conformational changes of the complex.

Homologues of yeast Tim14 and Tim16 were also identified in humans (DNAJC19 and Magmas respectively) [110,111]. DNAJC19 and Magmas are of great current interest since they are both associated with several human disorders. A novel autosomal recessive disorder called “DCMA syndrome” has already been associated with a mutation in the DNAJC19 protein [110], while Magmas is suspected to be involved in increased rates of anaerobic metabolism, resistance to apoptosis and altered growth-factor sensitivity, that are characteristic

of cancer cells [111,112]. Recent studies showed that human Tim14/Tim16 and their yeast homologues are structurally and functionally conserved [113,114]. A hint as to the evolutionary origin of this protein was provided when a homologue of Tim14 was also identified in α-proteobacteria. While its function in bacteria is unknown, mutating a single amino acid (A139N) enables the bacterial homologue (TimB) to complement the phenotype of a Tim14 deletion in yeast [82].

8. Interactions between the motor proteins

8.1. The interaction of Tim44 with mtHsp70

At the heart of the import motor resides the essential pair of proteins mtHsp70 and Tim44. It is their coordinated interaction that promotes the translocation of precursor proteins into the matrix. Such coordination is achieved by the modulation of the nucleotide status of mtHsp70, which is controlled by various co-chaperones. The nucleotide dependence of the Tim44–mthsp70 interaction is one of the issues that is central for understanding how the complex functions at the molecular level. Consequently it has been the subject of several studies. Upon solubilization of mitochondria it was shown that a stable complex is formed in presence of ADP, whereas the complex becomes unstable in presence of high ATP concentrations [18–20]. *In vitro* studies using purified components have provided deeper insights into the workings of this interaction and showed that in the presence of mitochondrial targeting presequence and Mge1, the Tim44–mtHsp70 complex dismantles, regardless of the nucleotide bound to mtHsp70 [21,115].

Another issue that is central to understanding the molecular function of the translocation motor is the structural basis of the mtHsp70–Tim44 interaction. Consequently, several studies have addressed this issue, and attempted to define the mtHsp70 binding site on Tim44. As noted previously, Tim44 is composed of two domains: a C-terminal domain and an N-terminal domain. Mutagenesis analysis carried out both *in vivo* and with isolated components showed that the N-terminal domain harbors the major mtHsp70 binding site on Tim44 [115,116]. Further studies endeavored to determine precise residues involved in the interaction. One report described the isolation of a lethal mutation of Tim44 in which Glutamate 67, a highly conserved residue, was changed to Alanine. Notably, using crosslinking with purified proteins, this Tim44 mutant was shown to exhibit an impaired interaction with mtHsp70, in particular in the presence of Mge1 [115]. An additional amino acid in the N-terminus of Tim44, which was identified as an important mediator of complex formation with mtHsp70, is Arginine 180. Mutation of Arginine to either Alanine or Lysine, did not disrupt the interaction of Tim44 with mtHsp70, however, dissociation in response to a substrate peptide did not occur. Since a regulated interaction between Tim44 and mtHsp70 is important *in vivo*, yeast cells carrying the mutant protein exhibited a temperature sensitive phenotype. A similar interaction pattern with mtHsp70, and *in vivo* phenotype, was observed for another Tim44 mutant, in which four consecutive amino acids were changed to Alanine: Tim44–174_{AAAA}. This indicates that the region surrounding amino acids 174 is important for a regulated Tim44–mtHsp70 interaction (Fig. 3) [116]. In summary, it is widely accepted that the N-terminal domain of Tim44 constitutes the direct binding site for mtHsp70. However, since it is possible that the impaired interaction of mtHsp70 with the above-described mutants of Tim44 arose due to global changes in the structure of Tim44, the determination of the precise binding site between the two proteins requires further confirmation.

Finally, the nature of the regions of the mtHsp70 structure that mediate the interaction with Tim44 has been the subject of extensive studies. Using the yeast two hybrid system and the import of intact domains of mtHsp70, it was suggested that Tim44 interacts with the nucleotide binding domain of mtHsp70 [117]. A different study showed that Tim44 interacts with the β-sandwich core of the peptide binding domain [90]. However, additional studies using purified components showed that both domains of mtHsp70 participate in complex formation [22,118]. Future studies yielding higher resolution data will no doubt serve as a basis for resolving these conflicting views.

8.2. The interaction between Tim44 and the Tim14–Tim16 complex

In contrast to the significant progress made in reconstituting the Tim44–mtHsp70 complex from purified proteins, no study to date has succeeded in showing a direct interaction between purified Tim14–Tim16 and Tim44. However, there are several *in vivo* indications for the existence of such an interaction. First, Tim14 and Tim16 can be crosslinked *in organello* to Tim44 [23,26]. Although such *in organello* crosslinking is not necessarily an indication for a direct binding, it suggests that the Tim14–Tim16 complex is located in close proximity to Tim44, which abuts the TIM23 channel. A second piece of evidence suggesting direct interaction is obtained from experiments which show that the depletion of Tim44 results in the complete dissociation of Tim14–Tim16 from the channel [26]. Hence, it can be reasonably assumed that direct binding to Tim44 is required for the recruitment of the Tim14–Tim16 complex to the import channel. A significant indication for direct interaction with Tim44 was obtained from studies of Tim16 mutants, which are impaired in their interaction with the TIM23 complex. It was shown that specific amino acid alterations in Tim44 can restore the binding. Those Tim44 suppressors reside in its N-terminal domain, all in the region of amino acids 76–99 (Fig. 3) [66]. This finding strongly supports the direct binding of Tim16 to Tim44. Moreover it may imply that this region of Tim44 contains the binding site for Tim16.

What are the regions of Tim16 that bind to Tim44? It was shown that a deletion of the hydrophobic N-terminal domain of Tim16 causes the dissociation of Tim14–Tim16 from the translocase [119]. Furthermore, the previously described Tim44 suppressors restored the binding to the translocon that was caused by a mutation in Tim16 N-terminal domain. Hence, it was suggested that this region participates in anchoring the J-domain complex to the channel. Since it was shown that deletion of a Tim14 transmembrane segment destabilized the Tim14–Tim16 association with the translocase, an additional interaction between Tim14 and Tim44 cannot be excluded. However, given that binding to the channel is more severely impaired in the Tim16 deletion than in the Tim14 deletion, it appears that Tim16 functions as the main anchor of the complex to the translocase while Tim14 helps to stabilize this interaction.

9. The mechanism of function of the import motor

Two fundamental questions can be posed regarding the mechanism of the translocation process through the TIM23 channel. The first question originates from the size of the TIM23 channel. The second question deals with the nature of the driving force for vectorial movement of the precursor through the channel. The size of the import channel was assessed using several techniques and it was calculated to possess a diameter of ~20 Å [12,120]. Since this pore size can accommodate only one α-helix, proteins that move through the channel must be at least partially unfolded [121]. Because mitochondria can import folded proteins which possess a matrix targeting signal, it became apparent that mitochondria must unfold proteins [122,123]. How do mitochondria carry out this unfolding? Based on the observation that preproteins in transit in the import channel can undergo bidirectional movement, it was suggested that Brownian motion is utilized for translocation into the mitochondria. The Brownian ratchet model suggests that mtHsp70 binds to a polypeptide segment inside the matrix, thereby abolishing its retrograde

movement, and shifts the equilibrium towards import into the organelle. An extension of the Brownian ratchet model suggests that the natural thermal breathing of proteins, which are in constant equilibrium between folded and partially unfolded states, is exploited when mtHsp70 binds to a polypeptide segment in the matrix. According to this model, mtHsp70 binding shifts the equilibrium of the protein undergoing thermal breathing towards unfolded forms [14]. The need for the ATP-hydrolyzing chaperone mtHsp70 for import, and the fact that tightly folded proteins are imported rapidly, led to the endorsement of the power stroke model. MtHsp70, which stands at the center of this model, is assumed to undergo a conformational change upon ATP hydrolysis which generates a mechanical pulling force perpendicular to the inner membrane. The directionality of the pulling force is achieved by anchoring mtHsp70 to the membrane. Additional modifications for the models have been suggested over the years. For example, it has been proposed that both mechanisms are utilized by the motor: loosely folded proteins use the Brownian ratchet mechanism, while tightly folded proteins use the power stroke. The most recent modification is an elaboration of the Brownian ratchet model, and is called entropic pulling. Entropic pulling is a general mechanism explaining the diverse functions of Hsp70 chaperones in the cell, including protein disaggregation and translocation, and is based on entropy loss due to excluded-volume effects. In translocation, it means that when the chaperone binds to the preprotein, the preprotein has restricted freedom of movement due to steric limits of the membrane and the channel. However, as the chaperone moves away from the channel its freedom to move increases. This results in an increase in entropy which generates a pulling force. This pulling force is used to reduce the free energy barrier for unfolding and hence accelerates the thermal breathing of the protein outside the channel [124,125]. The active pulling function of mtHsp70, according to the power stroke model, requires the use of a fulcrum which is essential for the generation of a unidirectional force. The central anchor of the translocation motor to the TIM23 core complex, Tim44, has been suggested to serve as a fulcrum for mtHsp70 [20]. Hence, active pulling by the mitochondrial translocation motor requires the anchoring of mtHsp70 to Tim44 while being simultaneously bound to a precursor (Fig. 4). Since the Brownian ratchet mechanism suggests that the central function of mtHsp70 is to provide unidirectional movement, a perquisite for this model is the

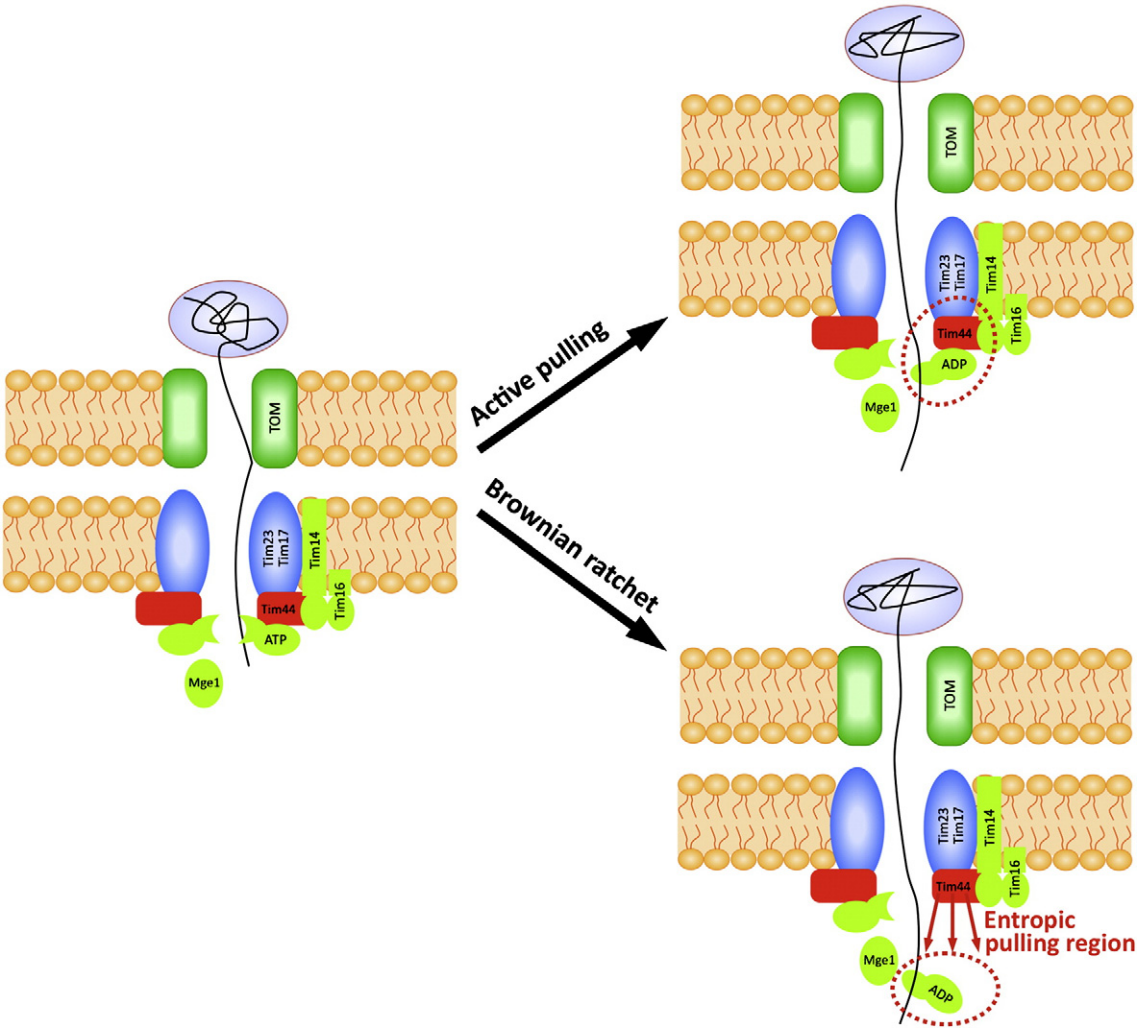

Fig. 4. Steps that distinguish between proposed models for the function of the import motor. The different models proposed for the function of the import motor. In the first step (the acceptor state), which is similar among all models, mtHsp70-ATP is recruited to the channel via Tim44, and binds to the preprotein. Under the latter conditions, the binding to Tim44 is weak and is not expected to be captured easily from purified components. Next the models diverge; according to the active pulling model, upon ATP hydrolysis, mtHsp70 undergoes a conformational change which generates a pulling force. The ternary complex between mtHsp70, Tim44 and peptide, which is unique to the motor model, is circled. In the Brownian ratchet model, mtHsp70-ADP that is bound to the precursor dissociates from Tim44, which is a necessary step for the forward movement of the preprotein. This step, which is unique to this model is circled. The entropic pulling mechanism suggests that the release of mtHsp70 from Tim44, and its forced movement away from the membrane, is facilitated by entropy loss, which generates a pulling force. This force is active in regions close to the membrane (indicated by red arrows) termed entropic pulling regions.

dissociation of mtHsp70 from its membrane anchor at some point, while still bound to the preprotein, in order to allow the protein to move forward into the matrix (Fig. 4).

Over the years, a large body of data has been accumulated supporting one or another of the above models. Data from experiments carried out with purified mitochondria and their lysates have been reviewed in the past and will not be detailed here [126–129]. Instead, we will elaborate here on recent results obtained from experiments carried out with purified components [21,22,115]. In an *in vitro* reconstitution experiment, purified mtHsp70 was allowed to form a complex with Tim44 and complex formation was examined using either pull-down or crosslinking with bifunctional reagents [115]. Two types of experiments were carried out. In the first one, the effect of nucleotides on complex formation between Tim44 and mtHsp70 was examined. Despite the disputed differences on the extent of binding in the presence of ATP, it is clear that the complex formation between mtHsp70 and Tim44 is strongest in the presence of ADP. Notably, upon the addition of a presequence, the interaction between Tim44 and mtHsp70 is destabilized regardless of the nucleotide found in the system. This may explain why a ternary complex containing mtHsp70–Tim44 and a preprotein has not yet been identified *in vitro* [115]. These results argue against the possibility that Tim44 can serve as a fulcrum for mtHsp70 upon performing its function. While the possibility of another protein serving as a fulcrum for mtHsp70 cannot be excluded (e.g. Tim14/Tim16), the results obtained so far, using purified components, are difficult to reconcile with active pulling, in its classical version, and are compatible with the mechanism of the Brownian ratchet or its modified version suggested by the entropic pulling model. Reconstitution experiments which include all components of the TIM23 complex will be required to settle this long standing dispute.

10. Future perspectives

Despite the fact that almost two decades passed since the discovery of the first TIM23 components, the research in the field seems now more interesting than ever. The list of the identified TIM23 components doubled in the last couple of years and we are beginning to understand the mechanism of function of the complex at the molecular level. The first high resolution insights were obtained from the structures of four of its components. However, there is still a long way to go. The entire complex is still notoriously difficult to purify and, except for the Tim14–Tim16 subcomplex, the structures of all other subcomplexes are still missing. No doubt, research directed towards obtaining more high resolution structures of components of the TIM23 complex is warranted in the next years. An additional advance to the field will be gained by exploring the dynamics of protein–protein interactions using methods like NMR, FRET and molecular dynamics. However, only with more structures in hand we will be able to exploit the full power of those biochemical and biophysical techniques to understand the molecular mechanisms which enable the TIM23 complex to use energy of the membrane potential and ATP for transport of proteins into two different mitochondrial subcompartments.

Acknowledgements

This work was supported by the German–Israeli Foundation for Scientific Research and Development (GIF) (grant no. 1012/08), the Israel Science Foundation (ISF 452/09) and SFB 594 grant B18. We thank Dr. Celeste Weiss for critically reading this manuscript.

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