



Review

Molecular chaperones as essential mediators of mitochondrial biogenesis

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Abstract

Chaperone proteins have been initially identified by their ability to confer cellular resistance to various stress conditions. However, molecular chaperones participate also in many constitutive cellular processes. Mitochondria contain several members of the major chaperone families that have important functions in maintaining mitochondrial function. The major Hsp70 of the mitochondrial matrix (mtHsp70) is essential for the translocation of cytosolic precursor proteins across the two mitochondrial membranes. MtHsp70 interacts with the preprotein in transit in an ATP-dependent reaction as it emerges from the translocation channel of the inner membrane. Together with two essential partner proteins, Tim44 and Mge1, mtHsp70 forms a membrane-associated import motor complex responsible for vectorial polypeptide movement and unfolding of preprotein domains. Folding of newly imported proteins in the matrix is assisted by the soluble chaperone system formed by mtHsp70 and its partner protein Mdj1. For certain substrate proteins, the protected folding environment that is offered by the large oligomeric Hsp60 complex facilitates further folding reactions. The mitochondrial Hsp70 Ssq1 is involved in the assembly of mitochondrial Fe/S clusters together with another member of the DnaJ family, Jac1. Chaperones of the Clp/Hsp100 family mediate the prevention of aggregation under stress conditions and eventually the degradation of mitochondrial proteins. Together, the chaperones of the mitochondrial matrix form a complex interdependent chaperone network that is essential for most reactions of mitochondrial protein biogenesis.

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Molecular chaperones or heat shock proteins (Hsp) occur in all organisms and are indispensable for the survival of the cell. Initially, Hsp were identified by the strong induction of protein expression under heat stress or other stress conditions. As more information about Hsp functions became available, it turned out that they are involved in or even required for many cellular functions also under normal growth conditions. The cellular activities of chaperones both in housekeeping tasks and stress protection are based on their ability to interact with proteins that are either unfolded or have not acquired their native conformation. Generally, substrates for chaperone interaction are mainly hydrophobic protein segments that are exposed to the surrounding solution. Aptly designated as chaperones, Hsp stabilize and protect bound polypeptides by preventing

irregular interactions leading to denaturation and aggregation [1]. Reactions like ribosomal translation or intracellular protein transport are prone to expose either unfolded protein segments to the environment or require posttranslational folding and/or unfolding events [2]. Hence, most essential cellular functions of chaperones are connected with the biogenesis of proteins or organelles.

Chaperones have been classified into groups according to their molecular weight. In general, five classes of chaperone proteins have been distinguished: Hsp70, Hsp60, Hsp90, Hsp100 and small Hsp [3–5]. Members of the Hsp70, Hsp60 and Hsp100 families have been identified in mitochondria. Although the basic reactivity of all chaperone types is quite similar, their structural characteristics are quite different and they participate in a multitude of very diverse cellular processes. Hsp70 are monomeric proteins that bind to unfolded, mainly hydrophobic segments of substrate proteins in an ATP-regulated manner. Interaction with Hsp70 stabilizes partially unfolded proteins, resulting in a higher efficiency of refolding or in prevention of aggregation. Hsp60 forms a large homo-oligomeric protein complex with an inner cavity that provides a protected environment

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Table 1
Chaperones and co-chaperones of the mitochondrial matrix of *S. cerevisiae*

Chaperone class	Protein	Function	Interaction partners
Hsp70	mtHsp70/Ssc1	Preprotein translocation, protein folding, stress protection	Tim44, Mdj1, Mge1
	Ssq1	Protein assembly; Fe/S cluster biogenesis	Mge1, Jac1?
	Ecm10/Ssc3	?	Mge1
Cochaperone (DnaJ type)	Mdj1	Protein folding, stimulation of mtHsp70 activity, mitochondrial DNA replication	Ssc1
	Mdj2	?	?
	Jac1	Protein assembly; Fe/S cluster biogenesis	Ssq1?
	Tim44	Preprotein translocation, membrane anchor for mtHsp70	Ssc1, Tim23, Tim17
Cochaperone (GrpE type)	Mge1	Nucleotide exchange factor for Hsp70 in preprotein translocation and folding	Ssc1, Ssq1, Ecm10
	Hsp60	Protein folding	Hsp10
Hsp60	Hsp10	Regulation of Hsp60 activity	Hsp60
	Hsp100/Clp	Hsp78	Prevention of aggregation, refolding of denatured proteins, thermotolerance
Cyclophilins	Mxc1	?	?
	Cpr3	Protein folding, peptidyl-proline-isomerisation	?

for the ATP-dependent folding of unfolded or newly synthesized single proteins or protein domains. Proteins of the Hsp100 family, also named Clp proteins, also form large homo-oligomeric protein complexes, usually rings of six or seven subunits. In contrast to Hsp60, Hsp100 are implicated in the re-solubilization of protein aggregates and in the unfolding of proteins. Hsp100 also cooperate closely, both in structure as in function, with specific proteases mediating protein turnover. The function of small Hsp is not well understood so far. Their major function seems to be the protection of soluble cellular proteins against heat stress. Members of the Hsp90 class seem to perform a more specific chaperone role restricted to certain specific substrate proteins.

Most information supplied in the following chapter is based on studies obtained with mitochondria from *Saccharomyces cerevisiae*. Mitochondrial function in this model organism is studied most extensively (Table 1). In almost all cases, conclusions on the mechanisms of protein import and folding obtained with yeast mitochondria can be generalized.

2. The mitochondrial Hsp70 system in preprotein translocation

2.1. MtHsp70 (Ssc1)

Mitochondria of *S. cerevisiae* contain three species of Hsp70 encoded by the genes *SSC1*, *SSQ1* and *ECM10* as soluble proteins of the mitochondrial matrix [6]. All three proteins show significant sequence homology to the major bacterial Hsp70, DnaK. The most abundant Hsp70 and also the most important for the function of mitochondria is Ssc1 (mtHsp70). Even if yeast cells can survive without the energy produced by mitochondria, deletions of *SSC1* are lethal under all conditions [7]. Like most members of the Hsp70 family, the mitochondrial Hsp70 consist of an N-terminal ATPase domain and a C-terminal peptide binding domain. The ATPase domain influences the properties of the

peptide-binding domain by an as yet unknown mechanism of interdomain communication. MtHsp70 undergoes a specific conformational change that is induced by binding of ATP [8]. This conformational change is correlated to a change in substrate binding affinity. In the ATP-bound state, the peptide-binding domain is in an open form with a low substrate affinity; in the ADP-bound state the peptide-binding domain is closed and has a high binding affinity [9]. Specific partner proteins, also termed cochaperones, regulate the cellular activity of Hsp70s. Based on the extensive analysis of the bacterial DnaK, two types of cochaperones have been identified for Hsp70. Proteins of the GrpE family catalyze nucleotide exchange and members of the DnaJ family assist in substrate binding. Both proteins are required for an efficient chaperone function and increase the low intrinsic ATPase activity of Hsp70 substantially. The role of the mitochondrial homologs of DnaJ and GrpE will be discussed below.

As unrelated as protein folding and polypeptide membrane translocation may seem, both activities rely on two basic properties of Hsp70 class chaperones: an affinity to unfolded protein segments and ATPase activity. Precursor proteins in transit represent ideal substrates for mtHsp70. The molecular architecture of the translocation channel requires that preproteins have to cross the membranes in a completely extended conformation [10–12]. As was shown by cross-linking experiments, mtHsp70 already interacts directly with precursor proteins arrested as translocation intermediates spanning both membranes [13,14]. The interaction of mtHsp70 with the incoming preprotein is absolutely essential for the translocation reaction of matrix targeted precursor proteins [15]. Inactivation of mtHsp70 by a temperature-sensitive mutation in the ATPase domain leads to the complete failure of the translocation of preproteins across the inner membrane. Interestingly, the essential nature of Ssc1 is shared by other proteins that are required for key steps of the mitochondrial import reaction, like the membrane channels Tom40 and Tim23. The affiliation of mtHsp70 with that group emphasizes the significance of Ssc1 function for mitochondrial biogenesis.

Two energy sources have been identified for the translocation process (Fig. 1). One is the membrane potential supplied by the respiratory chain of the inner membrane [16,17]. Its electrical component is thought to exert an electrophoretic force on the mainly positively charged N-terminal presequence driving its translocation into the matrix (Fig. 1A). This mechanism, however, is only sufficient for the translocation of the extreme N-terminal part of the preprotein. Translocation of the bulk polypeptide requires hydrolysis of ATP in the mitochondrial matrix [18,19]. MtHsp70 has been the only ATPase demonstrated to be involved in mitochondrial preprotein import. It is therefore responsible for the coupling of protein translocation to ATP hydrolysis (Fig. 1B). Mutants of Ssc1 that abolish its reactivity to ATP have similar translocation defects as those generated by selective depletion of ATP in the matrix [15,20,21]. Outer membrane translocation and the membrane potential-dependent step are unaffected in Ssc1 mutants. As a result, preproteins accumulate spanning both membranes, with the bulk of the polypeptide chain still exposed to the cytosol and the N-terminal presequence reaching into the matrix and being processed by the processing peptidase.

Some results indicate the possibility for an additional function of mtHsp70 during import. Preprotein translocation across the inner membrane requires a complex gating

mechanism of the TIM23 complex. Despite forming an aqueous channel through the inner membrane, the overall functional integrity of the membrane and especially a sufficient membrane potential has to be retained. Interestingly, mtHsp70 was also found in interaction with the inner membrane translocase component Tim17 without the involvement of Tim44 [22]. It cannot be excluded that mtHsp70 might be involved in the gating mechanism of the TIM23 complex.

2.2. The nucleotide exchange factor Mge1

As was already indicated above, Mge1, a homolog of the bacterial GrpE protein, regulates the activity of mtHsp70. The sequence conservation between GrpE and Mge1 is relatively high and Mge1 is supposed to enhance ATPase activity of its Hsp70 partner protein in a similar way as GrpE. Mge1 forms a very stable but ATP-sensitive interaction with mtHsp70 [23,24]. The interaction facilitates the release of ADP and P_i , thereby increasing the otherwise low intrinsic ATPase activity of mtHsp70 [25,26]. Interestingly, in contrast to the other mtHsp70 cochaperone Mdj1 (discussed below), Mge1 is much more important for cellular function since null mutations of *MGE1* are lethal while deletions of *MDJ1* are viable. In cooperation with mtHsp70, Mge1 has been shown to perform a prominent role in

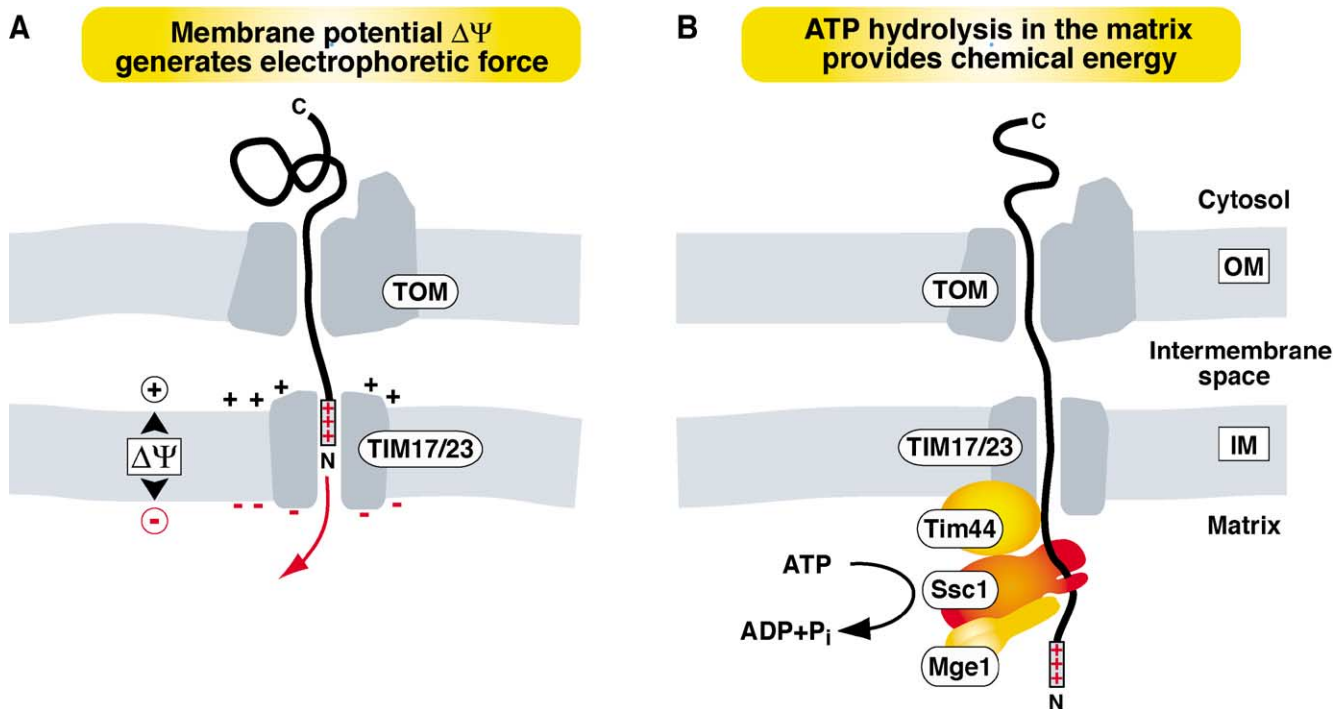


Fig. 1. Driving forces of protein translocation in mitochondria. (A) Proton pumping by the respiratory chain depletes the matrix of positive charges and generates an electrochemical gradient across the inner membrane (IM). The electric membrane potential ($\Delta\psi$) generates an electrophoretic force on the predominantly positively charged presequence once the preprotein in transit has crossed the outer membrane (OM). (B) ATP hydrolysis by matrix Hsp70 (encoded by the *SSC1* gene in yeast) provides the necessary energy for the complete translocation of the bulk polypeptide chain. Ssc1 forms a translocation motor in cooperation with the inner membrane protein Tim44 and the nucleotide-exchange factor Mge1. TOM: Translocase of the outer membrane; TIM17/23: Translocase of the inner membrane, consisting of Tim17 and Tim23. Relevant details are depicted in color.

preprotein translocation. Analysis of conditional *mge1* mutants demonstrated that an effective complex formation with mtHsp70 is important for an efficient translocation of precursor proteins [27,28]. Defects in Mge1 function also lead to an insufficient interaction of mtHsp70 with protein substrates [29]. Mge1 is involved at a very early step of the inner membrane translocation reaction. When the interaction of Mge1 with preproteins during import, via mtHsp70, was analyzed, complex formation was restricted to preproteins that have been arrested as intermediates spanning the mitochondrial membranes [30]. The arrested preproteins represent a situation where mtHsp70 is thought to perform its essential translocation function. Hence, Mge1 seems to be a key regulator of the mtHsp70 reaction cycle coordinating the affinity to substrate proteins and ATPase activity in order to obtain a productive translocation reaction [31]. The mitochondrial functions of Mge1 extend well beyond the involvement in preprotein import. As discussed below, Mge1 acts as a general cofactor in Hsp70-catalyzed reactions supporting the activity of Ssc1 as well as the other mitochondrial Hsp70s, Ssq1 and Ecm10 (Ssc3).

2.3. *Tim44 as membrane anchor for the soluble mtHsp70*

A crucial problem for the understanding of the import mechanism is how the soluble Hsp70 system can cooperate with the membrane-integrated TIM23 complex. The characterization of Tim44, the first import component identified as an inner membrane protein [32,33], provided a solution to this problem. Similar to the other key components of the mitochondrial import machinery, Tim44 is an essential protein residing closely attached to the inner face of the inner membrane [34,35]. The identification of a genetic interaction between both proteins in yeast indicated already a functional interaction between Tim44 and mtHsp70 [36]. A direct physical interaction between Tim44 and mtHsp70 could be confirmed by co-immunoprecipitation experiments using antibodies against mtHsp70. By this interaction with the membrane-associated Tim44, a portion of the soluble Hsp70 proteins becomes reversibly attached to the inner face of the inner membrane [36–38]. A stretch of 18 amino acids in Tim44 has a limited amino acid similarity to the J-domain of DnaJ that has been shown to mediate the interaction with DnaK. Tim44 might perform a similar role as the Sec63 protein of the endoplasmic reticulum that also contains a membrane-associated J-domain [39]. A deletion of this 18-amino-acid stretch in Tim44 resulted in a lethal phenotype in yeast. When co-expressed with a wild-type version of Tim44, the Tim44 $_{\Delta 18}$ mutant showed a reduced interaction with mtHsp70, resulting in significant preprotein import defects [40].

The interaction between mtHsp70 and Tim44 is very specific but sensitive to the presence of nucleotides. The nucleotide-bound state of mtHsp70 determines the interaction properties with Tim44 [8,41]. It was shown that a stable complex is formed with the ADP-bound form of mtHsp70

while ATP-binding caused a dissociation of the interaction. Hydrolysis of ATP seemed not to be required to disrupt the binding between mtHsp70 and Tim44 [8]. Interestingly, apart from being a nucleotide exchange factor for mtHsp70, Mge1 seems to influence the interaction behavior of mtHsp70 by stabilizing the complex between Tim44 and the ATP-bound form of mtHsp70 [31]. Thereby, Mge1 might substantially alter the binding properties of mtHsp70 to polypeptides in transit. However, the details of the import-related reaction cycle of mtHsp70 remain to be established. The nucleotide-dependent nature of the Tim44–mtHsp70 interaction resembles in principle the interaction of mtHsp70 with substrate proteins. However, the possibility that Tim44 is bound by mtHsp70 simply as a substrate protein has been excluded by several independent experiments. The conditional mtHsp70 mutant Ssc1-2 shows a significantly increased interaction with newly imported substrate proteins [42,43]. Under similar conditions, the interaction of Ssc1-2 with Tim44 is reduced and destabilized, clearly demonstrating distinct properties of mtHsp70 concerning substrate binding and Tim44 interaction [36,38]. Recent experiments addressed the question which domains of mtHsp70 mediate binding to Tim44. Each domain alone and different combinations were examined for their interaction efficiency with Tim44. Both by yeast two-hybrid assays and by co-immunoprecipitation assays it could be shown that the N-terminal ATPase domain is essential for the ATP-dependent interaction to Tim44, while the substrate binding domain alone is unable to bind to Tim44 [44]. Interestingly, while not binding directly to Tim44, the substrate-binding domain can determine the interaction behavior of the ATPase domain [45]. In Ssc1, the interaction with Tim44 is stabilized, while in the other mitochondrial Hsp70 homologs Ssq1 and Ecm10 (Ssc3), the binding to Tim44 is abolished by the influence of their substrate-binding domains.

Two different lines of evidence have supported the view that Tim44 performs a prominent role in the translocation of mitochondrial preproteins. First, it was shown by chemical cross-linking experiments that Tim44 is in direct association with polypeptide chains in transit across the mitochondrial inner membrane [13,34,40]. However, a possible direct involvement of Tim44 in the import reaction has not been clarified so far. Using immunoprecipitation and gel filtration of mitochondrial detergent extracts under mild conditions, Tim44 was found in association with the inner membrane translocase components Tim23 and Tim17 [22] but does not seem to be a constitutive component of the TIM23 complex. The major function of Tim44 in import is correlated with its complex formation with mtHsp70 [42,46]. By the protein interactions of Tim44, mtHsp70 is positioned directly at the site where the preprotein enters the matrix compartment. It is still controversial whether the interaction with Tim44 only serves to direct mtHsp70 to the immediate vicinity of the import site or whether Tim44 also serves as a leverage for the generation of a translocation force generated by

mtHsp70 (see below). Mutations in both proteins that compromise the interaction of mtHsp70 with Tim44 show a significant decrease in import efficiency. The Tim44 $_{\Delta 18}$ mutant protein that shows a reduced interaction with mtHsp70 displays a translocation defect *in vitro*. Even more pronounced were translocation defects in the conditional mutant *tim44-8* that is characterized by aggregation of Tim44 molecules at the non-permissive temperature [47]. However, import in *tim44* mutants analyzed so far was not inhibited completely. The strongest defects were found in the import of precursor proteins that contain conformational restrictions in form of tightly folded C-terminal domains and require unfolding by the mitochondrial import machinery during translocation (see below).

2.4. Molecular mechanism of mtHsp70 during translocation

The wealth of information accumulated on the function of Hsp70, and in other systems like bacteria, prompted the development of two different hypotheses for the molecular mechanism of mtHsp70 during translocation; the molecular ratchet or “trapping” model and the active motor or “pull-

ing” model (Fig. 2). The trapping mechanism was initially proposed as a general explanation of how polypeptide translocation through cellular membranes could be driven by protein interactions [48]. The main characteristic of the trapping mechanism is that polypeptide movement in the translocation channel is generated by Brownian motion. This random movement is converted into vectorial translocation by the consecutive trapping of exposed polypeptide sequences by a component inside the compartment. In the case of mitochondria, the N-terminal part of the preprotein is inserted into the inner membrane driven by the membrane potential. As soon as respective binding sites are exposed in the matrix, mtHsp70 will bind to the polypeptide in transit, thereby preventing the backward movement [46,49]. Polypeptide binding would activate the ATPase activity of mtHsp70, converting it into the high-affinity state for substrates. Several binding steps of mtHsp70 molecules would then eventually result in the complete translocation of the polypeptide. After the translocation is completed, the bound preproteins are released in the matrix leading to folding and assembly reactions. Preprotein membrane insertion and complete polypeptide

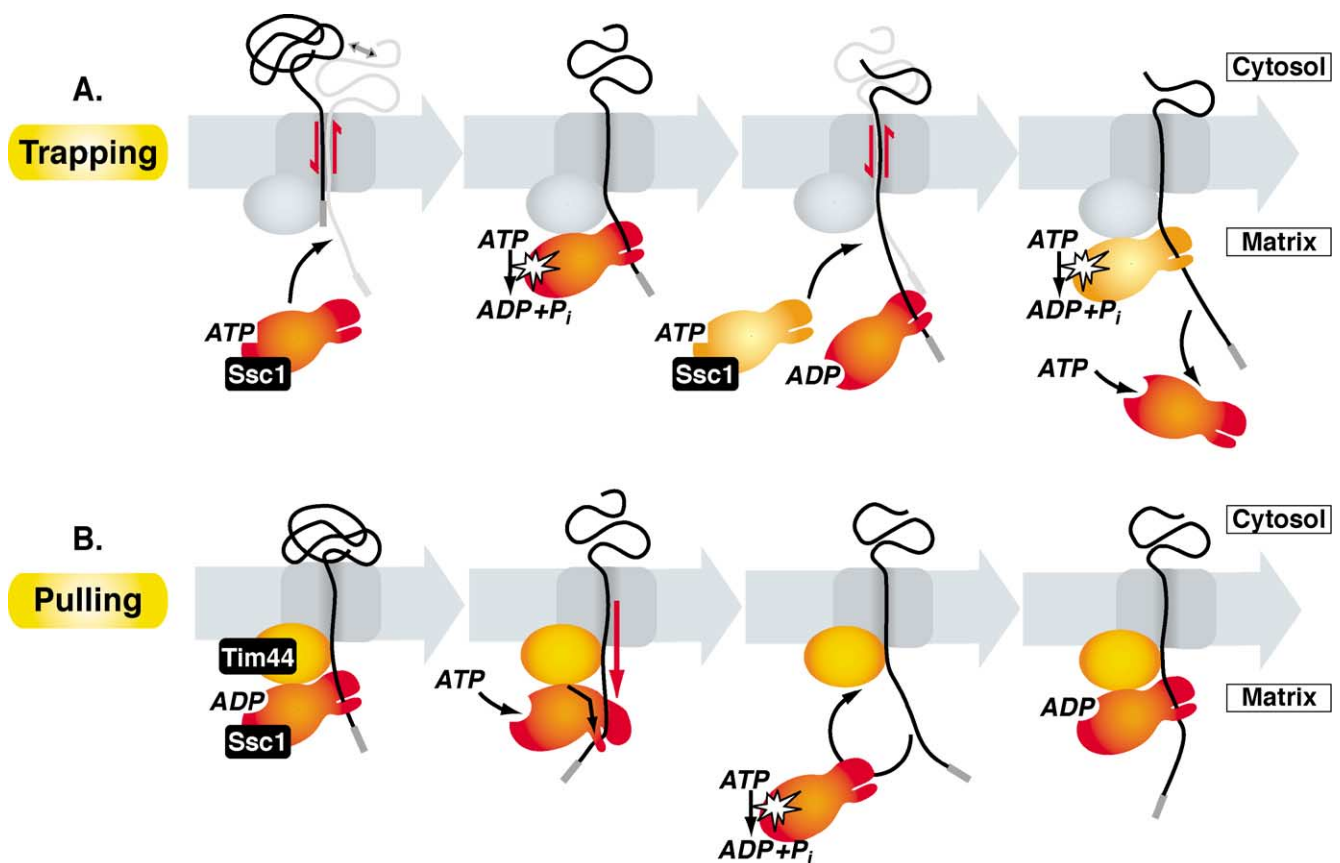


Fig. 2. Models of the mechanism of mtHsp70 during preprotein translocation. (A) Molecular ratchet (trapping): backward movement of a preprotein by Brownian oscillation in the translocation channel is inhibited once it is bound by matrix mtHsp70 (Ssc1 in yeast) located at the import site. Cycles of preprotein binding and release by mtHsp70 molecules, in response to the nucleotide-bound state of the chaperone, trap increasing lengths of preprotein in the matrix. (B) Active pulling: using Tim44 as a leverage at the import site, an ATP-induced conformational change of mtHsp70 bound to a preprotein generates an inward directed force that unfolds and pulls the preprotein into the matrix.

translocation are therefore functionally independent events catalyzed by separate polypeptide machineries [50]. The role of the cofactor Mge1 would be the recycling of active mtHsp70 molecules by the exchange of nucleotides. Tim44, possibly working as a dimer loosely associated with the inner membrane translocase, would be required to increase the local concentration of the soluble mtHsp70 in the direct vicinity of the import site [51]. Recent data provided direct evidence for a mtHsp70 import function via the trapping mechanism by demonstrating a tight correlation between enhanced preprotein binding by mtHsp70 and an increased import efficiency under conditions of a reduced membrane potential [52].

Although a trapping mechanism is sufficient to explain the basic import activity of mtHsp70, some studies indicated a more active role of mtHsp70 during translocation. Based on the importance of both ATP-hydrolysis by mtHsp70 and its interaction with the membrane component Tim44, the “pulling” model was proposed. This model postulates that mtHsp70 might be able to generate a direct force on the polypeptide during translocation, thus working like a translocation motor. The force would be generated by the ATP-induced conformational change of mtHsp70 while it is binding to the preprotein in transit. For this mechanism, a stable interaction of mtHsp70 with Tim44 is crucial to provide the necessary leverage for the force generation [53,54]. Experimental evidence supporting this active role for mtHsp70 has been based mainly on two observations: the requirement for preprotein unfolding during import and the generation of an inward directed translocation force. It was shown that due to the geometry of the import channel preproteins are required to cross the mitochondrial membranes in a completely unfolded or even stretched conformation [10,55]. The mitochondrial import machinery therefore must be able to unfold preproteins. Binding of the precursor protein to components of the outer membrane like surface receptors or the import pore can result only in a limited unfolding effect if at all [56,57]. Unfolding of tightly folded preprotein domains requires ATP hydrolysis in the matrix indicating a direct involvement of mtHsp70 [58]. This could be confirmed by the analysis of temperature-sensitive mutants of mtHsp70 that show defects in import of folded but not of unfolded preproteins [15,42]. A comparison between the rate of preprotein unfolding and the rate of preprotein translocation therefore could allow conclusions whether mtHsp70 acts via an active or passive mechanism. A prerequisite for import via the passive trapping mechanism is that the preprotein must unfold spontaneously before entering the translocation channel. Unfolded segments then move into the matrix and become trapped by mtHsp70. To be consistent with the passive trapping mechanism, the rate of spontaneous unfolding should be faster than the preprotein import rate [59]. However, recent results indicated that preprotein unfolding during translocation is both mechanistically and kinetically different from a spontaneous process. It could be shown that unfolding pathways, in other words,

the order by which protein segments unfold, are different between a preprotein in solution and during import [60]. Imported preproteins essentially unfold in the same direction as the import reaction occurs, from the N to the C terminus. In addition, detailed analysis of preprotein import kinetics argues against spontaneous unfolding as the rate-limiting step of the import reaction. A determination of maximal import rates under substrate saturating conditions demonstrated that the import rate is faster than preprotein unfolding in solution, indicating that preprotein unfolding is an active and mtHsp70-dependent process [61,62]. Interestingly, preproteins with short N-terminal extensions followed by a folded domain show an initial delay in the import rate that is dependent on the folding state of the preprotein. Since short N-terminal segments restrict the accessibility of the preprotein for mtHsp70 in the matrix, initial import rates become dependent on the slower spontaneous unfolding rates. The analysis of mtHsp70 mutants under these conditions confirmed that the activity of mtHsp70 largely determines the maximal import rate. However, it is not yet clear if the unfolding action of mtHsp70 is based on a direct pulling action on preprotein segments that cause the collapse of entire protein domains on the outside or if local unfolding fluctuations are trapped by binding and/or the conformational change of mtHsp70.

By a specific variation of the *in vitro* protein import assay, conclusions about an inward directed translocation force during the mitochondrial translocation reaction can be drawn. Preproteins with a tightly folded and stable C-terminal domain like dihydrofolate reductase fusion proteins in the presence of the ligand methotrexate can be completely blocked during the import reaction [63]. The resulting membrane-spanning translocation intermediates are partly resistant against external proteases since they are pulled close to the outer membrane surface by mtHsp70. Mitochondria containing the mtHsp70 mutant *ssc1-2* are unable to generate this inward directed translocation force [64]. *Ssc1-2* shows an increased binding to preprotein substrates but is deficient in the Tim44 interaction [8,42]. Intragenic suppressor mutants that rescue the temperature-sensitive growth defect also restore the Tim44 interaction and preprotein import [64]. Also in wild-type cells, it was shown that increased trapping of preproteins does not correlate with the generation of an active translocation force.

To summarize, mtHsp70 forms the core of a translocation motor that utilizes the energy of ATP hydrolysis to mediate preprotein movement and unfolding. MtHsp70 can function by two molecular mechanisms during preprotein translocation, the passive ‘trapping’ and the active ‘pulling’ mechanism. The basic translocation activity is based on the efficient binding to the preprotein in transit. The energy barrier imposed by folded C-terminal folded domains is likely lowered due to the generation of a translocation force by the mtHsp70 system. Current evidence indicates that both mechanisms cooperate in order to obtain maximal import efficiency [65].

3. Protein folding and assembly in the mitochondrial matrix

3.1. *Ssc1* and *Mdj1*

The ability and the necessity to import proteins from the outside are certainly a secondary evolutionary acquisition of mitochondria not existing in the ancestral bacterial cell. Accordingly, the unique role of mtHsp70 in preprotein membrane translocation is not its only function in mitochondrial biogenesis (Fig. 3). Not all mitochondrial proteins need to be imported from the cytosol, as a limited set of mainly hydrophobic proteins are synthesized by mitochondrial ribosomes. Indeed, it could be shown that mtHsp70 interacts directly with subunits of the ATP synthase after their synthesis by mitochondrial ribosomes and facilitates their assembly into the macromolecular complex [66]. Hence, mtHsp70 plays a prominent role in the biosynthesis of mitochondrially encoded proteins.

Direct support for a role of mtHsp70 in protein folding was provided by the identification of a genuine mitochondrial homolog of the bacterial DnaJ protein in yeast, termed Mdj1 [67]. Null mutations of *MDJ1* in yeast do not lead to a lethal phenotype but show respiratory defects. Mdj1 could not be found associated with preproteins that have been arrested as membrane-spanning translocation intermediates but only with fully imported preproteins [68]. Hence, it is unlikely that Mdj1 plays an important role in protein import, which was confirmed by the absence of translocation defects in the *mdj1Δ* mutant. However, newly imported proteins tend to misfold and aggregate in a *MDJ1* mutant [69], indicating a prominent role of Hsp70 and Mdj1 in mitochondrial protein folding. Interestingly, the role of mtHsp70 in protein folding seems to be independent from its role in translocation. MtHsp70 can be found either membrane-associated in a translocation complex with Tim44 or as a soluble folding complex together with Mdj1 [70]. A direct chaperone activity of the mitochondrial

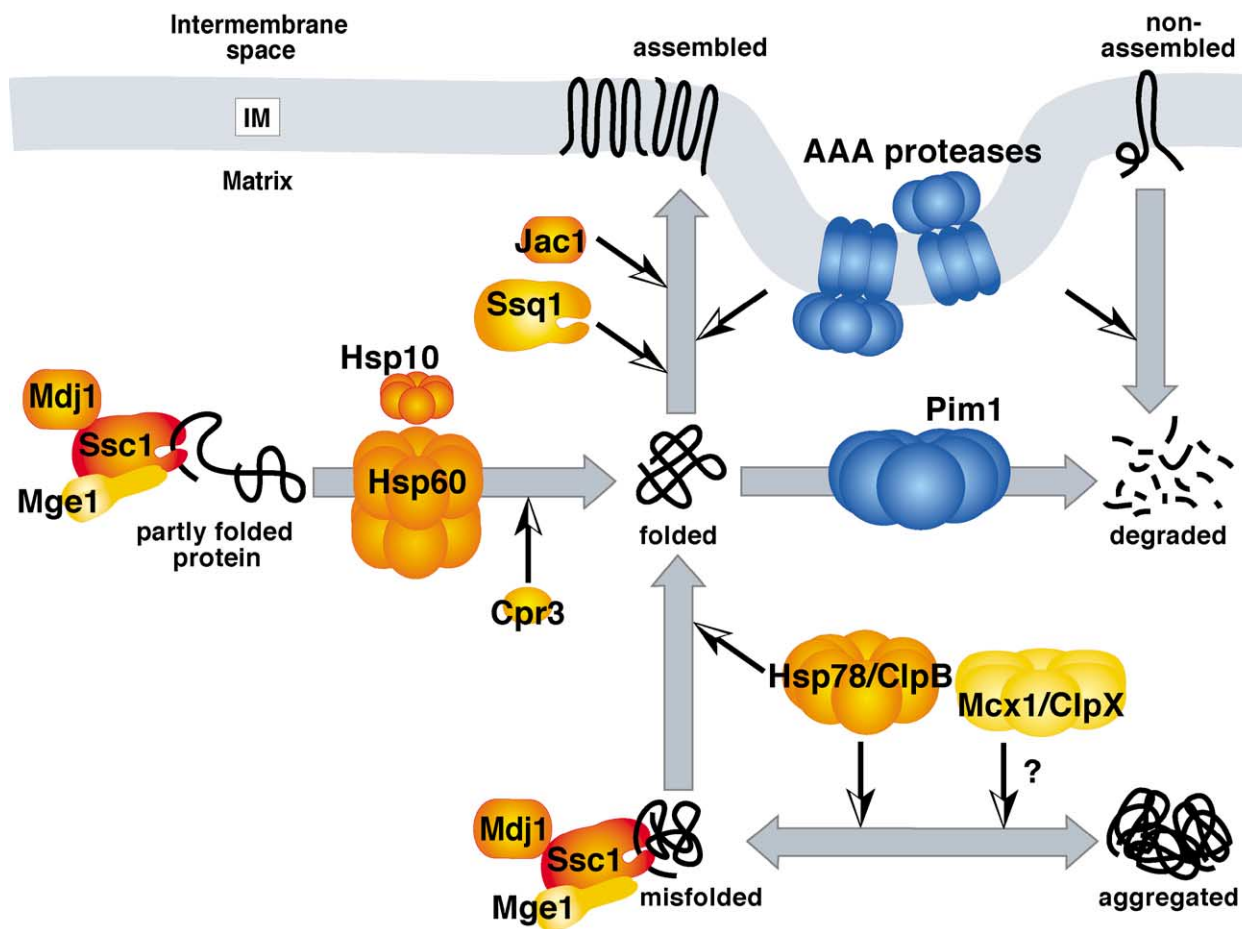


Fig. 3. Chaperone network in the mitochondrial matrix of *S. cerevisiae*. Chaperone proteins and cochaperones are depicted in yellow/red colors. Proteins with protease activity are shown in blue. The mtHsp70/Mdj1/Mge1 chaperone system in the matrix is associated with newly imported or synthesized, partly folded proteins, as well as with misfolded polypeptides. Hsp60 and its cochaperone Hsp10 are involved in protein folding of substrate proteins after release from mtHsp70. Chaperones of the Hsp100 family like Hsp78 as well as proteases such as Pim1 cooperate with the mtHsp70 system in refolding and degradation processes.

Hsp70 system could also be demonstrated by in vitro folding assays. Ssc1, Mdj1 and Mge1 were able to prevent heat-denatured luciferase from aggregation and increased the efficiency of refolding significantly [68,71]. In this assay, Mdj1 behaves similar to DnaJ in that it also shows chaperone activity on its own.

Apart from its role in the biogenesis of mitochondrial proteins, Mdj1 is also involved in the inheritance of mitochondrial DNA, even under normal growth conditions. It was shown that the activity of the mitochondrial DNA polymerase is influenced by Mdj1 [72]. This fact is most likely the reason for the respiratory deficiency of *mdj1* mutants. Apart from Mdj1 that shows high homology to DnaJ over the whole length of the protein, a membrane protein containing only the characteristic J-domain was identified in yeast mitochondria and named Mdj2 [68]. A deletion mutant did not show major defects in mitochondrial functions and its function remains unclear so far.

3.2. Ssq1 and Jac1

The genomic sequencing project revealed the existence of genes for two additional Hsp70 family proteins that contained N-terminal extensions characteristic for mitochondrial presequences. One of those Hsp70 homologs, Ecm10, has a very high sequence identity to the major mtHsp70, Ssc1. Surprisingly, *ECM10* was initially identified in a genetic screen looking for genes involved in yeast cell wall biogenesis. However, its expression level seems to be extremely low and since a gene deletion shows no phenotype, its functional relevance remains unclear. Recently, its mitochondrial localization has been confirmed and it was suggested that it can take over functions of Ssc1 when strongly overexpressed [73].

The second homolog, Ssq1, still shows significant sequence homology to Ssc1 but its overall conservation, especially in the substrate-binding domain, is significantly lower than in Ecm10. Deletion mutants of *SSQ1* show a distinct phenotype from mutations in *SSC1*. The mutants are defective in maintenance of the mitochondrial genome and have a cold-sensitive growth behavior [74]. They were also found to accumulate iron in the mitochondrial matrix. Subsequent studies demonstrated that levels of enzymes containing Fe/S cluster in vivo are decreased in *ssq1* mutants proposing a function of Ssq1 in the assembly of Fe/S cluster [75,76]. Mitochondria contain an elaborate machinery of enzymes assisting in the generation of Fe/S cluster and in their assembly into the functional protein complexes [77,78]. In addition, the maturation of another mitochondrial protein involved in iron metabolism, the yeast frataxin homolog Yfh1, is defective in *ssq1Δ* cells [75,79]. It is still unclear if Ssq1 plays a direct or only an indirect role in the biosynthesis of Fe/S cluster, since no specific protein substrates have been identified so far. Similar to Ssc1, Ssq1 activity is regulated by a specific interaction with the nucleotide exchange factor Mge1, exhibiting a unique

competitive reaction between two Hsp70s in one cellular compartment for the same cofactor [80]. However, as expected from the absent activity in preprotein translocation, Ssq1 is not able to interact with the translocase component Tim44.

Yeast genetic experiments first hinted at a relationship between Ssq1 and a new homolog of DnaJ, Jac1 [81]. Jac1 is an unusual member of the DnaJ-family, consisting mainly of the J-domain supposed to be responsible for DnaJ–Hsp70 interaction. In contrast to Ssq1, Jac1 is an essential protein, indicating an important function for the cell. Conditional mutants of *jac1* displayed similar defects in iron metabolism as were described for Ssq1 mutants [82,83]. Since a direct interaction between Ssq1 and Jac1 or an influence of Jac1 on Ssq1 activity has not been observed so far, the relevance of Jac1 for the function of Ssq1 remains to be determined.

3.3. Hsp60/Hsp10

A chaperone of the Hsp60 family is one of the most important components of the protein folding system in the mitochondrial matrix [84]. Members of the Hsp60 family are distinguished by their characteristic structure. They form a homo-oligomer of 14 subunits with seven subunits arranged in a ring, resulting in a characteristic “double doughnut” structure [85,86]. The double ring system forms a large inner cavity that is capable of accommodating proteins with a molecular weight of up to 50 kDa. Proteins that enter this cavity are protected from interactions with other components of the surrounding environment. Preferential substrates for Hsp60 are folding intermediates that have not acquired their native structure. Substrate proteins bind to hydrophobic amino acid residues that are exposed on the inner wall of the cavity. Binding of ATP induces a large conformational change in the single subunits that open up the cavity and reduce the overall hydrophobicity of the cavity surface. Thereby, bound proteins are released from the Hsp60 complex and can undergo new folding attempts. The cochaperone Hsp10 [87,88], the homolog of the *E. coli* GroES, can form a lid at the top of the double ring system, closing the opening of the central cavity [89]. Hsp10 is supposed to coordinate the behavior of the single Hsp60 monomers and regulate the ATPase cycle [90].

The important role of Hsp60 in mitochondrial biogenesis was initially identified by the analysis of temperature-sensitive mutants [91]. Null mutations of Hsp60 in *S. cerevisiae* are inviable due to the severe defects in folding of mitochondrial proteins. Conditional mutants accumulate unfolded proteins in the matrix that are not able to assemble into active enzyme complexes. It was shown that newly imported mitochondrial preproteins physically interact with Hsp60 shortly after reaching the matrix compartment [92,93]. The Hsp60-bound proteins then acquire their native state in an ATP-dependent reaction. A study using total yeast proteins translated from mRNA for in vitro import

reactions analyzed the range of natural substrate protein for Hsp60 in the matrix. The requirement for folding catalysis by Hsp60 and Hsp10 can vary substantially depending on the particular properties of the substrate protein [94]. At least for a subset of mitochondrial proteins, folding catalysis by Hsp60 is essential for the acquisition of the native conformation [95]. The two major chaperone classes in the mitochondrial matrix, Hsp70 and Hsp60, most likely cooperate in the folding reaction of imported proteins in a sequential order. Due to its role in translocation, preproteins first encounter mtHsp70. Only after being released from Hsp70, preproteins interact with the Hsp60 complex [96].

Protein folding is a biological problem that involves also other enzymes, in addition to molecular chaperones. A major component of the mitochondrial folding system is the peptidyl-prolyl-isomerase Cyclophilin, encoded by *CPR3* in yeast. Cyclophilins assist protein folding by bringing prolyl bonds in a conformation suitable for further folding reactions. Cpr3 was shown to be important for efficient folding of newly imported preproteins [97,98]. It acts in a cooperative manner with Hsp60 to obtain a high folding efficiency [99].

3.4. Hsp78

The Hsp70 and Hsp60 chaperone systems are the main machineries required for folding of newly imported and mitochondrially encoded preproteins. Members of the third family of mitochondrial chaperones, the Clp or Hsp100 proteins, perform important roles during the later stages of the life cycle of a protein. Functional proteins may become damaged by various stress situations leading to misfolding, aggregation and eventually degradation [100]. Recent results obtained mainly with the bacterial system indicate that members of the Hsp100 family mediate protective reactions, preventing cellular damage caused by the accumulation of aggregated proteins. It was shown that bacterial members of the Hsp100, mainly ClpB and ClpA, are capable to dissolve protein aggregates in an ATP-dependent reaction [101,102]. With the help of the Hsp70 system, the disaggregated proteins can then refold into their functional conformation. The ClpB homolog Hsp104, the major protein conferring resistance to extreme temperature levels in eukaryotic cells, fulfills a similar function in the cytosol [103,104]. On the other hand, Hsp100 also assist the degradation of irreversible damaged polypeptides by specific proteolytic machineries. It is thought that Hsp100 in cooperation with Hsp70 supply an unfolding activity required for the efficient and complete proteolytic degradation of misfolded substrate proteins. In both reactions, Hsp70 and Hsp100 proteins may have largely overlapping functions depending on the specific cellular environment and/or the properties of the individual substrate protein.

The first member of the Hsp100 family identified in mitochondria has been Hsp78, a homolog of the bacterial ClpB [105]. Under normal growth conditions, mutants of

Hsp78 do not show major defects. A possible role for Hsp78 in mitochondria emerged when it was shown that overexpression of Hsp78 can partially rescue the temperature-sensitive growth defect of the mtHsp70 mutant *ssc1-3* [106]. Double mutants of *hsp78Δ* and *ssc1-3* showed a significantly reduced membrane potential and a tendency to lose mitochondrial DNA [107]. These experiments confirm the close functional interdependence of both chaperone systems. Under severe temperature stress, Hsp78 becomes more important for the maintenance of mitochondrial function. Especially reactivation of the mitochondrial protein synthesis machinery is dependent on the presence of Hsp78 [108]. This role in cellular thermotolerance could be directly confirmed since the main cytosolic chaperone involved in heat stress protection, Hsp104, could be substituted by its mitochondrial relative Hsp78. Recently, a chaperone activity of Hsp78 could be demonstrated directly by in vitro refolding assays [109]. Again, maximal refolding activity could only be obtained when the Hsp70 system consisting of Ssc1, Mdj1 and Mge1 was present.

In addition to Hsp78, mitochondria contain other Hsp100 class proteins like Mcx1 in yeast [110] and ClpP in mammalian and plant mitochondria [111]. Their function is not well defined so far but in analogy to the bacterial homologs, it is assumed that they perform both functions in mitochondrial protein folding and proteolysis [100]. An additional set of proteins that exhibit chaperone activity are members of the so-called AAA protein family [112]. The major function of these proteins is the ATP-dependent proteolytic degradation of soluble and membrane proteins (see lower chapter).

4. Conclusion

Proteins from a variety of chaperone families have indispensable functions for the biogenesis of mitochondria. The core protein of the import motor complex, mtHsp70, is responsible for the coupling of ATP hydrolysis with polypeptide movement and unfolding during preprotein membrane translocation. The elucidation of the molecular mechanism of mtHsp70, the structural basis of its cooperation with the partner protein Tim44 and the functional interaction with the TIM23 complex will be the center of further research efforts. Mitochondrial chaperones form a complex machinery cooperating as a “chaperone network” involved in multiple reactions of protein biogenesis in the matrix (Fig. 3). This chaperone system is tailored to the folding and assembly problems presented by newly imported and also by mitochondrially synthesized substrate proteins. In addition, mitochondrial chaperones also perform crucial functions in thermotolerance, prevention of aggregation and protein degradation. The characterization of the multiple functional and structural interactions of mitochondrial chaperones will be the basis for the understanding of cellular protein biogenesis.

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