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The ATP-driven protein translocation-motor of mitochondria

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Summary

The majority of mitochondrial proteins are encoded in the nucleus. In the cytosol they are synthesized as precursor proteins which are transported into mitochondria. Protein import into mitochondria requires a concerted action of a variety of different proteins. For the transport of precursor proteins across the mitochondrial inner membrane and their folding in the matrix the mitochondrial hsp70 (mhsp70) chaperone plays an essential role. Mhsp70 is found in at least two protein complexes within mitochondria: together with Tim44 and mGrpE, mhsp70 forms the import-complex and together with Mdj1 and mGrpE it forms a folding-complex. This review focuses on the function of the import-complex. It is believed that mhsp70 can act as a mechano-chemical enzyme that actively pulls precursor proteins across the inner membrane.

I. Introduction

Mitochondria do contain their own DNA. However, this DNA encodes only about a dozen proteins. The majority of the mitochondrial proteins are encoded in the nucleus and are imported from the cytosol. These proteins are synthesized in the cytosol as precursor proteins containing usually an N-terminal mitochondrial targeting signal. They are co- or post-translationally transported into mitochondria. Cytosolic members of the hsp70 family as well as a mitochondrial import stimulating factor facilitate the transfer of the precursor to the receptor complex (Hachiya et al., 1995). Different precursor proteins bind to different subcomplexes of the heterotetrameric receptor. These precursors are then delivered to the insertion pore (Hachiya et al., 1995; Komiya et al., 1997). The translocation of precursor proteins across both mitochondrial membranes is facilitated by the components of the translocase found in the outer membrane (Tom) and the translocase found in the inner membrane (Tim). Translocation requires a loosely folded conformation of the precursor protein during import, an electrochemical potential across the mitochondrial inner membrane and ATP in the matrix space. Following translocation, the presequence is removed by the matrix localized processing peptidase. Finally, the precursor protein folds with or without the help of the matrix localized chaperones. Mitochondrial hsp70 (mhsp70) is essential for the translocation across the inner membrane for all matrix targeted precusor proteins as well as for the folding of some precursor proteins in the matrix space.

One of the most fascinating questions about protein import into mitochondria is how a matrix localized mhsp70 chaperone can facilitate import of precursor proteins from the cytosol into mitochondria. In this review we will focus on this aspect of mhsp70 function.

II. The HSP70 chaperone family

Heat shock proteins of the Hsp70 family are found in nearly all organisms. These proteins play an essential role in protein folding, transport into different cellular compartments, and regulation of the heat-shock response (reviewed in: Hightower et al., 1994; McKay et al., 1994). The members of this protein family are highly conserved from bacteria to man (Lindquist and Craig 1988; Boorstein et al., 1994). Some members of the hsp70 family are constitutively expressed whereas others are expressed only under stress conditions (Lindquist and Craig, 1988; Boorstein et al., 1994). Hsp70 proteins bind to unfolded,

hydrophobic surface-exposed segments of polypeptide chains (Pelham, 1986; Blond Elguindi et al., 1993; Flynn et al., 1991; Landry et al., 1992; Zhu et al., 1996). However, the actual mechanism of hsp70 function is still not completely understood. Binding and release of substrates (polypeptides or peptides) by hsp70 is regulated by adenine nucleotides. The conformation of hsp70 changes during the cyle of ATP-binding, ATP-hydrolysis, and release of ADP and Pi (Flaherty et al., 1990; Hightower et al., 1994; von Ahsen et al., 1996). Studies on DnaK, the hsp70 protein of *E. coli*, demonstrated that optimal functioning of this chaperone requires the co-chaperones DnaJ and GrpE (reviewed in Georgopoulos and Welch, 1993). DnaJ accelerates ATP hydrolysis by DnaK, whereas GrpE acts as an adenine nucleotide-exchange factor for DnaK (Liberek et al., 1991; McCarty et al., 1995). Homologs of DnaJ and/or GrpE cooperate with hsp70 proteins in the eukaryotic cytosol and in the lumenal spaces of mitochondria and the endoplasmic reticulum (Bolliger et al., 1994; Caplan et al., 1993; Ikeda et al., 1994; Laloraya et al., 1994; Schlenstedt et al., 1995; Horst et al., 1997a).

A. HSP70 chaperones involved in protein translocation

a. BiP

The lumen of the endoplasmic reticulum (ER) contains an abundant hsp70 chaperone: BiP (heavy chain binding protein) in mammals and Kar2p in yeast. The protein was initially identified because of its key role in the folding of newly-imported ER proteins (Brodsky and Schekman, 1993). Later it was found that this protein also mediates the ATP-dependent translocation of proteins into the yeast ER, and that this function involves the ATP-regulated interaction of BiP/Kar2p with the membrane protein Sec63p (reviewed in: Brodsky and Schekman, 1994; Brodsky, 1996). Kar2p is needed for co- as well as post-translational translocation into the yeast ER (Brodsky et al., 1995). When ER membranes are solubilized and fractionated in the absence of ATP, Kar2p copurifies with Sec63p (Brodsky and Schekman, 1993). Genetic studies have confirmed the functional importance of the Kar2p-Sec63p interaction (Scidmore et al., 1993). Kar2p appears to recognize a region in Sec63p that is homologous to the conserved "J domain" in members of the DnaJ protein family (Sadler et al., 1989; Ang et al., 1991).

b. Mitochondrial HSP70

In *Saccharomyces cerevisiae* the product of the *SSC1* gene (mitochondrial hsp70) is located within mitochondria. Genetic and biochemical evidence implicate mhsp70 as a component of the mitochondrial protein import machinery. Mhsp70 is essential for growth (Craig et al., 1987). In *vitro* import studies of mitochondria isolated from a temperature sensitive mhsp70 mutant showed defects in protein import and an accumulation of precursor proteins at contact sites (Kang et al., 1990). Furthermore, mhsp70 can be crosslinked to or co-immunoprecipitated with precursor proteins on their way to the matrix (Kang et al., 1990; Scherer et al., 1990). Subsequent experiments have shown that mhsp70 forms a transient, ATP-dependent interaction with newly imported precursor proteins (Manning-Krieg et al., 1991). Interestingly, it has been shown that a temperature sensitive mhsp70 allele fails to bind and to complete import of a partially translocated precursor protein (Gambill et al., 1993). These data and others suggest that mhsp70 is playing a major role in mitochondrial protein import and protein folding (reviewed in: Langer and Neupert, 1994; Rassow et al., 1996). Mhsp70 performs these different functions together with several different partner proteins.

The first identified mhsp70 complex was the site-specific endonuclease Endo.SceI, which is a dimer consisting of mhsp70 and a 50-kDa nuclease subunit (Morishima et al., 1990). In the heterotetrameric protein folding complex, mhsp70 works with two partner proteins (Horst et al., 1997a): Mdj1p, a homolog of bacterial DnaJ (Rowley et al., 1994); and a mitochondrial GrpE (mGrpE) dimer, a homolog of bacterial GrpE (bGrpE) (Bolliger et al., 1994; Laloraya et al., 1994; Nakai et al., 1994). Mdj1 is itself a chaperone (Prip-Buus et al., 1996). However, it also stimulates the ATPase-activity of mhsp70 suggesting that it supports mhsp70 function during protein folding (Horst et al., 1997a). MGrpE functions as a mitochondrial ADP-ATP exchange factor for mhsp70 (Azem et al., 1997). In *E. coli* the hsp70 chaperone system comprises of three proteins: DnaK, DnaJ, and bGrpE (Georgopoulos and Welch, 1993; Szabo et al., 1994). The mitochondrial hsp70 folding system is the only one in eukaryotes that contains a DnaJ as well as a bGrpE homolog, suggesting that the mitochondrial system is the closest eukaryotic homolog of the bacterial system (Bolliger et al., 1994; Laloraya et al., 1994; Nakai et al., 1994; Rowley et al.,1994; Westermann et al., 1995; Prip-Buus et al., 1996; Horst et al. 1997a). In the import complex, mhsp70 forms a heterotetrameric complex with Tim44 and a mGrpE dimer (Kronidou et al., 1994; Rassow et al., 1994; Schneider et al., 1994). Tim44 functions in this complex as a membrane anchor for mhsp70. Tim44 spans the mitochondrial inner membrane, appears to be intimately associated with the translocation channel (Maarse et al., 1992; Scherer et al., 1992) and contains a domain that is weakly homologous to the J domain of Sec63p. It was suggested that the J-like domain of Tim44 is the binding site for mhsp70 (Rassow et al., 1994). If so, the

translocation systems of mitochondria and the ER would be rather similar (see however, Brodsky, 1996).

III. Protein translocation models

What is the mechanism for the translocation of precursor proteins across the mitochondrial membranes? Two translocation mechanisms were suggested: a "Brownian-Ratchet" and "Translocation-Motor" (Neupert et al., 1990; Simon et al., 1992; Glick, 1995; Pfanner and Meijer, 1995; Horst et al., 1997b).

A. The "Brownian-Ratchet" model

In the "Brownian-Ratchet" model, precursor polypeptides randomly oscillate within the translocation channel. In the first version of the "Brownian-Ratchet" model this random oscillation was suggested to be only due to Brownian-molecular motion. When the precursor protein moves inward, a mhsp70 molecule binds to the emerging segment of the polypeptide chain in the matrix, thereby preventing its reverse movement. Repeating such a binding event will finally lead to the translocation of the entire precursor protein across the inner membrane into the matrix space (**Figure 1**). Tim44 can have two different functions: a more passive function just as a membrane anchor for mhsp70 or a more active one as a promotor for binding of mhsp70 molecules to the precursor chain. Tim44 could perform the latter function by acting in a DnaJ-like fashion to catalyze ATP hydrolysis by mhsp70. The observation that precursor chains can slide bidirectionally in the mitochondrial translocation channel strongly supports the "Brownian-Ratchet" model (Ungermann et al., 1994). This "sliding" was suggested to be due to random thermal motion of the polypeptide chain in the translocation channel (Neupert et al., 1990; Simon et al., 1992; Glick, 1995; Pfanner and Meijer, 1995; Horst et al., 1997b).

If Brownian motion was the sole factor involved in protein translocation a precursor protein which can not be fully imported due to a tightly folded domain should slide back out of the translocation channel in a few milliseconds. In reality this process takes some minutes (Ungermann et al., 1996). This suggests that some sort of interaction occurs between the translocating polypeptide and the components of the translocation channel, hindering free diffusion of the polypeptide chain in the translocation channel. The created "friction" between the polypeptide chain in the translocation channel could partly be responsible for the unidirectionality of the translocation process. This would assume that the "friction" for the inward movement of the polypeptide chain is smaller than the one for the opposite movement of the polypeptide chain.

An extended version of the "Brownian-Ratchet" model has been proposed to explain the import of precursor proteins containing a tightly folded domain. In this model the folded domain spontaneously unfolds at the mitochondrial surface due to random thermal movements ("breathing" of the molecule). This would allow an inward movement of the precursor chain (Stuart et al., 1994). Thus, the rate of import of such a folded precursor protein is limited by its spontaneous unfolding rate. For example, fusion proteins containing mouse dihydrofolate-reductase (DHFR) are imported into mitochondria within 10-20 minutes at room temperature (Endo and Schatz, 1988); and DHFR spontaneously unfolds, on average, once every few minutes at this temperature (Viitanen et al., 1991). Such import kinetics can easily be explained by the "Brownian-Ratchet" model. However, the DHFR unfolds spontaneously very fast, much faster than the majority of other proteins. A single unfolding transition usually requires hours or even days (Creighton, 1993). For example, this is true for the heme-binding domain of cytochrome b_2, which is already tightly folded after release of the nascent chain from the ribosome. Nevertheless, the precursor is imported *in vitro* within only a few minutes (Glick et al., 1993). Interestingly, in contrast to DHFR fusion proteins, cytochrome b_2 absolutely requires both matrix ATP and functional mhsp70 for import (Glick et al., 1993; Voos et al., 1993; Stuart et al., 1994), suggesting that mhsp70 uses the energy of ATP hydrolysis to accelerate unfolding of the heme-binding domain at the mitochondrial surface.

B. The "ATP-dependent translocation-motor" model

The above mentioned results and others have lead to another model for the translocation across the mitochondrial inner membrane, the so called "ATP-dependent Translocation-Motor" model (Glick, 1995; Pfanner and Meijer, 1995, Horst et al., 1997). In this model the precursor bound mhsp70 undergoes a conformational change upon ATP hydrolysis, thereby generating an inward force on the precursor chain. As a result the polypeptide is pulled into the matrix space (**Figure 1**). The role of Tim44 in both translocation models would be different: in the "Brownian-Ratchet" model Tim44 positions mhsp70 just close to the import site, whereas in the "Translocation-Motor" model Tim44 would not only position mhsp70 close to the import pore, but would furthermore allow for force generation by serving as a membrane anchor for mhsp70 during its "powerstroke". In both models, Tim44 could also promote

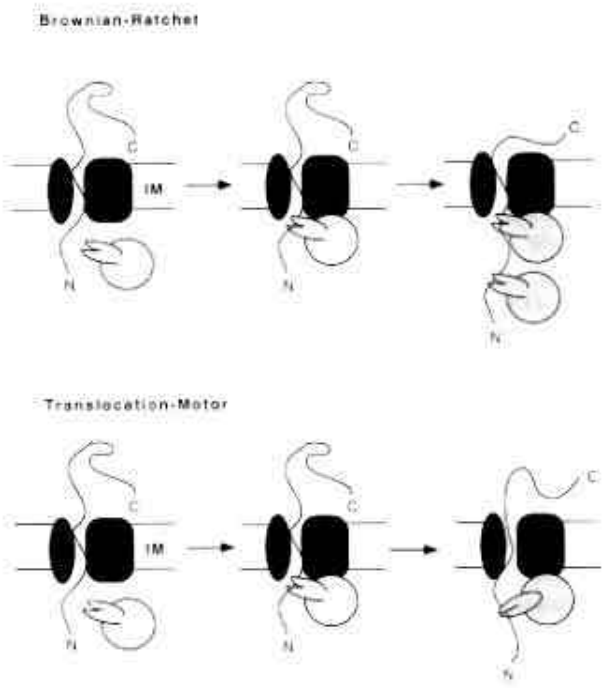

Fig.1 Comparison of the "Brownian-Ratchet" and the "Translocation-Motor" model for the translocation of polypeptides across the mitochondrial inner membrane (adapted from: Glick et al, 1995). Dark grey: translocation channel; light grey: mhsp70; IM: Mitochondrial inner membrane.

the binding of mhsp70 to the precursor chain. In the "ATP-dependent Translocation-Motor", the action of mhsp70 during protein import is analogous to the ATP-dependent translocation of actin filaments by myosin (Spudich, 1994).

To test the prediction that mhsp70 can accelerate unfolding of a precursor protein the following experiments using a precursor protein containing the DHFR-domain fused to the cytochrome b_2 presequence of variable length were performed. Precursor whose presequence is not long enough to span both mitochondrial membranes, and having a folded DHFR-domain, are imported slowly. Precursor proteins with a longer presequence are imported orders of magnitude faster (Matouschek et al., 1997). It seems therefore that if the presequence of these precursor proteins is long enough to span both mitochondrial membranes, mhsp70 can actively unfold the tightly folded heme-binding domain on the mitochondrial surface. Precursor proteins with a short presequence can not be unfolded as the presequence can not interact with mhsp70. Mhsp70 may have an unfolding activity linked to a conformational change which generats a power stroke on the precursor. If the "Translocation-Motor" model should hold true it has to be ruled out that the environment at the entrance of the mitochondrial import channel can unfold precursor proteins. There is some evidence against that: import of DHFR fusion proteins with short presequences is not faster than the spontaneous unfolding of DHFR in solution. Furthermore, in ATP-depleted mitochondria the cytochrome b_2 presequence inserts proper into the outer membrane close to the import site and the heme-binding domain remains stably folded (Glick et al., 1993).

Another approach to test the two different models is to characterize the nucleotide-dependent interactions (ATPase-cycle) of mhsp70 with Tim44 and a precursor protein. According to the "Translocation-Motor" model the generation of a force on the translocating precursor requires that at some point hsp70 must be bound simultaneously to its membrane anchor and to the translocating polypeptide chain. In contrast the "Brownian-Ratchet" model requires that mhsp70 dissociates from the membrane anchor after ATP-hydrolysis so that the translocating polypeptide chain can continue to oscillate in the channel. ATP promotes the dissociation of hsp70 proteins from unfolded polypeptides due to nucleotide dependant conformational changes (Hightower et al., 1994). ATP also disrupts the Tim44-mhsp70 and Sec63p-BiP/Kar2p complexes (Brodsky and Schekmann, 1993; Kronidou et al., 1994; Schneider et al., 1994; Rassow et al., 1994). Recent kinetic studies have suggested that ATP binding rather than ATP hydrolysis leads to the release of hsp70 proteins from peptide substrates (Palleros et al., 1993; Prasad et al., 1994; Schmid et al., 1994; Azem et al., 1997). Therfore the effect of poorly hydrolysable ATP analogs on the interactions between mhsp70 and Tim44 was investigated (Horst et al., 1996). These analogs promote dissociation of mhsp70 from Tim44, whereas mhsp70 remains bound to Tim44 in the presence of ADP. Thus, the current evidence implies that the ATP-bound form of mhsp70 reversibly associates with Tim44 and incoming precursor proteins, whereas ATP hydrolysis

generates an ADP-bound form of mhsp70 that associates tightly with Tim44 and the precursor. A conformational change of the ADP-bound mhsp70 molecule would then exert an inward pulling force on the precursor chain

The presequence of a mitochondrial precursor is first translocated across both membranes into the matrix where it interacts with mhsp70. Experiments with different length precursors have shown that at least 50 residues are needed to span the two mitochondrial membranes (Rassow et al., 1990). Therefore some process other than mhsp70-dependent pulling must initiate the translocation process. It was suggested that the electrochemical potential across the inner membrane is essential for insertion of the mitochondrial presequence across the inner membrane (Schleyer and Neupert, 1985; Cyr et al., 1993). Many mitochondrial precursor proteins like DHFR fusion proteins with presequences as short as 12 amino acids can be imported, even if the DHFR moieties are initially folded (Hurt et al., 1985). In these cases the DHFR-domain is most likely spontaneously unfolded on the mitochondrial surface ("breathing" of the DHFR domain) followed by the translocation of the presequence into the matrix where it comes into contact with mhsp70. The

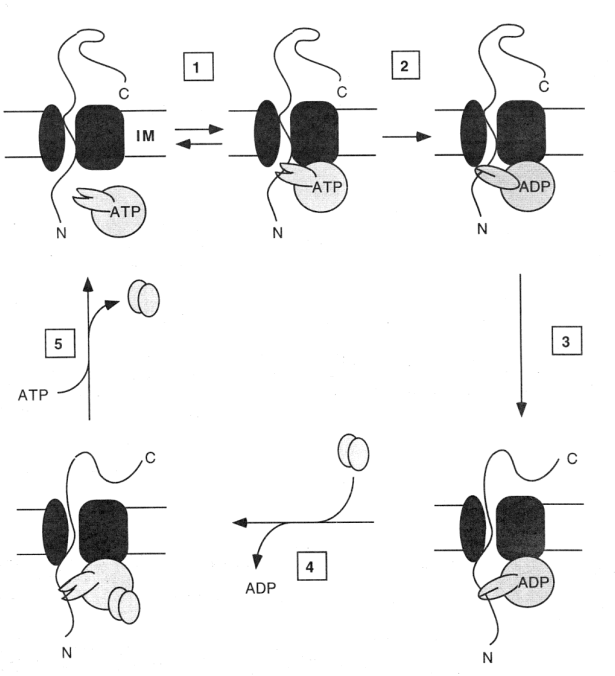

Fig.2 Model of the reaction cycle of the ATP-dependent "Translocation-Motor" (modified from: Horst et al., 1996). For details see paragraph IV. Dark grey: translocation channel; light grey: mhsp70 and mGrpE; IM: Mitochondrial inner membrane.

"Brownian Ratchet" model may therefore account for this initial step in the import process.

Taken together these results have lead us to propose the following schematic representation of the mhsp70 action (Glick, 1995; Pfanner and Meijer, 1995, Horst et al., 1997b; **Figure 2**).

IV. The function of the "translocation-motor"

Based on the observation that ATP is initially needed for the interaction of mhsp70 with the incoming precursor chain (Schneider et al., 1994) in stage 1, mhsp70-ATP associates transiently with Tim44 and the precursor. In the next stage mhsp70 hydrolyzes ATP and mhsp70-ADP associates stably with the precursor and Tim44. This is consistent with the observation that in the absence of ATP, mhsp70 copurifies with Tim44 (Kronidou et al., 1994; Schneider et al., 1994; Rassow et al., 1994) and that mhsp70, the precursor and Tim44 form a stable complex under these conditions (Horst et al., 1996). In stage 3, mhsp70 undergoes a conformational change thereby pulling a stretch of the precursor inwards. Indeed, it was recently shown that upon ATP-binding mhsp70 undergoes a conformational change (von Ahsen et al., 1996). In stage 4, an ADP-ATP exchange reaction, which is facilitated by mGrpE (Azem et al., 1997) takes place. Following the conformational change and the ATP-ADP exchange, mhsp70-ATP dissociates from Tim44 and the precursor (stage 5). This cycle (**Figure 2**) is repeated until the precursor protein is completely imported into the matrix. According to this model mhsp70 together with its associated proteins function remarkably similar to the proposed ATP-dependent mechanism of other hsp70 chaperones (Greene et al., 1995; McCarty et al., 1995) and to the conventional force generating systems such as the interaction found between myosin and actin cables (Spudich et al., 1994).

V. Outlook

Future experiments on the structure of this translocation complex and the interaction of its subunits at the molecular level should allow us to distinguish between the two translocation models. Most interestingly, in the intermembrane space of chloroplasts there is an hsp70 like protein anchored to the inner face of the outer membrane (Marshall et al., 1990; Schnell et al., 1994). This protein may also function by pulling precursor proteins across the chloroplast outer membrane. The "Translocation-Motor" may therefore be an universal mechanism for translocating proteins across organellar membranes.

It could also be possible to use techniques originally invented for studies in the motor protein field (Walker and Sheetz, 1993): mhsp70 could be coupled to a plane solid support so that all molecules are aligned. If mhsp70

functions as a molecular motor a precursor protein may move along the mhsp70 molecules.

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