The protein import motor of mitochondria: a targeted molecular ratchet driving unfolding and translocation

Koji Okamoto, Achim Brinker¹,
Stefan A. Paschen, Ismail Moarefi¹,
Manajit Hayer-Hartl¹, Walter Neupert² and
Michael Brunner³

Institut für Physiologische Chemie der Universität München,
Butenandtstraße 5, D-81377 München, ¹Max-Planck-Institute for
Biochemistry, Am Klopferspitz 18A, D-82152 Martinsried and
³Biochemie-Zentrum Heidelberg, Im Neuenheimer Feld 328,
D-69120 Heidelberg, Germany

²Corresponding author

e-mail: Neupert@bio.med.uni-muenchen.de

Unfolding and import of preproteins into mitochondria are facilitated by a molecular motor in which heat shock protein 70 (Hsp70) in the matrix plays an essential role. Here we present two different experimental approaches to analyze mechanisms underlying this function of Hsp70. First, preproteins containing stretches of glutamic acid (polyE) or glycine (polyG) repeats in front of folded domains were imported into mitochondria. This occurred although Hsp70 cannot pull on these stretches to unfold the folded domains, since it does not bind to polyE and polyG. Secondly, preproteins containing titin immunoglobulin (Ig)-like domains were imported into mitochondria, despite the fact that forces of >200 pN are required to mechanically unfold these domains. Since molecular motors generate forces of ~5 pN, Hsp70 could not promote unfolding of the Ig-like domains by mechanical pulling. Our observations suggest that Hsp70 acts as an element of a Brownian ratchet, which mediates unfolding and translocation of preproteins across the mitochondrial membranes.

Keywords: Brownian ratchet/Hsp70/mitochondria/protein import/unfolding

Introduction

Energy-coupled translocation motors play an essential role in unidirectional movement of preproteins across membranes (Matlack *et al.*, 1998). Molecular chaperones of the heat shock protein 70 (Hsp70) family act as core components of the translocation motors in the lumen of the endoplasmic reticulum (ER) and in the matrix of mitochondria (Rapoport *et al.*, 1999; Strub *et al.*, 2000). How Hsp70s drive preprotein unfolding and translocation across the membranes of these organelles is a fundamental problem in cell biology (Simon and Blobel, 1993; Wickner, 1994; Glick, 1995; Pfanner and Meijer, 1995; Jensen and Johnson, 1999; Bauer *et al.*, 2000).

The ATP-dependent mitochondrial import motor has been investigated in some detail (Bauer *et al.*, 2000; Matouschek *et al.*, 2000; Pfanner and Geissler, 2001). The

central part of the motor is mitochondrial Hsp70 (mtHsp70) in the matrix (Ssc1p in yeast; Craig *et al.*, 1989). The import motor relies on repeated cycles of interaction of mtHsp70 with the extended polypeptide chain of a translocating preprotein, in a reaction supported by two cooperating proteins, Tim44 and Mge1p (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994, 1996; Ungermann *et al.*, 1996). A dimer of Tim44 is associated with the inner membrane translocase and recruits two molecules of ATP-bound mtHsp70 from the soluble pool in the matrix to the translocation channel (Moro *et al.*, 1999). This specifies the molecular chaperones for preprotein import. Recruitment by Tim44 mediates binding of mtHsp70 to segments of a translocating preprotein as soon as they emerge from the import channel (Rassow *et al.*, 1994; Schneider *et al.*, 1994, 1996; Ungermann *et al.*, 1996). Binding to the polypeptide chain is believed to trigger ATP hydrolysis by mtHsp70 and thereby promote stable association of mtHsp70 with the incoming preprotein (Schneider *et al.*, 1994, 1996). Subsequently, the mtHsp70–preprotein complex dissociates from Tim44 and moves further into the matrix space. A second molecule of mtHsp70, which is associated with the Tim44 dimer, then binds to the next segment of the preprotein that newly emerges from the import channel (Moro *et al.*, 1999). Mge1p, a nucleotide exchange factor in the matrix, appears to facilitate dissociation of mtHsp70 from Tim44 and eventually catalyzes release of ADP from mtHsp70 (Schneider *et al.*, 1996). This allows mtHsp70 to bind ATP, which leads to dissociation of the polypeptide chain from the chaperone.

It is now widely believed that movement of unfolded polypeptide chains in the import channel is driven by thermal fluctuations and that the import motor can function as a ratchet that prevents retrograde movements by trapping incoming segments of the precursor directly at the outlet of the channel (Schneider *et al.*, 1994, 1996; Matlack *et al.*, 1998; Voisine *et al.*, 1999). However, there is no agreement on the mode of how mtHsp70 in the matrix drives unfolding of folded preproteins on the surface of mitochondria. Two fundamentally different models have been proposed: the ‘power stroke’ (‘pulling’) model and the ‘Brownian ratchet’ (‘trapping’) model. The ‘power stroke’ model proposes a conformational change of mtHsp70 perpendicular to the plane of the inner membrane, generating a pulling force on the extended polypeptide chain, which actively drives unfolding of a folded domain on the surface of mitochondria (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Voos *et al.*, 1996; Matouschek *et al.*, 1997; Chauwin *et al.*, 1998; Huang *et al.*, 1999; Voisine *et al.*, 1999; Geissler *et al.*, 2001; Lim *et al.*, 2001). In the ‘Brownian ratchet’ model, import of folded domains is explained by their ability to undergo spontaneous reversible local unfolding due to thermal

Fig. 1. Modeling of the DnaK peptide binding domain with substrate peptides. (A) The backbone of the peptide binding domain with the peptide NR (indicated in yellow) (Zhu *et al.*, 1996). The view faces the C-terminal end of the peptide. (B) Surface electrostatic potential of the peptide binding domain with NR replaced by E7. Surface color: red, negatively charged; blue, positively charged; and gray, neutral. The peptide is shown in a stick representation. (C and D) Close-up of the peptide binding clefts with the peptide E7 (C) and the peptide G7 (D). The negatively charged residue E402 of DnaK is located close to the cleft.

breathing. The thermal fluctuations allow steps of translocation of unfolded segments through the import channel. Interaction of mtHsp70 with these segments in the matrix ('trapping') would make these movements vectorial. Thus, when an N-terminal segment is unfolded, refolding and translocation of the unfolded segment would be competing reactions (Schneider *et al.*, 1994, 1996; Ungermann *et al.*, 1996; Gaume *et al.*, 1998; Moro *et al.*, 1999; Milisav *et al.*, 2001). The equilibrium is then shifted towards unfolding and translocation by the action of mtHsp70. Regardless of the mechanism of unfolding and forward movement, both models require a second molecule of mtHsp70 to trap the newly translocated segment of the incoming polypeptide chain, and thereby prevent backsliding and refolding of the preprotein on the surface of mitochondria.

To address how mtHsp70 drives unfolding of folded preproteins, we constructed preproteins containing glycine and glutamic acid repeats (polyG and polyE). Our results show that mtHsp70 does not bind to polyE and may bind to polyG with very low affinity. However, preproteins containing segments of polyG or polyE followed by folded protein domains were efficiently imported into mitochondria, despite the fact that pulling of mtHsp70 on polyG or polyE in order to unfold the folded passenger domain cannot occur. However, the forces required to unfold a folded preprotein on the surface of mitochondria are not known. We constructed preproteins containing immunoglobulin (Ig)-like domains of the human muscle protein titin. These preproteins were imported into mitochondria although forces of 200–260 pN have been shown to be required for mechanical unfolding of the

Ig-like domains by atomic force microscopy (AFM) (Carrion-Vazquez *et al.*, 1999; Marszalek *et al.*, 1999; Li *et al.*, 2000). Since bona fide molecular motors such as kinesin and myosin generate maximal forces of ~5 pN (Spudich, 1994), it is unlikely that mtHsp70 promotes unfolding of the Ig-like domains by mechanical pulling. Rather, the kinetics suggest that import relies on spontaneous unfolding of the Ig-like domains. Together, our observations strongly support the notion that unfolding and translocation of mitochondrial preproteins are mediated by a molecular ratchet mechanism.

Results

Unfavorable substrates of mtHsp70

We searched for protein substrates to which mtHsp70 may not bind. Glutamic acid and glycine residues rarely occur in binding sites for *Escherichia coli* DnaK, which is homologous to mtHsp70 (Gragerov *et al.*, 1994; Rüdiger *et al.*, 1997). To estimate whether DnaK would interact with repeats of glutamic acid and glycine residues, we used the three-dimensional image of the peptide binding domain of DnaK complexed with the peptide NR (NRLLLTG) (Zhu *et al.*, 1996; Figure 1A). We substituted the side chains of NR for those of seven glutamic acids (E7) or glycines (G7). The side chains of E7 do not fit into the hydrophobic peptide binding cavity (Figure 1B and C). In addition, the negatively charged side chain of the C-terminal residue is in close proximity to that of E402 of DnaK. Thus, DnaK and its homolog mtHsp70 would appear to be unable to bind a polyE peptide without

Fig. 2. mtHsp70 does not bind to glycine or glutamic acid repeats *in vitro*. (A) Binding of mtHsp70 to peptides C-NR, C-G7 or C-E7 on a CM5 biosensor chip analyzed by SPR. The association (Ass.) phase contained mtHsp70 and ADP in buffer D and the dissociation (Diss.) phase was in buffer D. (B) Binding of mtHsp70 to C-NR in the presence of competitor peptides. mtHsp70 was pre-incubated without peptide (–competitor) or with 500 μM NR, G7 or E7 in buffer D containing 5 mM ADP at 25°C for 10 min. The binding followed that described in (A). (C) Binding of mtHsp70 to C-NR in the presence of different concentrations of competitor peptides. SPR measurements were carried out as described in (B) without peptide or with NR, G7 or E7 as competitors. Response units at the end of the association phase from a reaction without competitor were set to 100%.

disrupting the structure of the peptide binding domain. The polyG peptide is also unlikely to interact with the surface of the DnaK peptide binding cavity because of the lack of side chains (Figure 1D).

mtHsp70 does not bind to polyG and polyE *in vitro*

To test whether mtHsp70 binds to polyG and polyE, we employed a surface plasmon resonance (SPR) assay. DnaK binds with high affinity to the NR peptide (Gragerov *et al.*, 1994). The peptide was immobilized via an N-terminal cysteine (C-NR) on a CM5 biosensor chip. mtHsp70 purified from *Neurospora crassa* bound to the C-NR peptide in the presence of ADP (Figure 2A). However, when the peptides CGGGGGGG or CEEEEEEE were immobilized, no binding of mtHsp70 was observed (Figure 2A). We examined binding of mtHsp70 to polyG

and polyE using competition assays (Figure 2B and C). Pre-incubation of mtHsp70 with a 2-fold molar excess of the NR peptide resulted in inhibition of binding of mtHsp70 to the immobilized C-NR peptide, indicating high affinity of the NR peptide to mtHsp70 (Figure 2C). In contrast, pre-incubation of mtHsp70 with either E7 or G7 peptides in 60- or 100-fold molar excess had no significant effect on subsequent binding of mtHsp70 to the C-NR peptide (Figure 2B and C). These data suggest that polyG and polyE are not substrates for mtHsp70.

It has been reported that the J-domain of Sec63p stimulates ATP hydrolysis of BiP, resulting in rapid trapping of peptides that would not bind to BiP in the absence of the co-chaperone (Misselwitz *et al.*, 1998). However, it is unlikely that polyE binding of mtHsp70 would be stimulated by the action of Tim44, since this substrate does not fit into the binding pocket of Hsp70 and since Tim44 does not belong to the DnaJ family (see Figures 1C and 4). Furthermore, preliminary experiments show that polyE, when pre-incubated with *E.coli* DnaJ and DnaK, did not compete for binding of DnaK to immobilized C-NR peptide in the SPR assay (A.Brinker and K.Okamoto, unpublished observations). In agreement, recent reports show that the affinity of DnaK for substrates determines the efficiency of DnaJ-modulated ATP hydrolysis (Mayer *et al.*, 2000).

Preproteins containing polyG and polyE are imported into mitochondria

We asked whether mtHsp70 would unfold a folded protein domain on the surface of mitochondria when the domain is preceded by segments of polyG or polyE. To generate appropriate preproteins, the N-terminal 75 residues of the precursor of *N.crassa* ATPase subunit 9 (Su9) were fused to stretches of 50 glycine or glutamic acid residues. Mouse dihydrofolate reductase (DHFR) was fused to the C-terminus of these stretches via a linker of 10 amino acid residues, yielding the precursors pG50-D and pE50-D, respectively (Figure 3A). Upon import of pG50-D and pE50-D, stretches of polyG and polyE will emerge from the import channel when the folded DHFR domain is about to be unfolded on the mitochondrial surface (Ungermann *et al.*, 1996; Kanamori *et al.*, 1997; Gaume *et al.*, 1998). For a control, we constructed pX50-D, which contains 60 residues derived from cytochrome *b*₂ between the presequence and DHFR (Figure 3A).

Radiolabeled pX50-D, pG50-D and pE50-D were synthesized *in vitro*, incubated with isolated mitochondria, and import was monitored by following the two consecutive processing steps of the presequence by the mitochondrial processing peptidase (MPP). The first processing step after residue 35 converts the precursor (p) form to the intermediate (i) form, and the second processing after residue 66 generates the mature (m) form. pX50-D, pG50-D and pE50-D were processed to the m-forms, which were protected from degradation by externally added proteinase K (PK) (Figure 3B). After conversion of mitochondria into mitoplasts, the m-forms were still protected from degradation, demonstrating their import into the matrix. Maturation and protease resistance of the preproteins were dependent on both the inner membrane potential ($\Delta\psi$) and ATP in the matrix (data not shown).

Fig. 3. Preproteins containing polyG or polyE are imported into mitochondria. (A) The N-terminal 75 amino acids of the precursor of *N.crassa* ATPase Su9 were fused via short linkers of two or three amino acids to stretches of 50 glycine (G50) or glutamic acid residues (E50). Mouse DHFR was fused via a linker of 10 amino acids C-terminal of these stretches. pX50-D contains 60 amino acids derived from yeast cytochrome *b*₂ between the Su9 presequence and the DHFR domain. This corresponds formally to 50 random amino acids (X50) followed by a linker of 10 amino acids. Arrowheads indicate cleavage sites for MPP after amino acids 35 and 66. pE50-H contains the HBD of cytochrome *b*₂. (B) pG50-D, pE50-D and pE50-H are imported into the mitochondrial matrix. ³⁵S-labeled precursors were incubated with mitochondria for 10 min at 25°C. When indicated, the samples were treated with PK or mitoplasts were generated by swelling (SW) and treated with PK. p-, i- and m-forms are indicated. Asterisks indicate internal translation products initiated at Met15, Met50 and Met75 of the Su9 presequence. (C) Kinetics and efficiency of import. ³⁵S-labeled precursors were incubated with isolated mitochondria at 25°C. Import reactions were stopped after the indicated time periods and mitochondria were subjected to swelling and PK treatment. The samples were quantified using a phosphoimaging system. The amount of added preprotein was set to 100%. (D) Schematic outline of import of pE50-D. The folded DHFR domain and the polyE stretch are indicated by a shaded oval and a bold line, respectively. mtHsp70 binds in front and after the polyE segment. OM, outer membrane; IM, inner membrane; 70, mtHsp70.

These results demonstrate that pX50-D, pG50-D and pE50-D are imported into the matrix of mitochondria. A significant fraction of E50-D accumulated as the i-form (Figure 3B). The electrophoretic mobility of the i-form of E50-D was similar to that of the p-form. The identity of the i-form was confirmed by *in vitro* processing of pE50-D with purified MPP (data not shown). The i-form was protected from degradation by PK in mitochondria but not in mitoplasts (Figure 3B). It was detected after short and long incubation periods (data not shown), suggesting that it was not a productive translocation intermediate. A fraction of the i-form of E50-D was apparently sliding back and accumulated as an off-pathway intermediate in

the intermembrane space, supporting the notion that mtHsp70 does not bind to the polyE segment.

To test whether tightly folded preproteins containing polyE can be imported into mitochondria, the DHFR domain of pE50-D was replaced with the heme binding domain (HBD) of cytochrome *b*₂, yielding the precursor pE50-H (Figure 3A). In the presence of heme, HBD is a tightly folded moiety (Gaume *et al.*, 1998). When *in vitro* synthesized pE50-H was treated with 1 mg/ml PK, the HBD moiety was protease resistant, indicating that it was tightly folded (data not shown). Upon import into isolated mitochondria, pE50-H was processed to the mature form, which was resistant to PK treatment in mitochondria and

Fig. 4. MTX-arrested pG50-D and pE50-D are inefficiently matured by MPP. (A) ^{35}S -labeled precursors were incubated with mitochondria in the presence or absence of MTX/NADPH (25°C, 20 min). When indicated, mitoplasts were generated (SW) and treated with PK. p-, i- and m-forms are indicated. Asterisks indicate internal translation initiation at Met15. (B) MTX-arrested membrane-spanning translocation intermediates of X50-D and E50-D. The polyE stretch is indicated by a bold line.

in mitoplasts (Figure 3B, lower panel). This indicates that the HBD becomes unfolded on the surface of mitochondria and is fully imported into the matrix of mitochondria under conditions when the polyE segment emerges from the inner membrane import channel.

Next, we measured the import kinetics and efficiency of pX50-D, pG50-D, pE50-D and pE50-H at 25°C (Figure 3C). Import was half-maximal between 1 and 3 min. The efficiencies of import of the precursors varied between 15 (pE50-D) and 40% (pG50-D), which is comparable to the import efficiency of many other preproteins synthesized *in vitro*.

In summary, pG50-D, pE50-D and pE50-H can be imported into mitochondria, suggesting that binding sites for mtHsp70 can be 50 residues apart (Figure 3D). During the translocation process, polyG or polyE emerges from the inner membrane import channel in the matrix when the folded DHFR domain or the HBD are about to be unfolded on the surface of mitochondria. Since mtHsp70 does not bind to polyG and polyE, it appears that unfolding of the domains occurs without 'pulling' by mtHsp70.

MTX-arrested pG50-D and pE50-D are not completely matured by MPP

Although mtHsp70 does not bind to polyG and polyE *in vitro*, it may interact with those repeats in the

mitochondrial matrix. To test this possibility, import reactions were carried out in the presence of the folate antagonist methotrexate (MTX) to stabilize the DHFR domain and to arrest the preproteins in a membrane-spanning fashion. Binding of mtHsp70 to a membrane-spanning precursor prevents retrograde movement of the polypeptide chain (Ungermann *et al.*, 1996). Segments of the polypeptide N-terminal to the mtHsp70 binding site are then exposed in the matrix space. If the exposed segments contain a processing site, it is cleaved by MPP. Processing of the arrested precursor, therefore, indicates binding of mtHsp70 to the segment C-terminal to the processing site.

pX50-D, pG50-D and pE50-D were incubated with energized mitochondria in the absence of MTX to control the import reaction. As expected, these preproteins were imported into the matrix and processed to the m-forms (Figure 4A). In the presence of MTX, pX50-D was also processed to the m-form. However, when mitoplasts were generated and ATP levels in the matrix were lowered, the majority of the m-form was degraded by added PK. This indicates that X50-D was arrested in a membrane-spanning fashion (Figure 4A). The polypeptide segment preceding the folded DHFR was sufficiently long to span both mitochondrial membranes, and mtHsp70 was binding to a segment of the precursor C-terminal of the processing site after amino acid residue 66, which allowed processing of the preprotein to the m-form (Figure 4B, left). A small fraction of the precursor escaped the MTX block and was fully imported into the matrix, resulting in protease resistance of the m-form of X50D.

MTX-arrested G50-D accumulated predominantly as the i-form, which was arrested in a membrane-spanning fashion (Figure 4A). A fraction of the preprotein (m-form) escaped the MTX-block; apparently it was not properly folded. The data suggest that mtHsp70 was not binding to the polyG segment C-terminal of the presequence.

When import of pE50-D was arrested by MTX, it accumulated exclusively as the i-form (Figure 4A). The i-form of E50-D was arrested in a membrane-spanning fashion, as indicated by its sensitivity to PK treatment of mitoplasts (Figure 4A). This demonstrates that mtHsp70 did bind to segments C-terminal of residue 35 of the presequence, but not to segments C-terminal of residue 66 (Figure 4B).

Together, these results show that, *in organello*, mtHsp70 does not bind to segments of polyG and polyE that emerge from the import channel.

Preproteins containing titin Ig-like domains are unfolded and translocated into mitochondria

It is not known whether mtHsp70 can generate a mechanical force to drive unfolding of a folded protein domain on the surface of mitochondria and how high the force would have to be. Human muscle protein titin contains ~300 copies of tightly folded Ig-like and fibronectin-like domains. Forces of 200–260 pN are required to unfold the Ig-like domains 27 (I27) and 28 (I28), as shown by AFM (Carrion-Vazquez *et al.*, 1999; Marszalek *et al.*, 1999; Li *et al.*, 2000).

To investigate whether these tightly folded domains could be unfolded and imported by the mitochondrial import machinery, we constructed the chimeric preproteins pI27I28 and pI27I28-C containing the presequence

Fig. 5. Titin Ig-like domains are imported into mitochondria. (A) Outlines of pI27I28, pI27I28-C and pI27 consisting of the N-terminal 94 amino acids of the Su9 presequence fused to titin Ig-like domain I27 or to I27 plus I28. pI27I28-C contains a 23 amino acid extension (C) at the C-terminus. Arrowheads indicate cleavage sites for MPP. (B) pI27I28 and pI27I28-C are imported into the mitochondrial matrix. ^{35}S -labeled precursors were incubated with isolated mitochondria. When indicated, samples were treated with PK or mitoplasts were generated (SW) and treated with PK. p-, i- and m-forms are indicated. i* denotes an intermediate from which the C-terminal extension was clipped off by PK. Asterisks indicate internal translation products. (C) Outline of the topology of I27I28-C i*- and m-forms. The folded I27I28 domain and the C-terminal extension are indicated by dotted ovals and a black box, respectively. (D) pI27 is imported into the mitochondrial matrix. Import was carried out and the samples were analyzed as described in (B), except for the incubation time. (E) Import kinetics of pI27, pI27I28 and pI27I28-C. Precursors were incubated with isolated mitochondria. Import reactions were stopped after 0, 1, 3, 6, 10, 20 and 30 min and subjected to PK treatment. The samples were quantified with a phosphorimager. The amount of protein added was set to 100%.

and N-terminal portion of *N.crassa* ATPase Su9 (residues 1–94) fused to I27I28 (Figure 5A). pI27I28-C carries, in addition, a short C-terminal extension of 23 amino acid residues to allow localization of the Ig-like domains on or within the mitochondria. We also constructed the precursor pI27, which contains the Su9 presequence and the I27 domain (Figure 5A). The Ig-like domains of these preproteins, synthesized *in vitro*, were resistant to treat-

ment with 1 mg/ml PK at 37°C, indicating that they were tightly folded (data not shown).

pI27I28 and pI27I28-C were incubated with energized mitochondria. After 1 min, the chimeric precursors were almost completely processed to the i- and m-forms, indicating that the Su9 presequence was efficiently and rapidly targeted to the mitochondria and that the N-terminus had reached the matrix (Figure 5B, upper

panel, lanes 2 and 5). The distances between the folded I27 domain and the first and second MPP processing sites comprise 61 and 30 amino acid residues, respectively. Thus, accumulation of the i-forms of I27I28 and I27I28-C indicates that the I27 domains were still folded on the surface of mitochondria. When mitochondria were treated with PK, the i-form of I27I28 was resistant to protease treatment, the C-terminal extension of I27I28-C was clipped from the i-form and the protease-resistant i*-form was generated (Figure 5B, upper panel, lanes 3 and 6). When mitoplasts were generated to allow access of PK to the intermembrane space, both the i-forms of I27I28 and I27I28-C were degraded, demonstrating that these intermediates had accumulated in a membrane-spanning fashion (Figure 5B, upper panel, lanes 4 and 7). Together, these results demonstrate that translocation of the precursors pauses when the I27 domain is about to be translocated. Thus, after a 1 min incubation, the majority of the preprotein was processed to the i-form, and I27I28 domains were tightly folded and exposed on the surface of mitochondria in such a manner that the C-terminal extension was accessible to the added PK (Figure 5C). In contrast, the C-terminus of the m-form of I27I28-C was not accessible to PK; it was completely translocated across the mitochondrial membranes (Figure 5B and C). After a 20 min incubation, most of I27I28 and I27I28-C were converted to the m-forms, which were resistant to PK treatment of mitochondria and mitoplasts (Figure 5B, lower panel). This indicates that the preproteins were completely imported into the matrix of the mitochondria.

pI27 was also efficiently imported into isolated mitochondria (Figure 5D). The preprotein was almost completely processed to the m-form after a 0.5 min incubation. Only a small fraction accumulated as the i-form. The i- and m-forms were resistant to PK treatment of mitochondria, but were accessible when mitoplasts were generated. They were obviously kinetic intermediates not yet fully translocated across the inner membrane. After 10 min of import, pI27 was completely converted to the m-form, which was completely imported into the matrix.

To measure the kinetics and efficiency of import of pI27I28, pI27I28-C and pI27, we determined the amounts of m-forms that were resistant to PK treatment as a function of the time of import (Figure 5E). Import of pI27I28 and pI27I28-C was half-maximal after ~5 min and approached a plateau after 30 min. The import kinetics of pI27 was faster than that of pI27I28 and pI27I28-C. Import was half-maximal after 2 min and reached a plateau after 10 min. The import efficiency of pI27I28, pI27I28-C and pI27 was ~38, 56 and 95%, respectively.

For further analysis, we constructed the chimeric precursor pSu9(1-69)I27-DHFR (Figure 6A). *In vitro* synthesized radiolabeled pSu9(1-69)I27-DHFR and, as controls, pSu9(1-69)DHFR and pSu9(1-94)DHFR were incubated with energized mitochondria in the absence or presence of MTX (Figure 6B). In the absence of MTX, the three precursors were efficiently processed to the m-forms, which were completely imported into the matrix. In the presence of MTX, pSu9(1-69)DHFR accumulated in the p-form and pSu9(1-94)DHFR accumulated in the i-form (Figure 6B). As expected, these forms accumulated in a membrane-spanning fashion. pSu9(1-69)I27-DHFR was efficiently processed to the m-form when unfolding of

Fig. 6. Unfolding of titin I27 domain by the import machinery. (A) pSu9(1-69)I27-DHFR contains a 21 amino acid linker between I27 and DHFR. (B) 35 S-labeled precursors were incubated with isolated mitochondria in the presence or absence of MTX/NADPH. When indicated, samples were subjected to PK treatment under swelling conditions. (C) Outline of MTX-arrested translocation intermediates of pSu9(1-69)DHFR, pSu9(1-94)DHFR and pSu9(1-69)I27-DHFR. The stretch of the unfolded I27 domain is indicated by a dotted box. (D) Schematic outline of pE50-I27I28 and import into isolated mitochondria.

DHFR was blocked by MTX (Figure 6B, lower panel). The m-form was present as a membrane-spanning fashion intermediate.

Thus, as outlined in Figure 6C, the presequence of pSu9(1–69)DHFR is too short to be processed when import is arrested with MTX, while the first MPP processing site of pSu9(1–94)DHFR is exposed into the matrix. In pSu9(1–69)I27–DHFR, the second MPP processing site is located five residues in front of the titin I27 domain. Since an unfolded segment of ~50 residues is required to span both membranes and to allow processing by MPP in the matrix, I27 must be present in an unfolded conformation to allow maturation of MTX-arrested pSu9(1–69)I27–DHFR.

We then constructed pE50–I27I28, a chimeric precursor in which the titin domains are preceded by 50 glutamic acid residues and the Su9 presequence (Figure 6D). When radiolabeled pE50–I27I28 was incubated with energized mitochondria, it was imported into the matrix and processed to the m-form (Figure 6D). A significant fraction was processed to the i-form and accumulated as a dead-end intermediate in the intermembrane space.

Together, the data show that preproteins containing tightly folded titin Ig-like domains are unfolded and translocated into the mitochondrial matrix, even when preceded by segments of polyE to which Hsp70 cannot bind. In contrast, the import machinery cannot unfold the MTX-stabilized DHFR domain.

Cycling of mtHsp70 at the import site

ATP consumption by mtHsp70 at the import site is not strictly coupled to forward movement of the translocating polypeptide chain (Gaume *et al.*, 1998). Thus, when import of DHFR fusion proteins is arrested with MTX, mtHsp70 cycles in an ATP-dependent manner on and off the segment of the arrested translocation intermediate that emerges from the import channel. To characterize translocation intermediates in more detail, pSu9(1–69)DHFR was incubated with mitoplasts in the absence and presence of MTX (Figure 7A). In the absence of MTX, pSu9(1–69)DHFR was imported into the matrix and processed to the m-form. In the presence of MTX, the i-form of Su9(1–69)DHFR was generated, indicating that the presequence was spanning the inner membrane and the first MPP processing site was exposed into the matrix. The i-form was not generated when pretreated (37°C) mitochondria from the *ssc1-3* strain were used (Figure 7A), which harbors a temperature-sensitive mutant form of mtHsp70 (Craig *et al.*, 1989). Thus, the generation of the i-form was dependent on mtHsp70. To assess the kinetics of cycling of mtHsp70, pSu9(1–69)DHFR was first incubated for 20 min with mitoplasts in the presence of MTX to accumulate the membrane-spanning i-form (Figure 7B). Then, valinomycin and oligomycin were added to one half of the sample to dissipate the inner membrane potential $\Delta\psi$ and promote hydrolysis of matrix ATP by the F_1 -ATPase. The other half was left untreated. The resulting decrease in the ATP level (Gaume *et al.*, 1998) should affect the kinetics of cycling of mtHsp70. The mitoplasts were then re-isolated by centrifugation, and the pellet and supernatant fractions were analyzed (Figure 7B). Re-isolation was completed within <45 s. In the absence of valinomycin/oligomycin, the i-form of Su9(1–69)DHFR was recovered with the mitoplast pellet, indicating that it was held in a membrane-spanning fashion by mtHsp70, as outlined in Figure 7C (left panel). In

Fig. 7. ATP-dependent cycling of mtHsp70 at the import site. (A) pSu9(1–69)DHFR was incubated with mitoplasts from wild-type (WT) and *ssc1-3* in the presence or absence of MTX/NADPH. When indicated, PK was added. (B) pSu9(1–69)DHFR was incubated with mitoplasts (WT) in the presence of MTX/NADPH. Samples were kept on ice for 3 min in 10 vols of HS buffer with or without 20 μ M oligomycin/1 μ M valinomycin and mitoplasts were pelleted within <45 s. The supernatant (sup) was subjected to $(\text{NH}_4)_2\text{SO}_4$ precipitation. (C) Backsliding of MTX-arrested iSu9(1–69)DHFR upon lowering the levels of ATP in the matrix.

contrast, in the presence of valinomycin/oligomycin, the i-form was released from the mitoplasts and recovered in the supernatant fraction. This indicates that mtHsp70 rapidly binds and releases the short segment of the translocation intermediate that emerges from the import channel. Apparently, when the matrix ATP level is lowered, mtHsp70 cannot efficiently rebound to the translocation intermediate. As a consequence, the i-form slides back in the import channel and is released from the mitoplasts (Figure 7C, right panel). Dissociation of mtHsp70 from the translocating polypeptide chain appears to be rapid, since the vast majority of the arrested i-form was released from the mitoplasts within 45 s.

Discussion

Hsp70 in the mitochondrial matrix is part of a preprotein import motor that is tethered to the outlet of the import channel. A number of studies over the past few years have indicated that this motor drives translocation of unfolded polypeptide chains by a targeted molecular ratchet mechanism: unfolded segments of a translocating polypeptide chain oscillate in the channel due to thermal fluctuations, and mtHsp70 traps incoming segments at the outlet of the translocation channel and thereby prevents retrograde movements (Schneider *et al.*, 1994, 1996; Moro *et al.*, 1999; Bauer *et al.*, 2000; Matouschek *et al.*, 2000). On the other hand, the mechanism by which mtHsp70 facilitates unfolding and import of tightly folded preproteins is still controversial. A representation of the two models that have been proposed, the 'power stroke' (or 'pulling') model and the 'Brownian ratchet' (or 'trapping') model, is given in Figure 8. These models are identical in some steps, but differ radically in the predicted mode of unfolding of folded domains and forward movement of the translocating preprotein (Figure 8, steps IV and V).

Initiation of translocation occurs by the $\Delta\psi$ -driven translocation of the N-terminal targeting signal across the TIM23 complex in the inner membrane (step I). Tim44 at the exit of the TIM channel recruits mtHsp70 to the import site. mtHsp70 binds to the incoming polypeptide chain as it emerges from the import channel (step II). Binding of mtHsp70 to the polypeptide chain facilitates ATP hydrolysis. ATP hydrolysis by mtHsp70 causes a conformational change that closes the substrate binding pocket and leads to a stable association with the unfolded polypeptide substrate (step III). Both models differ in the subsequent steps. The 'power stroke' model now proposes that mtHsp70, tightly associated with the preprotein, uses Tim44 as a fulcrum and undergoes a second major conformational change perpendicular to the plane of the inner membrane (step IVa). This would generate a pulling force on the extended polypeptide chain of the translocating preprotein that actively drives unfolding of a folded domain on the surface of mitochondria. Assuming that mtHsp70 may bind to a polypeptide approximately every 10 residues, a single power stroke must displace the substrate binding domain by at least 3.5 nm perpendicular to the membrane to allow another molecule of mtHsp70 to bind the translocating chain (step Va). Thus, the 'power stroke' model requires two consecutive major conformational changes in Hsp70 driven by hydrolysis of one molecule of ATP: the first one to close the substrate binding site in order to tightly bind to the incoming preprotein, and the second one to pull in order to mechanically unfold the folded domain on the surface of the mitochondria. A conformational change in Hsp70s subsequent to substrate binding has not been described.

The 'Brownian ratchet' model requires only one major conformational change in the chaperone leading to tight substrate binding. mtHsp70, stably bound to the incoming preprotein, would merely prevent retrograde movement of the polypeptide chain in the channel. The model assumes that unfolded segments of a preprotein slide in the TIM channel. In the case that a folded passenger domain is about to be translocated, it unfolds spontaneously (step IVb). At physiological temperatures, folded domains

unfold and refold locally on the time scale of milliseconds (thermal breathing) (Zhuang *et al.*, 2000). The same forces, i.e. random thermal fluctuations that drive refolding of the partially unfolded protein, would also drive Brownian oscillation of the unfolded segment in and out of the import channel (step Vb). Regardless of the mechanism of forward movement of the polypeptide chain in the import channel, both models require that a second mtHsp70 molecule, associated with the Tim44 oligomer, traps the newly translocated segment of the incoming polypeptide chain, thereby preventing backsliding and refolding of the preprotein on the surface of mitochondria (step VI).

One approach taken here to experimentally discriminate between both modes of import was to study import of polypeptide substrates that cannot be bound by mtHsp70. On such substrates, mtHsp70 would not be able to exert a 'pulling' force. We asked whether a folded domain could be unfolded when only segments of a precursor are available at the import site that cannot be bound by mtHsp70. We found that preproteins containing stretches of 50 glutamic acid or glycine residues in front of a folded domain were rapidly and efficiently imported into mitochondria. Even very tightly folded proteins, such as HBD and titin Ig-like domains, were imported with a stretch of polyE in front. As mtHsp70 cannot pull on polyE or polyG to unfold the folded passenger domain, the folded domains must unfold and move into the translocation channel in a spontaneous (Brownian) reaction. Our results also show that binding sites for mtHsp70 in a preprotein can be 50 amino acid residues apart.

It is essential for deriving these conclusions from our experiments that, in fact, mtHsp70 does not bind to the sequences used. The following data exclude binding of mtHsp70 to polyG and in particular to polyE. (i) Molecular modeling of polyE into the peptide binding domain of DnaK, the closest homolog to mtHsp70, shows that the side chains of glutamic acid residues do not fit into the binding pocket and that nearby negative charges strongly disfavor the binding of glutamic acid residues. (ii) Binding studies of mtHsp70 to peptides using a SPR assay do not show any affinity for polyE and polyG peptides. In competition experiments, even a vast excess of these peptides, as compared with a peptide with high affinity, did not lead to inhibition of mtHsp70 binding to an immobilized specific substrate peptide. (iii) Interaction of mtHsp70 with preproteins arrested upon entry into the matrix was studied in an *in organello* assay. mtHsp70 was found to be unable to hold on to polyE and polyG segments that were exposed in the matrix. As a result of this, preprotein domains underwent retrograde movement into the intermembrane space.

In conclusion, it is clear from our data that mtHsp70 could not 'pull' on polyE or polyG stretches.

Experiments put forward in support of the 'power stroke' model were based on the study of a mutant mtHsp70, Ssc1-2p in yeast (Voisine *et al.*, 1999). At a non-permissive temperature, Ssc1-2p binds to precursor polypeptide chains, but interacts with Tim44 with reduced affinity (Gambill *et al.*, 1993; Schneider *et al.*, 1994; Voos *et al.*, 1996; Geissler *et al.*, 2001; Lim *et al.*, 2001). Mitochondria from cells carrying this mutation were reported to import loosely folded preproteins with a

Fig. 8. Models for unfolding and translocation of preproteins across the mitochondrial membranes. MPP, mitochondrial processing peptidase; OM, outer membrane; IM, inner membrane. See text for details.

reduced rate, but they did not import preproteins with the tightly folded HBD of cytochrome b_2 (Voos *et al.*, 1996; Voisine *et al.*, 1999). This led to the interpretation that, in contrast to wild-type Ssc1p, the mutant Ssc1-2p cannot pull on the polypeptide chain to unfold HBD since it cannot use Tim44 as a membrane fulcrum (Voos *et al.*, 1996; Voisine *et al.*, 1999). However, using a quantitative assay, it became apparent that Ssc1-2p is also defective in import of unfolded preproteins (Milisav *et al.*, 2001). Import studies *in vitro* using *sscl-2* mitochondria, therefore, do not allow discrimination between 'pulling' and 'trapping'. The interaction of mtHsp70 with Tim44 is a prerequisite for both unfolding of folded preproteins and translocation of unfolded polypeptide chains.

According to the 'power stroke' model, the free energy of ATP hydrolysis by mtHsp70 is transduced in the mechanical force responsible for unfolding of a folded preprotein. The mechanical force that could be theoretically generated by mtHsp70 in a single power stroke would result from hydrolysis of one ATP molecule. Therefore, import of folded preproteins could only occur when the mechanical force generated by pulling was higher than the force required for unfolding of the folded preproteins. Mechanical unfolding of I27 and I28 by AFM requires forces of 200–260 pN (Carrion-Vazquez *et al.*, 1999; Marszalek *et al.*, 1999; Li *et al.*, 2000). If the complete free energy of ATP hydrolysis could be used for a power stroke of 3.5 nm (see above), the motor could generate a maximal force of 14 pN ($30 \text{ kNm}/6 \times 10^{23}/3.5 \text{ nm}$). These forces are much lower than those reported for mechanical unfolding of I27 or I28 by AFM. However, the unfolding force varies as a function of the pulling speed and proteins will unfold at low applied force when the pulling speed is sufficiently slow (Carrion-Vazquez *et al.*, 1999; Smith and Radford, 2000). This is because the probability of spontaneous local unfolding and refolding events increases with time, and when a segment is unfolded, it can be fully extended with low force. Thus, mtHsp70 could mechanically unfold I27 or I28 when the conformational change leading to a power stroke would be very slow. Extrapolating AFM data, a single power stroke lasting for minutes would be required to mechanically unfold titin with forces in the low piconewton range. However, we have shown here and elsewhere (Gaume *et al.*, 1998) that, *in organello*, mtHsp70 rapidly releases and rebinds an arrested translocation intermediate in an ATP-consuming manner. This is inconsistent with hydrolysis of a single molecule of ATP followed by a slow power stroke. Finally, in order to unfold, I27 or I28 domains must overcome an activation energy barrier of 92 kJ/mol (Carrion-Vazquez *et al.*, 1999). Therefore, a power stroke driven by hydrolysis of a single molecule of ATP could not mechanically unfold these domains.

Translocation was observed to pause when an Ig-like domain was about to be unfolded (K. Okamoto, unpublished data), which is consistent with the notion that the domain has to unfold spontaneously, but inconsistent with an import device that could instantly unfold the domain by a 'power stroke'. Complete spontaneous unfolding of I27 and I28 is estimated to be slow (Li *et al.*, 2000; Smith and Radford, 2000) and cannot account for the observed import kinetics. However, as unfolding is a probabilistic event, spontaneous local unfolding occurs with a much

higher frequency than global unfolding. The kinetics of single molecule unfolding of titin revealed that spontaneous local unfolding and refolding events occur in the range of 10–50 ms (Zhuang *et al.*, 2000). Movement of an unfolded segment into the translocation channel does not require high forces and can easily be driven by Brownian motion. When the translocating polypeptide chain is trapped in the matrix, the segment in the channel cannot slide back and refold. Thereby, the initial local unfolding event will promote complete unfolding of the domain in a cooperative manner. Interaction of partially unfolded domains with lipids or chaperones on the surface of the mitochondria may further facilitate complete unfolding and import of folded preproteins. In this study, we investigated the import of tightly folded preproteins in order to unravel the functional properties of the import machinery, although in the cell, cytosolic chaperones may keep mitochondrial precursors in a loosely folded conformation.

Our results provide a consistent simple rationale for the various reactions involved in unfolding and translocation of preproteins across the mitochondrial membranes. One could argue that mtHsp70, in addition to its role in trapping segments of polypeptide chains, could weakly (or slowly) pull on incoming chains. This possibility cannot be excluded, however, if the forces involved in such a hypothetical reaction would not add to the efficiency of a Brownian ratchet based on chemical equilibria. Notably, a Brownian ratchet mechanism has been proposed for the post-translational translocation of proteins into the ER (Matlack *et al.*, 1999). Whether such a mechanism would be able to explain all types of protein transport across membranes involving Hsp70-based motors remains to be shown. In the case of mitochondria, the three-dimensional structures of mtHsp70 in the ATP and ADP forms, the structure of Tim44, as well as its precise mode of interaction with mtHsp70 and the membrane, remain to be investigated to understand the molecular movements of the components involved.

Materials and methods

SPR measurements

SPR measurements were performed on a BIAcore 2000 instrument at 25°C. The peptides CNRLLTG (C-NR), CGGGGGGG (C-G7) and CEEEEEEE (C-E7) were immobilized on a CM5 biosensor chip (BIAcore) via a thioether linkage. Ten millimolar HEPES–NaOH pH 7.5, 150 mM NaCl, 3 mM EDTA and 0.05% Tween-20 were used as a running buffer at a flow rate of 5 $\mu\text{l}/\text{min}$. A mixture containing 25 mM *N*-hydroxysuccinimide (NHS) and 0.1 M *N*-ethyl-*N'*-dimethylaminopropyl carbodiimide (EDC) was injected for 15 min to activate the carboxymethyl-derivatized dextran matrix. Amino groups were generated by coupling ethylenediamine (1 M, pH 6.0, 10 min) to the activated chip. Maleimido groups were subsequently introduced using the heterobifunctional cross-linker Sulfo-GMBS (Pierce) (50 mM), and C-NR, C-G7 or C-E7 (0.1 mM) was injected for an additional 10 min. Excess unreacted maleimido groups were inactivated by a 2 min pulse of 0.1 M NaOH.

Binding of mtHsp70 (see Supplementary data available at *The EMBO Journal* Online) to immobilized C-NR, C-G7 or C-E7 was followed at 25°C. mtHsp70 (5 μM) was passed over the chip at a flow rate of 20 $\mu\text{l}/\text{min}$ in the presence of 5 mM ADP. For competition experiments, NR (CNRLLTG), G7 (CGGGGGG) or E7 (EEEEEEE) was pre-incubated at 25°C for 10 min at a final concentration of 10, 300 or 500 μM with 5 μM mtHsp70 and 5 mM ADP. The mixture was then passed over the C-NR derivatized chip. Regeneration of the surface was performed in all cases by two 1-min pulses of 6 M GdnCl pH 3.0. Signals due to unspecific

interactions with an unmodified biosensor matrix were <10% of the specific signals, and were subtracted.

Protein import into isolated mitochondria

Mitochondrial precursors were synthesized *in vitro* using TNT coupled reticulocyte lysate systems (Promega) in the presence of [³⁵S]methionine. Reticulocyte lysate containing ³⁵S-labeled precursors was incubated with isolated mitochondria (1 mg protein/ml) from the yeast strain D273-10B in import buffer (0.6 M sorbitol, 50 mM HEPES-KOH pH 7.4, 80 mM KCl, 10 mM MgCl₂, 2 mM KH₂PO₄, 0.1% fatty acid-free bovine serum albumin) in the presence of 2 mM ATP and 4 mM NADH at 25°C. If indicated, 10 mM creatine phosphate and 0.1 mg/ml creatine kinase were added to the import buffer. After incubation, the samples were split into three aliquots: one aliquot was diluted 10-fold with HS buffer (0.6 M sorbitol, 20 mM HEPES-KOH pH 7.4); the second aliquot was diluted 10-fold with HS buffer containing 0.1 mg/ml PK; the third aliquot was diluted 10-fold with 20 mM HEPES-KOH pH 7.4 and 0.1 mg/ml PK. The samples were incubated on ice for 25 min, and then treated with PMSF at a final concentration of 2 mM on ice for 5 min. Mitochondria or mitoplasts were isolated by centrifugation and washed with HS buffer containing 1 mM PMSF. MTX arrest of precursors containing a DHFR domain was performed as described previously (Ungermann *et al.*, 1996).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

We thank Dr M.Gautel (Max-Planck-Institute, Dortmund, Germany) for plasmids encoding the Ig-like domains of human titin, and C.Kotthoff and N.Okamoto for expert technical assistance. K.O. was supported by a long-term fellowship from the Human Frontier Science Program Organization (HFSP). A.B. and S.A.P. were supported by a predoctoral fellowship from the Boehringer Ingelheim Fonds. This work was supported by grants from the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 184 (B2 and B12), the Human Frontiers of Science Program, the Fonds der Chemischen Industrie and funds from the German-Israeli foundation and the HFSP (to W.N.).

References

- Bauer,M.F., Hofmann,S., Neupert,W. and Brunner,M. (2000) Protein translocation into mitochondria: the role of TIM complexes. *Trends Cell Biol.*, **10**, 25–31.
- Carrion-Vazquez,M., Oberhauser,A.F., Fowler,S.B., Marszalek,P.E., Broedel,S.E., Clarke,J. and Fernandez,J.M. (1999) Mechanical and chemical unfolding of a single protein: a comparison. *Proc. Natl Acad. Sci. USA*, **96**, 3694–3699.
- Chauwin,J.-F., Oster,G. and Glick,B.S. (1998) Strong precursor–pore interactions constrain models for mitochondrial protein import. *Biophys. J.*, **74**, 1732–1743.
- Craig,E.A., Kramer,J., Shilling,J., Werner-Washburne,M., Holmes,S., Kosc-Smiths,J. and Nicolet,C.M. (1989) SSC1, an essential member of the yeast HSP70 multigene family, encodes a mitochondrial protein. *Mol. Cell. Biol.*, **9**, 3000–3008.
- Gambill,B.D., Voos,W., Kang,P.-J., Miao,B., Langer,T., Craig,E.A. and Pfanner,N. (1993) A dual role for mitochondrial heat shock protein 70 in membrane translocation of preproteins. *J. Cell Biol.*, **123**, 109–117.
- Gaume,B., Klaus,C., Ungermann,C., Guiard,B., Neupert,W. and Brunner,M. (1998) Unfolding of preproteins upon import into mitochondria. *EMBO J.*, **17**, 6497–6507.
- Geissler,A., Rassow,J., Pfanner,N. and Voos,W. (2001) Mitochondrial import driving forces: enhanced trapping by matrix hsp70 stimulates translocation and reduces the membrane potential dependence of loosely folded preproteins. *Mol. Cell. Biol.*, **21**, 7097–7104.
- Glick,B.S. (1995) Can Hsp70 proteins act as force-generating motors? *Cell*, **80**, 11–14.
- Gragerov,A., Zeng,L., Zhao,X., Burkholder,W. and Gottesman,M.E. (1994) Specificity of DnaK-peptide binding. *J. Mol. Biol.*, **235**, 848–854.
- Huang,S., Ratliff,K.S., Schwartz,M.P., Spenner,J.M. and Matouschek,A. (1999) Mitochondria unfold precursor proteins by unraveling them from their N-termini. *Nat. Struct. Biol.*, **6**, 1132–1138.
- Jensen,R.E. and Johnson,A.E. (1999) Protein translocation: is Hsp70 pulling my chain? *Curr. Biol.*, **9**, R779–R782.
- Kanamori,T., Nishikawa,S., Shin,I., Schultz,P.G. and Endo,T. (1997) Probing the environment along the protein import pathways in yeast mitochondria by site-specific photocrosslinking. *Proc. Natl Acad. Sci. USA*, **94**, 485–490.
- Kronidou,N.G., Oppliger,W., Bolliger,L., Hannavy,K., Glick,B.S., Schatz,G. and Horst,M. (1994) Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. *Proc. Natl Acad. Sci. USA*, **91**, 12818–12822.
- Li,H., Oberhauser,A.F., Fowler,S.B., Clarke,J. and Fernandez,J.M. (2000) Atomic force microscopy reveals the mechanical design of a modular protein. *Proc. Natl Acad. Sci. USA*, **97**, 6527–6531.
- Lim,J.H., Martin,F., Guiard,B., Pfanner,N. and Voos,W. (2001) The mitochondrial Hsp70-dependent import system actively unfolds preproteins and shortens the lag phase of translocation. *EMBO J.*, **20**, 941–950.
- Marszalek,P.E., Lu,H., Li,H., Carrion-Vazquez,M., Oberhauser,A.F., Schulten,K. and Fernandez,J.M. (1999) Mechanical unfolding intermediates in titin modules. *Nature*, **402**, 100–103.
- Matlack,K.E.S., Mothes,W. and Rapoport,T.A. (1998) Protein translocation: tunnel vision. *Cell*, **92**, 381–390.
- Matlack,K.E.S., Misselwitz,B., Plath,K. and Rapoport,T.A. (1999) BiP acts as a molecular ratchet during posttranslational transport of prepro- α factor across the ER membrane. *Cell*, **97**, 553–564.
- Matouschek,A., Azem,A., Ratliff,K., Glick,B.S., Schmid,K. and Schatz,G. (1997) Active unfolding of precursor proteins during mitochondrial protein import. *EMBO J.*, **16**, 6727–6736.
- Matouschek,A., Pfanner,N. and Voos,W. (2000) Protein unfolding by mitochondria. The Hsp70 import motor. *EMBO rep.*, **1**, 404–410.
- Mayer,M.P., Schröder,H., Rüdiger,S., Paal,K., Laufen,T. and Bukau,B. (2000) Multistep mechanism of substrate binding determines chaperone activity of Hsp70. *Nat. Struct. Biol.*, **7**, 586–593.
- Milislav,I., Moro,F., Neupert,W. and Brunner,M. (2001) Modular structure of the TIM23 preprotein translocase of mitochondria. *J. Biol. Chem.*, **276**, 25856–25861.
- Misselwitz,B., Staack,O. and Rapoport,T.A. (1998) J proteins catalytically activate Hsp70 molecules to trap a wide range of peptide sequences. *Mol. Cell*, **2**, 593–603.
- Moro,F., Sirrenberg,C., Schneider,H.-C., Neupert,W. and Brunner,M. (1999) The TIM17-23 preprotein translocase of mitochondria: composition and function in protein transport into the matrix. *EMBO J.*, **18**, 3667–3675.
- Pfanner,N. and Geissler,A. (2001) Versatility of the mitochondrial protein import machinery. *Nat. Rev. Mol. Cell. Biol.*, **2**, 339–349.
- Pfanner,N. and Meijer,M. (1995) Pulling in the proteins. *Curr. Biol.*, **5**, 132–135.
- Rapoport,T.A., Matlack,K.E.S., Plath,K., Misselwitz,B. and Staack,O. (1999) Posttranslational protein translocation across the membrane of the endoplasmic reticulum. *Biol. Chem.*, **380**, 1143–1150.
- Rassow,J., Maarse,A.C., Krainer,E., Kubrich,M., Muller,H., Meijer,M., Craig,E.A. and Pfanner,N. (1994) Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. *J. Cell Biol.*, **127**, 1547–1556.
- Rüdiger,S., Germeroth,L., Schneider-Mergener,J. and Bukau,B. (1997) Substrate specificity of the DnaK chaperone determined by screening cellulose-bound peptide libraries. *EMBO J.*, **16**, 1501–1507.
- Schneider,H.-C., Berthold,J., Bauer,M.F., Dietmeier,K., Guiard,B., Brunner,M. and Neupert,W. (1994) Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature*, **371**, 768–774.
- Schneider,H.-C., Westermann,B., Neupert,W. and Brunner,M. (1996) The nucleotide exchange factor MGE exerts a key function in the ATP-dependent cycle of mt-Hsp70–Tim44 interaction driving mitochondrial protein import. *EMBO J.*, **15**, 5796–5803.
- Simon,S.M. and Blobel,G. (1993) Mechanisms of translocation of proteins across membranes. *Subcell. Biochem.*, **21**, 1–15.
- Smith,D.A. and Radford,S.E. (2000) Protein folding: pulling back the frontiers. *Curr. Biol.*, **10**, R662–R664.
- Spudich,J.A. (1994) How molecular motors work. *Nature*, **372**, 515–518.
- Strub,A., Lim,J.H., Pfanner,N. and Voos,W. (2000) The mitochondrial protein import motor. *Biol. Chem.*, **381**, 943–949.
- Ungermann,C., Guiard,B., Neupert,W. and Cyr,D.M. (1996) The $\Delta\psi$ - and Hsp70/MIM44-dependent reaction cycle driving early steps of protein import into mitochondria. *EMBO J.*, **15**, 735–744.
- Voisine,C., Craig,E.A., Zufall,N., von Ahsen,O., Pfanner,N. and Voos,W. (1999) The protein import motor of mitochondria:

- unfolding and trapping of preproteins are distinct and separable functions of matrix Hsp70. *Cell*, **97**, 565–574.
- Voos,W., von Ahsen,O., Müller,H., Guiard,B., Rassow,J. and Pfanner,N. (1996) Differential requirement for the mitochondrial Hsp70–Tim44 complex in unfolding and translocation of preproteins. *EMBO J.*, **15**, 2668–2677.
- Wickner,W.T. (1994) How ATP drives proteins across membranes. *Science*, **266**, 1197–1198.
- Zhu,X., Zhao,X., Burkholder,W.F., Gragerov,A., Ogata,C.M., Gottesman,M.E. and Hendrickson,W.A. (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. *Science*, **272**, 1606–1614.
- Zhuang,X., Ha,T., Kim,H.D., Centner,T., Labeit,S. and Chu,S. (2000). Fluorescence quenching: a tool for single-molecule protein-folding study. *Proc. Natl Acad. Sci. USA*, **97**, 14241–14244.

*Received February 28, 2002; revised May 14, 2002;
accepted May 21, 2002*