THE PROBLEM OF how proteins are translocated across biological membranes is a salient feature in biochemistry and cell biology. In the case of the bacterial plasma membrane, the endoplasmic reticulum (ER), chloroplasts and mitochondria, many of the components involved in mediating protein translocation have been identified. However, many open questions exist with regard to several aspects of these translocation events. For example, proteins appear not to traverse membranes in a folded state, but rather as extended polypeptide chains. How is this unfolding achieved? How is energy harnessed to cause unfolding? How is the unfolded state maintained? On completion of translocation how does the protein then refold to its native structure? Another important question concerns the nature of the driving force for the movement of proteins across membranes. The translocation process is generally observed to be unidirectional; what is the basis of this phenomenon? Some insights into these questions have recently been gained. Increasing evidence indicates that the translocation process is integrally coupled to the action of a set of molecular chaperones located in the cytosol and in the mitochondrial matrix (Table I).

Mitochondria represent an attractive system in which to study the basic mechanisms and energetics of protein translocation events, for a number of reasons. First, powerful in vitro protein import systems have been established in which the pathway of protein translocation can be experimentally dissected into a series of distinct steps. Second, the bioenergetics are well documented and can be easily manipulated, enabling independent modulation of both membrane potential and levels of ATP in the matrix. Furthermore, many mutants defective in mitochondrial function have been identified, particularly in yeast. These mutants have not only resulted in the cloning of genes encoding proteins essential for mitochondrial function, but have also proved invaluable for analysis of the import process.

With the exception of a small percentage of proteins that are encoded by the mitochondrial genome, the majority of proteins are synthesized in the cytoplasm as precursor proteins with amino-terminal presequences, which are proteolytically removed in the mitochondrial matrix. The precursor proteins are imported along a multistep pathway, and this process normally occurs by a post-translational mechanism for a recent review see Ref. 1). Precursors are initially recognized by, and bind to, receptor proteins on the outer surface of mitochondria. The passage of preproteins across the inner membrane (IM) is dependent on both a membrane potential, ΔΨ, across the IM, and ATP hydrolysis in the matrix. It requires the participation of a proteinaceous machinery localized in the IM, the composition of which has recently begun to be unravelled. In addition, the mitochondrial chaperone Hsp70 (mt-Hsp70), encoded by the SSCI gene in Saccharomyces cerevisiae, appears to play a decisive role in the translocation of preproteins across the mitochondrial membranes. Incoming polypeptide chains are thought to represent a substrate for the matrix-localized mt-Hsp70, and, through a series of binding and release cycles, mt-Hsp70 mediates the passage of the protein into the matrix.

In this review, we will summarize the progress made over the past year or so that has increased our understanding of the mechanism of protein translocation into mitochondria. New observations have, in particular, shed light on the importance of molecular chaperones.

Table I. Molecular chaperones implicated in mitochondrial protein translocation and folding in yeast

<table>
<thead>
<tr>
<th>Chaperone</th>
<th>Cellular location</th>
<th>Essential</th>
<th>Ref(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSA1/SSA2</td>
<td>Cytosol</td>
<td>Yes</td>
<td>16</td>
</tr>
<tr>
<td>YDJ1</td>
<td>Cytosol</td>
<td>No*</td>
<td>17,18</td>
</tr>
<tr>
<td>SSCI (mt-Hsp70)</td>
<td>Mitochondrial matrix</td>
<td>Yes</td>
<td>2.3</td>
</tr>
<tr>
<td>MDJ1</td>
<td>Mitochondrial matrix</td>
<td>No*</td>
<td>c</td>
</tr>
<tr>
<td>MGE1</td>
<td>Mitochondrial matrix</td>
<td>Yes</td>
<td>d</td>
</tr>
<tr>
<td>Hsp60</td>
<td>Mitochondrial matrix</td>
<td>Yes</td>
<td>25.26</td>
</tr>
<tr>
<td>Hsp10</td>
<td>Mitochondrial matrix</td>
<td>Yes</td>
<td>28.6</td>
</tr>
</tbody>
</table>

*Viable at 23°C but not at 37°C.

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which appear to function at several distinct steps of the import pathway.

**Manipulation of mt-Hsp70 action**

Two important tools exist for studying the actions of mitochondrial chaperones, in particular mt-Hsp70; these are (1) modulation of matrix ATP concentrations to levels that adversely affect the ATP-dependent action of mt-Hsp70, and (2) mutations in the SSC1 gene resulting in altered mt-Hsp70 proteins that are temperature sensitive for function.

In addition to ΔΨ, hydrolysis of matrix ATP constitutes (at least part of) the driving force for the vectorial movement of preproteins into the matrix. A critical role of matrix ATP in protein import appears to be through its facilitating the reversible binding of mt-Hsp70 to incoming polypeptides. In isolated mitochondria, matrix ATP can be stringently reduced to experimentally definable levels; thus it is possible to directly address which specific stages of import are affected when ATP levels become limiting for mt-Hsp70 function. Other ATP-requiring chaperones, such as Hsp60, do not play a role in translocation per se, and hence are not determining factors in these energetic studies.

Temperature-sensitive (ts) mutants of the SSC1 gene have also proved invaluable for the study of mt-Hsp70 functions. In addition to the previously published sscl-2 allele, a second ts allele of the Hsp70 gene, sscl-3, was recently identified. Import of preproteins into mitochondria was inhibited both in vitro and in vivo in sscl-2 at nonpermissive temperatures. In sscl-3 mitochondria, mt-Hsp70 function was more severely affected than in the sscl-2 mutant (see below). The sscl-3 mutation mapped to the amino-terminal ATPase domain, in contrast with the sscl-2 mutation, which was localized to the putative peptide-binding domain at the carboxy-terminal portion of mt-Hsp70. Mt-Hsp70 from the sscl-2 mutant displays the ability to bind substrates (release being proposed to be affected), whereas it was suggested that the function of mt-Hsp70 in the sscl-3 mutant was compromised at the initial level of binding to substrates.

Comparison of these two mutants, together with the studies of ATP-depleted mitochondria, has revealed new insights into the role of mt-Hsp70, and, more specifically, has implicated the direct involvement of mt-Hsp70 in three distinct stages of precursor protein import: (1) binding to amino-terminal regions of the incoming polypeptide in order to stabilize the targeting sequence on the trans-side of the IM, thereby making the initial import step irreversible; (2) unfolding of tightly folded segments of preproteins on the cis-side of the outer membrane (OM), facilitated by binding of mt-Hsp70 to the matrix-exposed domains of the polypeptide; and (3) completion of translocation across the IM by binding to further domains of the preprotein.

In the following sections, we summarize the findings that gave rise to these conclusions, and present a model for the action of mt-Hsp70 that includes the involvement of other recently identified mitochondrial chaperones.

**Matrix ATP and mt-Hsp70 are required for translocation of the presequence across the IM**

The passage of the targetting presequence across the IM in response to ΔΨ represents the first committed step of translocation across the IM. It has recently become clear that mt-Hsp70 plays an important role in this early step of import, trapping the presequence in the matrix following its initial translocation triggered by ΔΨ (Fig. 1).

Drastic reduction of matrix ATP to low micromolar levels caused not only complete inhibition of import, but also of presequence translocation across the IM and subsequent processing in the matrix, despite the presence of ΔΨ. Thus, in the absence of mt-Hsp70 function, the precursors accumulated as import intermediates, being both unprocessed by matrix processing peptidase (MPP) and accessible to exogenously added proteases. These import intermediates were arrested at an early stage of import, since their further chase required not only the presence of ATP in the matrix, but also the ΔΨ across the IM. Similar accumulation of unprocessed external import intermediates was observed (with the same precursors) in the sscl-3 mutant (D. M. Cyr, R. A. Stuart and W. Neupert, unpublished).

Presence translocation across the IM therefore appears to occur in response to ΔΨ. However, it seems to be a reversible process (Fig. 1). Mt-Hsp70, by binding to the incoming polypeptide upon their exposure to the matrix, makes this import event unidirectional by preventing the polypeptide from slipping back to the cis-side of the IM. Binding of mt-Hsp70 could occur through direct interaction of mt-Hsp70 with the presequence, or with amino-terminal mature regions of the preprotein. It appears that the mt-Hsp70 is competent for binding substrates in the matrix when it is in its ATP-bound state, and is thus compromised under conditions of matrix-ATP depletion. The
The role of cytosolic chaperones in mitochondrial protein import

It is generally agreed that precursor proteins cannot traverse membranes in a folded conformation. This assumption is based on several observations, in particular: (1) when precursors were bound to antibodies or Fab fragments, import was abolished; (2) chimeric preproteins consisting of mitochondrial sequences and mouse cytosolic dihydrofolate reductase (DHFR) are not imported if the DHFR domain is stabilized by binding of a ligand; and (3) a series of chimeric proteins, consisting of a mitochondrial presequence fused to increasingly large segments of mature proteins containing DHFR at the carboxyl terminus, stall with the folded DHFR domain on the surface of the OM and the amino terminus in the matrix. These fusion proteins have been used to map the minimum polypeptide length required to span the outer and inner membranes, a distance of ~10–12 nm. Roughly 50 amino acids are required to span both membranes. Taken together, these data would suggest that a precursor protein passes through the mitochondrial import channels as an extended polypeptide chain.

At this stage, one might ask what the conformation of a mitochondrial preprotein in the cytosol is. The following findings are relevant in this respect. (1) Preproteins in the cytosol are not usually tightly folded, as they are extremely sensitive to digestion by added proteases. (2) However, domains of some preproteins, such as the DHFR fusion proteins discussed above, can be correctly folded, and these preproteins are still imported efficiently. (3) The presence of a correctly folded domain may, however, at least in certain cases, present a problem for the efficiency of import. Interestingly, fusion proteins carrying mutations in DHFR that destabilize the native state show an increased rate of import. Furthermore, additional stabilization of the DHFR domain by a ligand (see above) completely blocks import. Thus, during import, preproteins must unfold...
Figure 3

Hypothetical model for the sequential action of mitochondrial chaperone proteins in the translocation and folding of mitochondrial matrix–destined precursor proteins. Cytosolic Hsp70 (ct-Hsp70), together with YDJ, has been suggested to maintain some precursors in an unfolded, translocation-competent conformation before membrane translocation. Import of precursors into the matrix is mediated by mt-Hsp70 in an ATP-dependent manner. The involvement of MDJ and MGE is presently under investigation (see text for details). Following translocation, precursors are folded directly or become transferred from mt-Hsp70 to Hsp60. Both processes are proposed to require MDJ and MGE. Folding by Hsp60 requires both ATP hydrolysis and the action of a further chaperone, Hsp10. Other abbreviations are as for Fig. 1.

either from a native folded state or from a more loosely folded state. In some instances, it may be necessary to prevent folding of precursors, since unfolding cannot be managed by the transport system. Also, in many cases, it may be necessary to prevent aggregation of precursors, since loosely folded regions have a tendency to aggregate, which may abrogate the ability to become unfolded. How does a cell deal with all these problems? In particular, what is the mechanism that leads to unfolding during import?

Cytosolic chaperones, especially of the Hsp70 family (ct-Hsp70; the SSA gene family in S. cerevisiae) have been postulated to interact with precursors in the cytosol. These chaperones, by interacting with precursors, most likely prevent them from folding into a conformation that cannot be unfolded, and also prevent aggregation14. Depletion of ct-Hsp70s in vivo resulted in accumulation of precursors of the β-subunit of the mitochondrial protein F1-ATPase. In addition to members of the Hsp70 family, another chaperone, YDJ1, which is a cytosolic homologue of the bacterial DnaJ, has been implicated in playing a role in efficient post-translational import into mitochondria, especially at elevated temperatures14–19 (the role of this chaperone will be discussed in detail in another article in this series, by D. M. Cyr and M. C. Douglas). The presence of an unfolded activity, either in the cytosol or on the mitochondrial surface, that would actively support the unfolding of precursors, has not been demonstrated.

ATP–mt-Hsp70 promotes unfolding of precursors outside mitochondria

Inhibition of precursor import into mitochondria of ssc1-2 mutants could be overcome if the precursor was (artificially) unfolded in 8 M urea before import11. This suggested that the translocation of an unfolded protein could be driven by this defective mt-Hsp70, whereas translocation of a partially folded protein could not11. Further observations supporting a role of mt-Hsp70 in precursor unfolding come from studies of the import pathway of cytochrome b2 (Ref 7, 8). Cytochrome b2 is targeted to its functional location, the intermembrane space, by a bipartite signal sequence. How this signal operates is a matter of debate, and two models are currently being discussed, the 'conservative sorting' and the 'stop-transfer' models20–21. Irrespective of the sorting pathway used, a recent study of the energetics of import has demonstrated a role for mt-Hsp70 in unfolding tightly folded segments of precursor proteins outside the mitochondria. The import of cytochrome b2 displayed a very strong requirement for matrix ATP. In the absence of matrix ATP, the precursor accumulated on the outer surface of mitochondria as an unprocessed species that was accessible to externally added proteases. This necessity for matrix ATP reflected dependence on the action of mt-Hsp70, as very similar results were observed in the above-mentioned ssc1-2 and ssc1-3 mutants (Ref 22; R. A. Stuart and W. Neupert, unpublished). If, however, the precursors of cytochrome b2 and derived fusion proteins, which also displayed strong mt-Hsp70 requirements were denatured in 8 M urea before import, they could be very efficiently imported into both ATP-depleted mitochondria and ssc1-3 mutant mitochondria22.

Cytochrome b2 (l-lactate dehydrogenase) contains both flavin and haem as prosthetic groups. About the first 100 residues of the mature polypeptide chain form a tightly folded structure which forms the cytochrome b2– or haem-binding domain20. This folded domain most probably prevents the stable insertion of the precursor in the matrix, unless unfolded through the action of mt-Hsp70 (see below). Hence, precursors containing this domain require ATP-dependent binding of mt-Hsp70 to the matrix-exposed part of the protein to mediate its unfolding. By contrast, precytochrome b2 constructs in which this domain has been deleted or disrupted can be processed with very high efficiency in the apparent absence...
of mt-Hsp70 activity. Taken together, the data suggest a function of mt-Hsp70 in supporting the unfolding of precursors on the mitochondrial surface. A possible mechanism is that, by binding to segments of precursors on the matrix side, stabilization of an unfolded conformation on the mitochondrial surface is favoured.

**Model for the action of Hsp70 in the translocation of proteins into mitochondria**

To summarize, we propose the following model for the translocation of polypeptides into the matrix and the involvement of mt-Hsp70. Mt-Hsp70 appears to participate in at least three distinct stages of import which, together, serve to confer unidirectionality and provide the driving force for the import process.

The transport apparatus of the OM guides the amino-terminal presequence of the precursor into the intermembrane space. The translocation machinery of both the OM and the IM is proposed to form proteinaceous channels that mediate the passage of an unfolded polypeptide chain. In response to ΔΨ, the matrix-targeting presequence of preproteins crosses the IM in a reversible manner. Binding of mt-Hsp70 to the incoming polypeptide upon exposure to the matrix serves to anchor this motif in the matrix and, by so doing, prevents the escape of the precursor back to the cis side of the IM. In this manner, the translocation of the preprotein in vivo, the matrix is committed, and mt-Hsp70 has thus conferred unidirectionality on the process (Fig. 1).

Spontaneous relaxing, or 'breathing', of the remaining mature parts of the polypeptide chain outside the mitochondrion would then render segments of the protein free to pass across the membrane. In this manner, the protein is translocated in a stepwise manner where further binding of mt-Hsp70 commits increasing amounts of the protein to the matrix and thus shifts the equilibrium of the protein structure outside the mitochondrion towards the unfolded state, which ultimately draws it towards the matrix; however, mt-Hsp70 could not drive the equilibrium towards the matrix (Fig. 2). This sequence of events would share a number of features with the 'Brownian ratchet' model proposed recently.

**Figure 4**

Hypothetical model for the nucleotide-modulated binding and dissociation of unfolded proteins to mt-Hsp70, involving MDJ and MGE. Mt-Hsp70—ATP and MDJ are thought to interact with the unfolded newly imported polypeptide, to form an MDJ—mt-Hsp70—ATP substrate complex. Substrate-stimulated ATP hydrolysis converts mt-Hsp70 to a form with high affinity for substrate, mt-Hsp70—ADP. Dissociation of the substrate occurs following the interaction of MGE with this complex. Existence of a stable DnaK—DnaJ—GroE—substrate complex has not been demonstrated in bacteria, so the analogous mitochondrial complex is depicted here in parentheses. MGE might function by exchanging the bound ADP from mt-Hsp70 for ATP. (It is also possible, however, that a nucleotide-free intermediate exists.) Exchange/release of the ADP from mt-Hsp70 results in dissociation of the complex, setting free the substrate and allowing its subsequent transfer to Hsp60 for folding.

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Recently, a mitochondrial DnaJ homologue, MDJ (mitochondrial DnaJ, termed MDJ1 in yeast), was identified, and is associated with the inner face of the IM (N. Rowley et al., unpublished). Deletion of the MDJ1 gene was not lethal; however, mdj1 null mutant cells could not grow at 37°C and were petite...
at all temperatures. Folding of newly imported proteins and stability against heat denaturation were reduced in the md1 null mutant. MD1 is thus not essential for folding and refolding of proteins, but makes these processes more efficient and becomes limiting at higher temperatures (N. Rowley et al., unpublished). MD1 therefore resembles bacterial DnaJ, which is not essential at normal temperatures but is required for refolding of denatured proteins at elevated temperatures ~9°~°. Furthermore, a GrpE homologue has been found in yeast mitochondria. Disruption of the gene encoding the mitochondrial GrpE (MGE, mitochondrial GroE) is lethal in yeast (S. Laloraya, B. D. Gambill and E. A. Craig, unpublished). The precise functions of MGE are not known. On the basis of published), the precise functions of MGE are not known. On the basis of

The question remains as to whether MD1 and MGE are also required for facilitating import of precursor proteins. With md1 mutants, no deficiency in import at normal or elevated temperature was observed, indicating that this chaperone is not necessary for import. However, it cannot be excluded that, under certain conditions, MD1 increases the efficiency of import. mt-Hsp70 seems to bind to incoming precursor proteins in the absence of MD1, and perhaps also to release precursors efficiently. The possible function of MGE in this process awaits further characterization of the protein.

Concluding remarks

Analysis of protein translocation into mitochondria has furthered our understanding and appreciation of the importance of molecular chaperones. The participation of these proteins at different stages ofochondrial protein import is vital. Molecular chaperones act in concert to ensure that preproteins to be translocated can attain, or be maintained in, a conformation compatible with passage across membranes. In addition, their action underlies the driving force (together with ΔΨ) and confers directionality on the translocation event. Can these new insights into chaperone function be relevant for other membrane systems? Already the Hsp70 homologue BIP (encoded by KAR2) in the ER has been implicated in the passage of proteins across the ER membrane. Interestingly, transport of secretory proteins was defective in kar2 mutants, and preproteins accumulated on the surface of the ER as unprocessed species. It remains to be seen whether similar chaperone-assisted mechanisms also facilitate the translocation of preproteins into chloroplasts and peroxisomes.

Acknowledgements

We wish to thank Thomas Langer for his support and for many constructive discussions. We are also grateful to those colleagues who have shared their unpublished results. This work was supported by grants from the DFG Schwerpunktprogramm ‘Heat shock response’, the Sonderforschungsbereich 184, Teilprojekt B2 and the Münchener Medizinische Wochenschrift.

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