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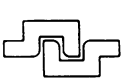

Series A: Life Sciences

New Developments in Lipid–Protein Interactions and Receptor Function

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RECOGNITION OF PRECURSOR PROTEINS BY THE MITOCHONDRIAL PROTEIN IMPORT APPARATUS

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1. BIOGENESIS OF MITOCHONDRIA

Eukaryotic cells are functionally and morphologically subdivided into a number of membrane-bounded compartments. Each of the subcompartments, or organelles, has a specific protein composition according to its function in the cell. Mitochondria and, in plant cells, chloroplasts are unique in that they have their own genetic systems including DNA, ribosomes, tRNAs. Both organelles are surrounded by a double membrane and have extended internal membrane systems. According to the endosymbiotic hypothesis of the origin of mitochondria and chloroplasts both organelles are derived from prokaryotic endosymbionts in the ancestor of the eukaryotic cell (Margulis, 1970; Schwartz and Dayhoff, 1978). A large number of the original prokaryotic features are retained in the organelles. In the course of evolution, both organelles have lost most of their genetic information, which has been transferred to the nucleus (Schwartz and Dayhoff, 1978).

Mitochondria fulfil several important functions in the metabolism of a cell. The most prominent are catabolic reactions yielding NADH or $FADH_2$, namely the tricarboxylic acid cycle and the ß-oxidation of fatty acids. NADH and $FADH_2$ are funneled into the electron transport chain which supplies the cell with ATP via oxidative phosphorylation thereby

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providing the energy required for metabolic processes. The enzymes of the respiratory chain reside in the inner mitochondrial membrane, predominantly in the cristae, which are the invaginated regions of the inner membrane. The genes of the mitochondrial genome code for some proteins of the respiratory chain complexes and mitochondrial tRNAs and rRNAs (Attardi, 1981).

The vast majority of mitochondrial proteins, however, are encoded by nuclear genes. Their translation takes place in the cytoplasm and subsequently they are imported into the organelle. This raises the question of how these precursors are correctly sorted. It is generally recognized that mitochondrial precursors carry specific targeting signals and these are recognized by the mitochondrial protein import apparatus. As a result of this, translocation into the organelle and sorting to the various mitochondrial subcompartments takes place. The specific targeting to and translocation into mitochondria has been analysed in some detail and will be reviewed in the following.

2. GENERAL PRINCIPLES OF MITOCHONDRIAL PROTEIN IMPORT

Most of the characteristics of mitochondrial protein import described in the following were analysed *in vivo* and *in vitro* with the fungi *Neurospora crassa* and *Saccharomyces cerevisiae* (Attardi and Schatz, 1988; Hartl et al., 1989; Wienhues et al., 1992). A general model of the mitochondrial protein import is shown in figure 1.

Protein translocation into mitochondria is independent of translational elongation, it can occur post-translationally *in vitro* (Korb and Neupert, 1978; Maccecchini et al., 1979) and *in vivo* (Hallermayer et al., 1977; Schatz, 1979; Wienhues et al., 1991).

Proteins destined to the mitochondria contain specific targeting sequences which are recognized by the mitochondrial protein import apparatus. After binding to a receptor in the mitochondrial outer membrane, precursor proteins are inserted into this membrane. Translocation proceeds at the contact sites where the two mitochondrial membranes are in close apposition. In the presence of a membrane potential the precursors are then transported across the inner membrane into the matrix space. This translocation step is dependent on mitochondrial hsp70 which directly interacts with the precursor proteins in transit.

Upon arrival in the matrix space, the presequence is cleaved by the matrix localized mitochondrial processing peptidase (MPP) (Arretz et al., 1991; Hawlitschek et al., 1988). Some precursors have to undergo a second processing step by the mitochondrial intermediate peptidase (MIP) (Kalousek et al., 1988) or by MPP (subunit 9 of the F_1-F_0-ATPase) to be processed to their mature size. Folding of the proteins and assembly into oligomeric structures are mediated by interaction with mitochondrial hsp60 in the matrix (Ostermann et al., 1989). After import, sorting to other intramitochondrial compartments (matrix space, inner membrane, intermembrane space) takes place. Precursors destined to

the inner membrane are inserted into this membrane or translocated across it (Mahlke et al., 1990). Some proteins destined to the intermembrane space have bipartite signal sequences, which consist of the matrix targeting sequence and a sorting signal to the intermembrane space. The sorting pathway of the Rieske iron-sulfur protein includes import into the matrix followed by retranslocation to the intermembrane space (Hartl et al., 1986). In the case of the cytochromes b_2 and c_1, two mechanisms have been proposed for their sorting to the intermembrane space: The "stop-transfer model" is based on the assumption that the second part of the bipartite presequence functions as a stop-transfer signal by which proteins get arrested at the inner membrane and then laterally diffuse into the intermembrane space (Glick et al., 1992; Van Loon and Schatz, 1987). According to the "conservative sorting model", proteins are first imported into the matrix space and then retranslocated to the intermembrane space by an export process resembling the general pathway of protein secretion in bacteria. This model takes into account the prokaryotic origin of mitochondria and the similarity between the intermembrane space targeting sequence and bacterial export signals (Hartl and Neupert, 1990; Koll et al., 1992).

There are a few exceptions to the above outlined general pathway of protein import into mitochondria. Some proteins use only part of the components by either deviating from the general pathway at an early stage (cytochrome c heme lyase, which uses only the receptor complex and GIP) (Lill et al., 1992b) or else getting inserted at a later stage (subunit Va of cytochrome oxidase, which is imported independently of receptors and inserted into the outer membrane presumably at the GIP-stage) (Miller and Cumsky, 1991).

Targeting of the intermembrane space protein cytochrome c is independent of any of the components of the general protein import apparatus: it is inserted into the outer membrane without the aid of protease-sensitive surface receptors and directly reaches the intermembrane space (Lill et al., 1992a; Stuart et al., 1990).

3. STEPS IN MITOCHONDRIAL PROTEIN IMPORT

3.1. Targeting Sequences Direct Precursor Proteins to Mitochondria

Proteins destined to the mitochondria carry targeting sequences which are recognized by the mitochondrial protein import apparatus. For most mitochondrial proteins the targeting sequence consists of an amino-terminal presequence which is cleaved upon arrival of the protein in the mitochondrial matrix. Mitochondrial presequences are characterized by being positively charged and having a tendency to form amphipathic helices in a hydrophobic environment (Von Heijne, 1986). Some mitochondrial preproteins, however, contain internal, sometimes multiple, noncleaved targeting sequences which have not yet been defined in detail (Pfanner et al., 1987).

The targeting function of mitochondrial presequences has been demonstrated by fusing mitochondrial targeting sequences to cytosolic proteins and showing that the fusion proteins are imported into mitochondria (Hurt et al., 1984; Rassow et al., 1990).

3.2. Precursors Are Recognized by Mitochondrial Import Receptors

An initial approach to the identification of mitochondrial protein receptors consisted in raising antisera to the various proteins of the outer membrane of *Neurospora crassa* mitochondria. IgGs and Fab fragments purified from these monospecific antisera were prebound to mitochondria, and inhibition of import of precursor proteins was tested.

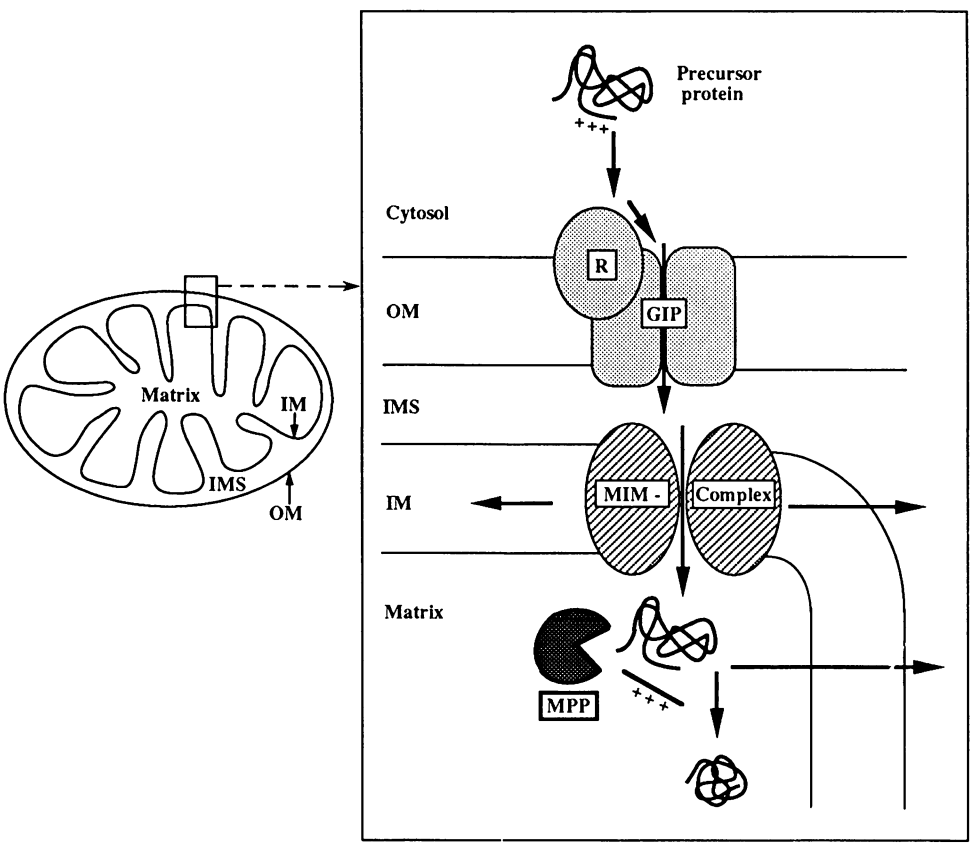

Fig. 1. Working model for the import of proteins into mitochondria
OM: Outer Membrane, IM: Inner Membrane, IMS: Intermembrane Space,
R: Receptor, GIP: General Insertion Pore, MIM-Complex: Inner Membrane Complex,
MPP: Mitochondrial Processing Peptidase

Antibodies directed against two outer membrane proteins of 19kDa and 72kDa, termed MOM19 and MOM72, were effective in inhibiting import. Antibodies to MOM19 inhibited import of most mitochondrial precursor proteins including all those containing cleavable presequences (Söllner et al., 1989). In contrast to this, antibodies to MOM72 strongly inhibited import of the AAC and only weakly inhibited import of other precursors (Söllner et al., 1990). These results suggested that MOM19 and MOM72 are mitochondrial protein import receptors of *Neurospora crassa*.

In yeast only one mitochondrial import receptor has been identified so far, named MAS70 or yeast-MOM72. Preincubation of yeast mitochondria with antibodies to this protein resulted in a decrease of the import of some precursor proteins (Hines et al., 1990). Since the MOM72 proteins of yeast and *Neurospora crassa* are homologous (32% identity, 46% similarity), they are supposed to fulfil similar functions in both organisms (Steger et al., 1990). A homolog of MOM19 in yeast has not yet been identified.

Following binding to the mitochondrial receptors MOM19 or MOM72, a precursor protein gets inserted into the outer membrane (Pfaller et al., 1988). At this step the pathways coming from the two different receptor proteins converge. This was demonstrated in competition experiments using several different precursor proteins. Precursors with different receptor specificity, like porin (MOM19) and AAC (MOM72), did not compete for binding to these receptors, as expected. But they were found to compete at a later stage of import, the embedding into the outer membrane (Pfaller et al., 1988). The corresponding activity was named General Insertion Pore (GIP). Beyond this step, import pathways were shown to diverge again depending on the final intramitochondrial localisation of the respective proteins.

3.3. A Protein Complex in the Mitochondrial Outer Membrane Contains Receptors and the General Insertion Pore

In an approach to identify components involved in binding and insertion of precursor proteins into the mitochondrial outer membrane, mitochondria from *Neurospora crassa* were lysed with the mild detergent digitonin and analysed for a protein complex containing the mitochondrial protein import receptors. A high molecular weight protein complex was found after fractionation by gel chromatography and subsequent coimmunoprecipitation with antibodies to MOM19. It contained the outer membrane proteins MOM19, MOM22, MOM38, and MOM72 (Kiebler et al., 1990). In another approach mitochondria were lysed with digitonin and directly subjected to immunoprecipitation using antibodies monospecific for MOM19. Several proteins were coimmunoprecipitated which were named according to their molecular weight: MOM7, MOM8, MOM19, MOM22, MOM30, MOM38, and MOM72 (Söllner et al., 1992) (see also figure 2). The proteins MOM19, MOM22, and MOM72 were very sensitive to treatment of mitochondria with proteases indicating that they were exposed at the mitochondrial surface and they have therefore been implicated in the

receptor function. MOM38 is resistant to protease treatment and behaves exactly as predicted for a GIP-component. Therefore MOM38 has been tentatively assigned to the GIP function (Kiebler et al., 1990).

The characterisation of the numerous components of the mitochondrial import apparatus was greatly facilitated by establishing techniques to generate translocation intermediates at various stages of the import pathway of precursor proteins. This was most extensively studied with the ADP-ATP-carrier (AAC), a protein of the mitochondrial inner membrane (Pfanner and Neupert, 1987).

Upon ATP-depletion and dissipation of the membrane potential, the AAC was not completely imported but instead remained bound to the surface of the mitochondria. If a membrane potential was regenerated and ATP added to the reaction, this bound precursor could be chased to complete its import and assembly into the inner membrane, showing that the arrested precursor was on the correct import pathway. The AAC arrested at this stage was crosslinked to MOM19 and MOM72 (Söllner et al., 1992), again indicating that MOM19 and MOM72 are mitochondrial import receptors. Additionally, the arrested AAC could be coimmunoprecipitated with antibodies to MOM72 showing a direct interaction between these two proteins.

In attempts to characterize the GIP-activity, the AAC was stalled at this stage by dissipating the membrane potential in the presence of ATP prior to addition of the protein. It could be crosslinked to several outer membrane proteins: MOM7, MOM8, MOM19, MOM30, MOM38 (Söllner et al., 1992). The crosslink products corresponding to MOM7, MOM8, and MOM30 were not generated with the AAC arrested at the receptor-bound stage, suggesting that they are related to the GIP-function. MOM7 and MOM8 were crosslinked with very high efficiency suggesting that they might be located very closely to the translocating polypeptide chain. Since, on the other hand, these two proteins are extractable at pH 11.5, indicating that they are associated with the outer membrane through interaction with other membrane proteins, they have been implied in being part of the putative translocation channel (Söllner et al., 1992).

A similar protein complex was found in yeast mitochondria (Moczko et al., 1992). It contains proteins tentatively named MOM7, MOM8, MOM19, MOM22, MOM30, MOM38, and MOM72 according to their putative *Neurospora*-counterparts. The MOM38 of yeast mitochondria (also termed ISP42 for Import Site Protein of 42kDa) was shown to be part of the translocation machinery of yeast mitochondria (Vestweber et al., 1989). A yeast mutant depleted of ISP42 is not viable indicating that this protein has a crucial function in mitochondrial protein import (Baker et al., 1990).

To summarize these data, the several components of the mitochondrial receptor complex can be assigned to two activities: receptor activity and GIP activity. MOM19 and MOM72 have been shown to be mitochondrial import receptors (Söllner et al., 1989; Söllner et al., 1990). MOM38 is believed to be a main component of the GIP function since it fulfils the criteria defined for the GIP-stage of protein import (Kiebler et al., 1990). MOM7 and

MOM8 may be part of the translocation channel in the outer membrane which would be functionally identical to GIP (Söllner et al., 1992). The roles of MOM22 and MOM30 are still being investigated. A tentative model of the mitochondrial receptor complex is shown in figure 2.

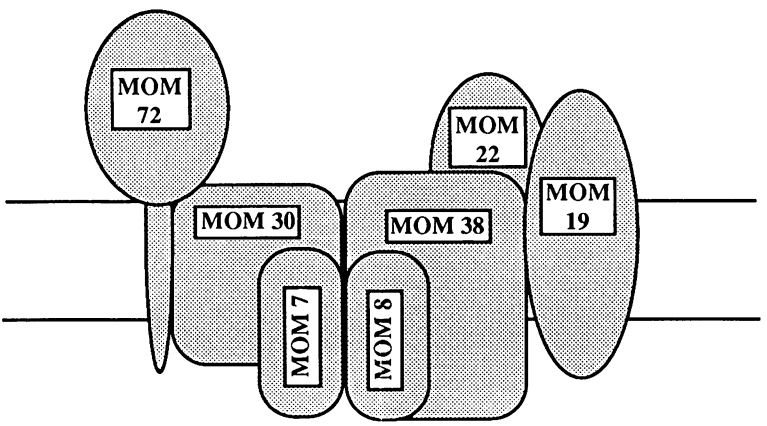

Fig. 2. Hypothetical working model for the mitochondrial receptor complex

3.4. The Inner Membrane Contains Independent Import Sites

Mitochondria from which the receptors have been removed by treatment with trypsin can import proteins only at a very low efficiency (Pfaller et al., 1989). Import into these mitochondria could be restored by removing the outer membrane (Ohba and Schatz, 1987). This showed that the mitochondrial inner membrane contains an independent translocation machinery. This inner membrane translocation machinery seems to be present in considerably higher numbers than the outer membrane machinery. This has been demonstrated by saturating the outer membrane import sites and the contact sites with two-membrane-spanning translocation intermediates which led to a complete block of the import of precursors into intact mitochondria. If, however, the outer membrane of these mitochondria was disrupted, import was restored (Hwang et al., 1989). This demonstrated that the inner membrane contains a fully functional import system which can operate independently of the outer membrane translocation machinery.

The existence of independent import sites in the inner membrane was also suggested by the following observations. In intact mitochondria, translocation intermediates were

characterized which were localized in such a way that the aminoterminus was already protruding into the matrix while the carboxyterminus was found to be exposed to the intermembrane space (Hwang et al., 1991; Rassow and Pfanner, 1991). The precursors arrested in this position could be chased to the fully imported forms showing that they were on the correct import pathway and that import could be accomplished by the inner membrane machinery alone. These experimental data strongly indicate that protein translocation is accomplished by two distinct yet cooperating protein translocation machineries in the outer and inner mitochondrial membrane rather than a continuous channel across both mitochondrial membranes (Glick et al., 1991; Pfanner et al., 1992).

This notion was also supported by *in vitro* experiments with membrane vesicles derived from the mitochondrial membranes. Inner membrane vesicles virtually free of outer membrane components imported several mitochondrial precursor proteins in an ATP- and membrane potential dependent manner (Hwang et al., 1989). Outer membrane vesicles which contained no detectable amount of inner membrane proteins were able to import proteins such as cytochrome c heme lyase, porin, MOM19, and MOM22 (A. Mayer, R. Lill, W. Neupert, in preparation). This again showed that both membranes contain protein translocation complexes which can function independently of each other.

3.5. Translocation Contact Sites are Dynamic Structures

Electron microscopic studies revealed that protein import takes place at the so-called contact sites (Kellems et al., 1975; Pfanner et al., 1990; Schwaiger et al., 1987). These are the regions of the mitochondrial envelope where the two membranes are in close proximity being only 2-8 nm apart. The parts of the inner membrane contained within these regions have been termed inner boundary membrane as opposed to the cristae which protrude into the matrix space. Around 90% of the outer membrane is adjacent to the inner boundary membrane in intact mitochondria (Pfanner et al., 1992; Van der Klei et al., submitted). Stable contacts between the two membranes have been demonstrated with mitochondria in which the matrix space was condensed due to hyperosmotic treatment (Hackenbrock, 1968). This caused a retraction of the inner membrane which stayed attached to the outer membrane only at these stable morphological contact sites which then comprised about 5-10% of the mitochondrial surface (Rassow et al., 1989).

Precursor proteins arrested during translocation in a two-membrane-spanning manner were demonstrated to be localized at or close to the morphological contact sites by immuno electron microscopy (Schleyer and Neupert, 1985; Schwaiger et al., 1987).

In apparent contrast, the receptor proteins were found to be distributed all over the mitochondrial outer membrane, although MOM72 was concentrated at morphological contact sites (Söllner et al., 1989). This suggests that the protein translocation machinery of mitochondria is dynamic in that the components are able to move laterally within the

membranes. The receptors and general insertion protein can assemble and disassemble again indicating a dynamic nature of the complexes (Kiebler et al., 1990). The translocation of proteins is facilitated by the proximity of the two membranes at the contact regions which ensures that a precursor protein can directly bind to an inner membrane complex upon emerging from the outer membrane import site. This is further facilitated by the higher number of the inner membrane complexes as compared to the outer membrane receptor complexes (Hwang et al., 1989; Pfanner et al., 1992).

In summary, protein import into the mitochondrial matrix appears to occur by successive translocation across the two mitochondrial membranes each of which contains a distinct import apparatus. Translocation can occur simultaneously across both membranes since an incoming polypeptide chain can directly be handed over to the inner membrane machinery upon emerging in the intermembrane space. This may be facilitated by the close proximity of the membranes and the higher number of inner membrane translocation complexes.

3.6. Mitochondrial Hsp70 is Essential for Translocation

Protein translocation into the matrix not only requires the electrical membrane potential, it is also dependent on mitochondrial hsp70, a heat shock protein of 70 kDa in the mitochondrial matrix. In a yeast mutant deficient of hsp70, import of precursor proteins into mitochondria was inhibited. In the mutant, precursors were arrested during translocation in a membrane-spanning manner (Kang et al., 1990). The block could be overcome by artificially unfolding the precursor protein in urea prior to import. The protein then was completely imported into the matrix but did not attain its native conformation. This suggested a dual role for hsp70 in import and folding of precursor proteins.

Hsp70 was shown to bind to incoming precursor proteins (Scherer et al., 1990). This binding keeps precursor proteins in an unfolded conformation. Release of the protein from hsp70 requires hydrolysis of ATP. Therefore, interaction with hsp70 was proposed to provide the driving force of mitochondrial protein import (Hartl et al., 1992). In addition, binding of hsp70 to segments of the precursor appearing on the matrix side may facilitate unfolding on the cytosolic side of the outer membrane (Neupert et al., 1990).

4. CYTOSOLIC FACTORS IN MITOCHONDRIAL PROTEIN IMPORT

How are precursor proteins targeted to the mitochondrial receptor complex? Precursors can only be recognized by the translocation machinery if the targeting sequence is exposed. But are the precursors, or their targeting sequences, directly recognized by the receptor proteins or are they targeted to the receptors by cytosolic factors analogous to the signal

recognition particle (SRP) of the endoplasmic reticulum (ER) or the SecB protein of bacteria?

The signal sequences of proteins destined to the ER are recognized by the SRP as soon as they emerge from the ribosome. Translation then may slow down and the precursor-SRP-complex is targeted to the ER membrane via recognition of the SRP receptor (docking protein) on the ER surface and donates the precursor protein to a translocation channel in the ER membrane whereas SRP is recycled (Walter et al., 1984; Meyer, 1991).

In bacteria such as *E. coli*, proteins have been identified that are able to keep precursor proteins in an import competent conformation *in vitro*, SecB, DnaK, and GroEL (Lecker et al., 1989). Proteins that are to be exported are recognized by SecB, a soluble cytoplasmic protein, or other components which target the precursor to the SecA protein, a peripheral protein of the export machinery of the plasma membrane. Translocation then involves the membrane proteins SecY, SecE, other membrane proteins, and lipids (Hartl et al., 1990; Wickner et al., 1991).

In mitochondria, however, little is known about the role of cytosolic factors in the import of precursor proteins. A number of cytosolic factors have been identified which stimulated import into isolated mitochondria, including cytosolic 70kDa-heat-shock proteins (hsp70s) (Deshaies et al., 1988b; Sheffield et al., 1990; Murakami et al., 1988a), a "presequence binding factor" (PBF) (Murakami et al., 1988b; Murakami and Mori, 1990) and a "cytosolic factor" (Ono and Tuboi, 1988). Two main functions for a putative cytosolic factor can be distinguished: a chaperone-like function which consists in preserving the import-competence of precursor proteins by preventing misfolding, and a targeting function which would direct the precursor protein to the target membrane, in this case the mitochondrial membrane. It is well established that cytosolic hsp70 proteins play an important role in the translocation of preproteins into mitochondria (and the ER) (Deshaies et al., 1988a; Murakami et al., 1988a). Much less is known about the targeting of precursor proteins to the mitochondria: are precursors directly recognized by the mitochondrial protein import receptors, or do the receptors recognize a "targeting factor" which binds to the precursors?

4.1. Unfolding of Precursors is Required for Import

With a number of protein translocation systems studied it was shown that unfolding is a prerequisite for translocation of most proteins (Randall and Hardy, 1986; Schleyer and Neupert, 1985; for a review see: Langer and Neupert, 1991).

Protein translocation into mitochondria also requires unfolding of the precursor proteins (Eilers and Schatz, 1986; Schleyer and Neupert, 1985). The unfolded conformation is stabilized by interaction with the cytosolic hsp70 protein and probably other factors, which probably binds to the precursor directly after synthesis (Nelson et al., 1992).

Conversely, a stable tertiary structure inhibits import of proteins into mitochondria. This was demonstrated *in vitro* by inducing a tightly folded tertiary structure to a precursor protein and showing that this precursor cannot be imported afterwards (Eilers and Schatz, 1986; Rassow et al., 1989). This is not due to an artificial situation in the *in vitro* system. When in intact yeast cells a protein containing DHFR (dihydrofolate reductase) was expressed in the presence of a folate analog, aminopterine (which induced tight folding of the protein) import was inhibited. The fusion protein was accumulated in a two-membrane-spanning manner. Upon removal of the aminopterine, the protein was unfolded and subsequently import was completed (Wienhues et al., 1991).

4.2. Targeting Sequences are Recognized by Mitochondrial Receptors

It has been stated before that the mitochondrial outer membrane protein MOM19 is a receptor for most mitochondrial precursor proteins, including all those carrying amino-terminal cleavable targeting sequences. This suggests that MOM19 might recognize the targeting sequence of the precursor proteins. To test this hypothesis we constructed a fusion protein consisting of the matrix targeting sequence of cytochrome b_2 fused to DHFR, a cytosolic protein. The construct was imported into mitochondria, as expected. Upon preincubation of the mitochondria with antibodies to MOM19, import of the fusion protein was almost completely inhibited showing that import occurred via MOM19 (Becker et al., 1992). This demonstrated that a cleavable presequence was sufficient for directing import of a fusion protein via MOM19 and suggests that MOM19 indeed recognizes the presequence of mitochondrial proteins.

4.3. Purification of Mitochondrial Precursor Proteins

For most of the experiments described above, precursor proteins were synthesized *in vitro* in a rabbit reticulocyte lysate in the presence of ^{35}S-methionine (Pelham and Jackson, 1976). These radiolabelled precursors were added to isolated mitochondria and their import into the organelles was analysed. Most characteristics of the mitochondrial import pathways known to date were established using this experimental system.

To be able to analyse if cytosolic targeting factors are essential for mitochondrial protein import we needed purified precursor proteins which were devoid of the cytosolic factors present in reticulocyte lysate. To achieve this we overexpressed in *E. coli* and purified several mitochondrial proteins.The proteins were produced as insoluble inclusion bodies in the bacteria. They were purified by several washing steps in the presence of detergent and solubilized in urea-containing buffer. The proteins were radiolabelled by adding ^{35}S-sulfate to the media for the bacteria during induction (Wienhues et al., 1992). A fusion protein consisting of the first 148 amino acids of cytochrome b_2 -including the complete matrix

targeting sequence- fused to the first half of the mature ß-subunit of the F_1-F_0-ATPase (pb_2(148)*) was chosen for the experiments described in the following section. This protein was produced in high amounts in the bacteria. It was purified to apparent homogeneity and solubilized in urea-containing buffer (Becker et al., 1992).

4.4. Import of a Purified Preprotein into Mitochondria is Independent of the Addition of a Cytosolic Signal Recognition Factor

The purified protein pb_2(148)* was imported into mitochondria independently of cytosolic hsp70 since it was present in an unfolded conformation due to its denaturation in urea. Import of the protein was dependent on the receptor MOM19 since antibodies to MOM19 inhibited import of the protein (Becker et al., 1992). Therefore this protein was used to test for the requirement for cytosolic targeting factors in mitochondrial protein import.

Mitochondria were washed in buffers containing up to 2M KCl to remove any attached cytosolic factors which might have been copurified during isolation of the mitochondria. These salt-washed mitochondria showed normal import activity.

The purified precursor protein pb_2(148)* was imported into the salt-washed mitochondria and showed the usual import characteristics: It was correctly processed in the matrix and the processed form was completely resistant to protease K added from outside showing that it had been completely imported. Preincubation of mitochondria with antibodies to MOM19 strongly inhibited the import of the protein showing that also in this case the import occurred via MOM19.

These results taken together suggest that MOM19 directly recognizes mitochondrial targeting sequences and that mitochondrial protein import *in vitro* is independent of the addition of cytosolic signal recognition factors (Becker et al., 1992). This, however, does not exclude that *in vivo* protein import into mitochondria can be mediated or enhanced by cytosolic factors which might increase the efficiency of targeting and translocation or prevent aggregation of precursor proteins.

5. ACKNOWLEDGMENTS

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6. REFERENCES

Arretz, M., Schneider, H., Wienhues, U. , and Neupert, W., 1991, Processing of mitochondrial precursor proteins, *Biomed. Biochim. Acta* 50: 403-412.

Attardi, G., 1981, Organisation and expression of the mammalian mitochondrial genome: a lesson in economy, *Trends Biochem. Sci.* 6:86-89, 100-103.
Attardi, G. and Schatz, G., 1988, Biogenesis of mitochondria, *Ann. Rev. Cell. Biol.* 4:289-333.
Baker, K.P., Schaniel, A., Vestweber, D. , and Schatz, G., 1990, A yeast mitochondrial outer membrane protein essential for protein import and cell viability, *Nature* 348:605-609.
Becker, K., Guiard, B., Rassow, J., Söllner, T. , and Pfanner, N., 1992, Targeting of a chemically pure preprotein to mitochondria does not require the addition of a cytosolic signal recognition factor, *J. Biol. Chem.* 267:5637-5643.
Deshaies, R.J., Koch, B.D. , and Schekman, R., 1988a, The role of stress proteins in membrane biogenesis, *Trends Biochem. Sci.* 13:384-388.
Deshaies, R.J., Koch, B.D., Werner-Washburne, M., Craig, E.A. , and Schekman, R., 1988b, A subfamily of stress proteins facilitates translocation of secretory and mitochondrial precursor polypeptides, *Nature* 332:800-805.
Eilers, M. and Schatz, G., 1986, Binding of a specific ligand inhibits import of a purified precursor protein into mitocondria, *Nature* 322:228-232.
Glick, B., Wachter, C. , and Schatz, G., 1991, Protein import into mitochondria: two systems acting in tandem?, *Trends Cell Biol.* 1:99-103.
Glick, B., Brandt, A., Cunningham, K., Müller, S., Hallberg, R.L. , and Schatz, G., 1992, Cytochromes c1 and b2 are sorted to the intermembrane space of yeast mitochondria by a stop-transfer mechanism, *Cell* 69:809-822.
Hackenbrock, C.R., 1968, Chemical and physical fixation of isolated mitochondria in low-energy and high-energy states, *Proc. Natl. Acad. Sci. USA* 61:598-605.
Hallermayer, G., Zimmermann, R. , and Neupert, W., 1977, Kinetic studies on the transport of cytoplasmically synthesized proteins into the mitochondria in intact cells of *Neurospora crassa*, *Eur. J. Biochem.* 81:523-532.
Hartl, F.-U., Schmidt, B., Wachter, E., Weiss, H. , and Neupert, W., 1986, Transport into mitochondria and intramitochondrial sorting of the Fe/S protein of ubiquinol-cytochrome c reductase, *Cell* 47:939-951.
Hartl, F.-U., Pfanner, N., Nicholson, D.W. , and Neupert, W., 1989, Mitochondrial protein import, *Biochim. Biophys. Acta* 988:1-45.
Hartl, F.-U. and Neupert, W., 1990, Protein sorting to mitochondria: evolutionary conservations of folding and assembly, *Science* 247:930-938.
Hartl, F.-U., Lecker, S., Schiebel, E., Hendrick, J.P. , and Wickner, W., 1990, The binding cascade of SecB to SecA to SecY/E mediates preprotein targeting to the E. coli plasma membrane, *Cell* 63:269-279.
Hartl, F.-U., Martin, J. , and Neupert, W., 1992, Protein folding in the cell: the role of molecular chaperones Hsp70 and Hsp60, *Ann. Rev. Biophys.* 21:293-322.
Hawlitschek, G., Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U. , and Neupert, W., 1988, Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein, *Cell* 53:795-806.
Hines, V., Brandt, A., Griffiths, G., Horstmann, H., Brütsch, H. , and Schatz, G., 1990, Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70, *EMBO J.* 9:3191-3200.
Hurt, E.C., Pesold-Hurt, B. , and Schatz, G., 1984, The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix, *EMBO J.* 3:3149-3156.
Hwang, S., Jascur, T., Vestweber, D., Pon, L. , and Schatz, G., 1989, Disrupted yeast mitochondria can import precursor proteins directly through their inner membrane, *J. Cell Biol.* 109:487-493.
Hwang, S.T., Wachter, C. , and Schatz, G., 1991, Protein import into the yeast mitochondrial matrix: a new translocational intermediate between the two mitochondrial membranes, *J. Biol. Chem.* 266:21083-21089.
Kalousek, F., Hendrick, J.P. , and Rosenberg, L.E., 1988, Two mitochondrial matrix proteases act sequentially in the processing of mammalian matrix enzymes, *Proc. Natl. Acad. Sci. USA* 85:7536-7540.
Kang, P.J., Ostermann, J., Shilling, J., Neupert, W., Craig, E.A. , and Pfanner, N., 1990, Requirement for hsp70 in the mitochondrial matrix for translocation and folding or precursor proteins, *Nature* 348:137-143.
Kellems, R.E., Allison, V.F. , and Butow, R.A., 1975, Cytoplasmic type 80S ribosomes associated with yeast mitochondria, *J. Cell Biol.* 65:1-14.
Kiebler, M., Pfaller, R., Söllner, T., Griffiths, G., Horstmann, H., Pfanner, N. , and Neupert, W., 1990, Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins, *Nature* 348:610-616.

Koll, H., Guiard, B., J., R., Ostermann, J., Horwich, A.L., Neupert, W. , and Hartl, F.-U., 1992, Antifolding activity of hsp60 couples protein import into the mitochondrial matrix with export to the intermembrane space, *Cell* 68:1163-1175.

Korb, H. and Neupert, W., 1978, Biogenesis of cytochrome c in *Neurospora crassa*, *Eur. J. Biochem.* 91:609-620.

Langer, T. and Neupert, W., 1991, Heatshock proteins hsp60 and hsp70: their roles in folding, assembly and membrane translocation of proteins, *Curr. Top. Microbiol. Immunol.* 160:3-30.

Lecker, S., Lill, R., Ziegelhoffer, T., Bassford, J.P.J., Kumamoto, C.A. , and Wickner, W., 1989, Three pure chaperone proteins of Escherichia coli - SecB, trigger factor and GroEL - form soluble complexes with precursor proteins in vitro, *EMBO J.* 8:2703-2709.

Lill, R., Hergersberg, C., Schneider, H., Söllner, T., Stuart, R. , and Neupert, W., 1992a, General and exceptional pathways of protein import into the sub-mitochondrial compartments, *in:* Membrane biogenesis and protein targeting, W. Neupert and R. Lill, ed., Elsevier Science Publishers.

Lill, R., Stuart, R.A., Drygas, M.E., Nargang, F.E. , and Neupert, W., 1992b, Import of cytochrome c heme lyase into mitochondria: a novel pathway into the intermembrane space, *EMBO J.* 11:449-456.

Maccecchini, M.-L., Rudin, Y. , and Schatz, G., 1979, Transport of proteins across the mitochondrial outer membrane: a precursor form of the cytoplasmically made intermembrane enzyme cytochrome c peroxidase, *J. Biol. Chem.* 254:7468-7471.

Mahlke, K., Pfanner, N., Martin, J., Horwich, A.L., Hartl, F.-U. , and Neupert, W., 1990, Sorting pathways of mitochondrial inner membrane proteins, *Eur. J. Biochem.* 192:551-555.

Margulis, L., 1970, Aerobiosis and the mitochondrion, *in:* Origin of eukaryotic cells, Yale University Press, New Haven and London.

Meyer, D.I., 1991, Protein translocation into the endoplasmic reticulum: a light at the end of the tunnel, *Trends Cell Biol.* 1:154-158.

Miller, B.R. and Cumsky, M.G., 1991, An unusual mitochondrial import pathway for the precursor to yeast cytochrome c oxidase subunit Va, *J. Cell. Biol.* 112:833-841.

Moczko, M., Dietmeier, K., Söllner, T., Segui, B., Steger, H.F., Neupert, W. , and Pfanner, N., 1992, Identification of the mitochondrial receptor complex in *Saccharomyces cerevisiae*, *FEBS Lett.* 310:265-268.

Murakami, H., Pain, D. , and Blobel, G., 1988a, 70-kD heat shock-related protein is one of at least two distinct cytosolic factors stimulating protein import into mitochondria, *J. Cell. Biol.* 107:2051-2057.

Murakami, K., Amaya, Y., Takigushi, M., Ebina, Y. , and Mori, M., 1988b, Reconstitution of mitochondrial protein transport with purified ornithine carbamoyltransferase precursor expressed in *Escherichia coli*, *J. Biol. Chem.* 263:18437-18442.

Murakami, K. and Mori, M., 1990, Purified presequence binding factor (PBF) forms an import-competent complex with a purified mitochondrial precursor protein, *EMBO J.* 9:3201-3208.

Nelson, R.J., Ziegelhoffer, T., Nicolet, C., Werner-Washburne, M. , and Craig, E.A., 1992, The translation machinery and 70kd heat schock protein cooperate in protein synthesis, *Cell* 71:97-105.

Neupert, W., Hartl, F.-U., Craig, E.A. , and Pfanner, N., 1990, How do polypeptides cross mitochondrial membranes?, *Cell* 63:447-450.

Ohba, M. and Schatz, G., 1987, Disruption of the outer membrane restores protein import to trypsin-treated yeast mitochondria, *EMBO J.* 6:2117-2122.

Ono, H. and Tuboi, S., 1988, The cytosolic factor required for import of precursors of mitochondrial proteins into mitochondria, *J. Biol. Chem.* 263:3188-3193.

Ostermann, J., Horwich, A.L., Neupert, W. , and Hartl, F.-U., 1989, Protein folding in mitochondria requires complex formation with hsp60 and ATP hydrolysis, *Nature* 341:125-130.

Pelham, H.R.B. and Jackson, R.J., 1976, An efficient mRNA-dependent translation system from reticulocyte lysates, *Eur. J. Biochem.* 67:247-256.

Pfaller, R., Steger, H.F., Rassow, J., Pfanner, N. , and Neupert, W., 1988, Import pathways of precursor proteins into mitochondria: multiple receptor sites are followed by a common insertion site, *J. Cell. Biol.* 107:2483-2490.

Pfaller, R., Pfanner, N. , and Neupert, W., 1989, Mitochondrial protein import: bypass of proteinaceous surface receptors can occur with low specificity and efficiency, *J. Biol. Chem.* 264:34-39.

Pfanner, N., Hoeben, P., Tropschug, M. , and Neupert, W., 1987, The carboxyterminal two-thirds of the ADP/ATP carrier polypeptide contains sufficient information to direct translocation into mitochondria, *J. Biol. Chem.* 262:14851-14854.

Pfanner, N. and Neupert, W., 1987, Distinct steps in the import of the ADP/ATP carrier into mitochondria, *J. Biol. Chem.* 262:7528-7536.

Pfanner, N., Rassow, J., Wienhues, U., Hergersberg, C., Söllner, T., Becker, K. , and Neupert, W., 1990, Contact sites between inner and outer membranes: structure and role in protein translocation into mitochondria, *Biochim. Biophys. Acta* 1018:239-242.

Pfanner, N., Rassow, J., van der Klei, I. , and Neupert, W., 1992, A dynamic model of the mitochondrial protein import machinery, *Cell* 68:999-1002.
Randall, L.L. and Hardy, S.J.S., 1986, Correlation of competence for export with lack of tertiary structure of the mature species: a study in vivo of maltose binding protein in E. coli, *Cell* 46:921-928.
Rassow, J., Guiard, B., Wienhues, U., Herzog, V., Hartl, F.-U. , and Neupert, W., 1989, Translocation arrest by reversible folding of a precursor protein imported into mitochondria. A means to quantitate translocation contact sites., *J. Cell. Biol.* 109:1421-1428.
Rassow, J., Hartl, F.-U., Guiard, B., Pfanner, N. , and Neupert, W., 1990, Polypetides traverse the mitochondrial envelope in an extended state, *FEBS Lett.* 275:190-194.
Rassow, J. and Pfanner, N., 1991, Mitochondrial preproteins en route from the outer membrane to the inner membrane are exposed to the intermembrane space, *FEBS Lett.* 293:85-88.
Schatz, G., 1979, How mitochondria import proteins from the cytoplasm, *FEBS Lett.* 103:203-211.
Scherer, P.E., Krieg, U.C., Hwang, S.T., Vestweber, D. , and Schatz, G., 1990, A precursor protein partly translocated into yeast mitochondria is bound to a 70 kD mitochondrial stress protein, *EMBO J.* 9:4315-4322.
Schleyer, M. and Neupert, W., 1985, Transport of proteins into mitochondria: translocational intermediates spanning contact sites between outer and inner membranes, *Cell* 43:339-350.
Schwaiger, M., Herzog, V. , and Neupert, W., 1987, Characterization of translocation contact sites involved in the import of mitochondrial proteins, *J. Cell. Biol.* 105:235-246.
Schwartz, R.M. and Dayhoff, M.O., 1978, Origins of prokaryotes, eukaryotes, mitochondria, and chloroplasts, *Science* 199:395-403.
Sheffield, W.P., Shore, G.C. , and Randall, S.K., 1990, Mitochondrial precursor protein. Effects of 70-kD heat shock protein on polypeptide folding, aggregation, and import competence, *J. Biol. Chem.* 265:11069-11076.
Söllner, T., Griffiths, G., Pfaller, R., Pfanner, N. , and Neupert, W., 1989, MOM19, an import receptor for mitochondrial precursor proteins, *Cell* 59:1061-1070.
Söllner, T., Pfaller, R., Griffiths, G., Pfanner, N. , and Neupert, W., 1990, A mitochondrial import receptor for the ATP/ADP carrier, *Cell* 62:107-115.
Söllner, T., Rassow, J., Wiedmann, M., Schlossmann, J., Keil, P., Neupert, W. , and Pfanner, N., 1992, Mapping of the protein import machinery in the mitochondrial outer membrane by crosslinking of translocation intermediates, *Nature* 355:84-87.
Steger, H.F., Söllner, T., Kiebler, M., Dietmeier, K.A., Pfaller, R., Trülzsch, K.S., Tropschug, M., Neupert, W. , and Pfanner, N., 1990, Import of ADP/ATP-carrier into mitochondria: two receptors act in parallel, *J. Cell Biol.* 111:2353-2363.
Stuart, R.A., Nicholson, D.W. , and Neupert, W., 1990, Early steps in mitochodrial protein import: receptor functions can be substituted by the membrane insertion activity of apocytochrome c, *Cell* 60:31-49.
Van der Klei, I., Veenhuis, M. , and Neupert, W., submitted, A morphological view on mitochondrial protein targeting.
Van Loon, A.P.G.M. and Schatz, G., 1987, Transport of proteins to the inner membrane: the "sorting" domain of the cytochrome c1 presequence is a stop-transfer sequence for the mitochondrial inner membrane, *EMBO J.* 6:2441-2448.
Vestweber, D., Brunner, J., Baker, A. , and Schatz, G., 1989, A 42K outer-membrane protein is a component of the yeast mitochondrial protein import site, *Nature* 341:205-209.
Von Heijne, G., 1986, Mitochondrial targeting sequences may form amphiphilic helices, *EMBO J.* 5:1335-1342.
Walter, P., Gilmore, R. , and Blobel, G., 1984, Protein translocation across the endoplasmic reticulum, *Cell* 38:5-8.
Wickner, W., Driessen, A.J.W. , and Hartl, F.-U., 1991, The enzymology of protein translocation across the Escherichia coli plasma membrane, *Ann. Rev. Biochem.* 60:101-124.
Wienhues, U., Becker, K., Schleyer, M., Guiard, B., Tropschug, M., Horwich, A.L., Pfanner, N. , and Neupert, W., 1991, Protein folding causes an arrest of preprotein translocation into mitochondria in vivo, *J. Cell. Biol.* 115:1601-1609.
Wienhues, U., Koll, H., Becker, K., Guiard, B. , and Hartl, F.-U., 1992, Protein targeting to mitochondria, *in:* A Practical Approach to Protein Targeting, IRL (Oxford University Press).