Transport of proteins into mitochondria

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Introduction

More than 90% of mitochondrial proteins are encoded by nuclear genes and are synthesized as precursor proteins on cytosolic polysomes [1]. The translocation of mitochondrial proteins from the cytosol to their functional destination in one of the four mitochondrial subcompartments (outer membrane, intermembrane space, inner membrane and matrix; Fig. 1) involves a complex series of steps. The field of mitochondrial protein import has undergone very rapid development in recent years. Most studies have used fungal mitochondria (the yeast *Saccharomyces cerevisiae* and *Neurospora crassa*) *in vitro* and *in vivo*. This development includes the characterization of properties of precursor proteins, the resolution of distinct translocation intermediates on the import pathways and the identification of components of the protein import machinery.

Specific recognition of mitochondrial precursor proteins

Mitochondrial precursor proteins contain positively charged targeting sequences. In many cases, targeting signals are found in N-terminal peptide extensions of about

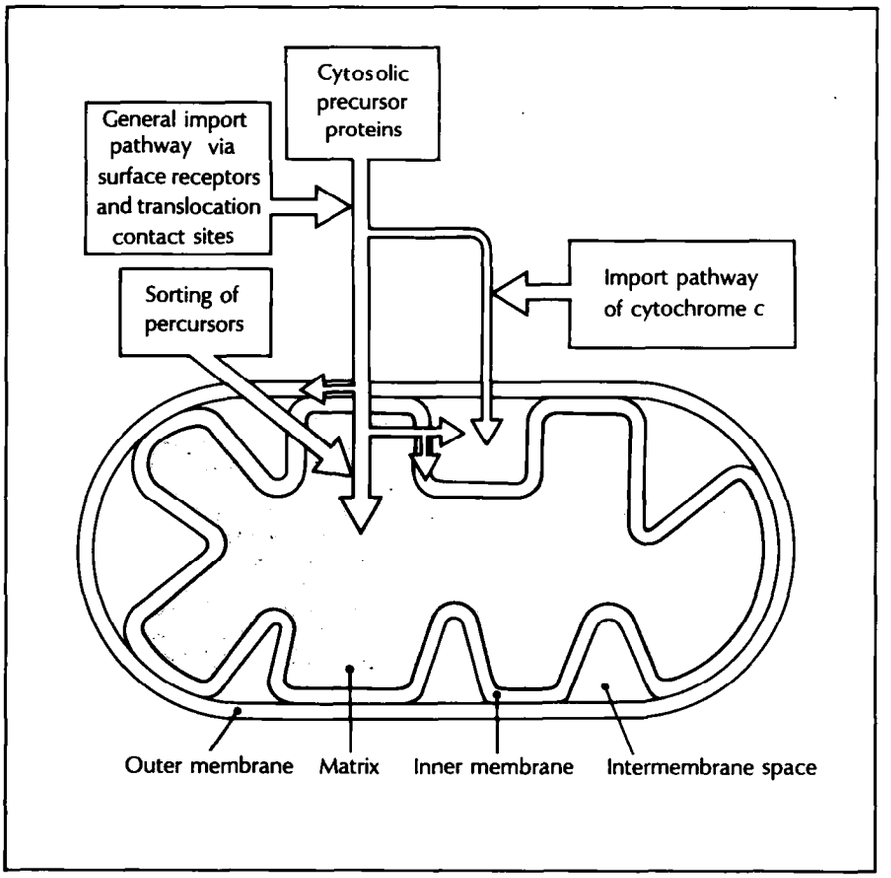

Fig. 1. Transport pathways of precursor proteins into mitochondria.

Abbreviations

GIP—general insertion protein; **hsp60**—heat-shock protein of 60 kD; **PEP**—processing enhancing protein.

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20–70 amino acid residues, termed presequences (Hurt and van Loon, *Trends Biochem Sci* 1986, 11:204–207) [2], which are cleaved off by specific peptidases inside the mitochondria. The main feature that mitochondrial targeting sequences have in common is the presence of positively charged amino acid residues and the nearly complete absence of negatively charged residues. In addition, targeting sequences may form amphiphilic structures under appropriate conditions [3]. So far, no particular amino acid sequence motif has been identified in the targeting peptides. This led to the suggestion that their secondary structure may have an important role in directing proteins into mitochondria. In several cases, the mature (non-cleavable) regions of precursor proteins may contribute to the specific high-affinity interaction of precursors with binding sites on the mitochondria, possibly via hydrophobic 'assistant sequences' [4].

Proteinaceous receptors on the mitochondrial surface are responsible for the specific recognition and high-affinity binding of precursor proteins [5,6] (Pfanner and Neupert, *J Biol Chem* 1987, 262:7528–7536). In the current working model, at least two distinct receptor proteins are required for the binding of the various precursor proteins (Fig. 2) [6]. High-affinity binding sites could be reconstituted into liposomes, providing an important tool for the purification of receptor sites [5]. Antibodies which are directed against 45 kD mitochondrial proteins inhibit import of precursor proteins, indicating that a 45 kD protein could be involved in translocation of precursors (Ohba and Schatz, *EMBO J* 1987, 6:2109–2115).

A detailed analysis of the role of the surface receptors gave rise to an explanation for a previously surprising result. Non-mitochondrial signals, such as chloroplast signal sequences, are able to direct proteins into mitochondria, albeit with a low efficiency (Hurt *et al., EMBO J* 1986, 5:1343–1350). It was observed that precursor proteins can bypass the proteinaceous surface receptors with a very low efficiency, i.e. can enter the mitochondrial import pathway at a later stage (Pfaller *et al., J Biol Chem* 1989, 264:34–39). This bypass transport appears to be the only pathway used by non-mitochondrial signals. The very low import rates make it likely that bypass import does not disturb the selectivity of mitochondrial protein uptake and the unique mitochondrial protein composition [7].

Translocation of precursors into and across the mitochondrial membranes

Precursor proteins with a stable tertiary structure cannot be inserted into mitochondrial membranes (Eilers and Schatz, *Nature* 1986, 322:228–232). Precursors are at least partially unfolded before, or during, membrane translocation. This can be clearly demonstrated by the reversible accumulation of precursors spanning both mitochondrial membranes. The N-terminal portion of the precursor enters the matrix space, whereas a (folded) C-terminal region remains in the cytosol (Schleyer and Neupert, *Cell* 1985, 43:339–350) [8]. Moreover, destabilization of the tertiary structure of precursor proteins ('unfolding'), e.g. by the introduction of point mutations, can increase the rates of import into mitochondria [9].

Import of mitochondrial precursor proteins requires hydrolysis of ATP (Pfanner and Neupert, *FEBS Lett* 1986, 209:152–156; for a summary, see [10]). ATP is involved in the generation or maintenance of a translocation-competent (loosely folded) precursor conformation. In particular, incompletely folded polypeptide chains require less ATP for import than their more strongly folded counterparts [11]. Translocation into the outer mitochondrial membrane of a chemically denaturated (unfolded) precursor protein is completely independent of ATP [12]. Since the specific import criteria, such as high-affinity binding to surface receptors, are fulfilled by the chemically unfolded precursor [5], the role of ATP seems to be (directly or indirectly) related to modulation of the conformation of precursors. Yeast mutants which are defective in a subset of stress proteins ('heat-shock proteins') of the 70 kD class have been shown to be deficient in import of some mitochondrial proteins [13]. Seventy-kilodalton stress proteins are generally thought to be involved in modification of the conformation of proteins in an ATP-dependent manner [14]. In fact, Murakami *et al.* [15] demonstrated that 70 kD stress proteins, and a further cytosolic component, stimulate transport of a precursor protein into mitochondria *in vitro*. The present working model includes the participation of 70 kD stress proteins, and possibly other factors, in the generation or maintenance of a transport-competent conformation of precursor proteins. It seems unlikely that cytosolic factors also have a role in other steps of mitochondrial protein import, such as targeting of precursors, since several precursors can be imported in the absence of any cytosolic factor [5,10].

The outer mitochondrial membrane contains apparently a common membrane insertion site used by all precursor proteins studied so far with the exception of cytochrome *c* (Fig. 2). This site, which is saturable with precursor proteins, is termed the 'general insertion protein' (GIP) [6]. Insertion into the outer membrane seems to require a high degree of unfolding of precursors and relatively high levels of ATP (Pfanner *et al., Cell* 1987, 49:815–823). After interaction with GIP, the precursors are inserted into the inner membrane. This step depends on the electrical potential across the inner membrane; the potential (negative inside) may exert an electrophoretic effect on the positively charged targeting sequences (Pfanner and Neupert, *EMBO J* 1985, 4:2819–2825) [3]. Butow and colleagues (Kellems *et al., J Cell Biol* 1975, 65:1–14) proposed that proteins are translocated at morphologically visible sites of close contact between mitochondrial outer and inner membranes. The experimental proof for translocation of precursor proteins via contact sites was provided by the demonstration of reversible trapping of precursors in contact sites (Schleyer and Neupert, 1985). Contact sites are stable structures which can be enriched in submitochondrial vesicles (Schwaiger *et al., J Cell Biol* 1987, 105:235–246). Precursor proteins which are inserted into contact sites are extracted from the membranes by pro-

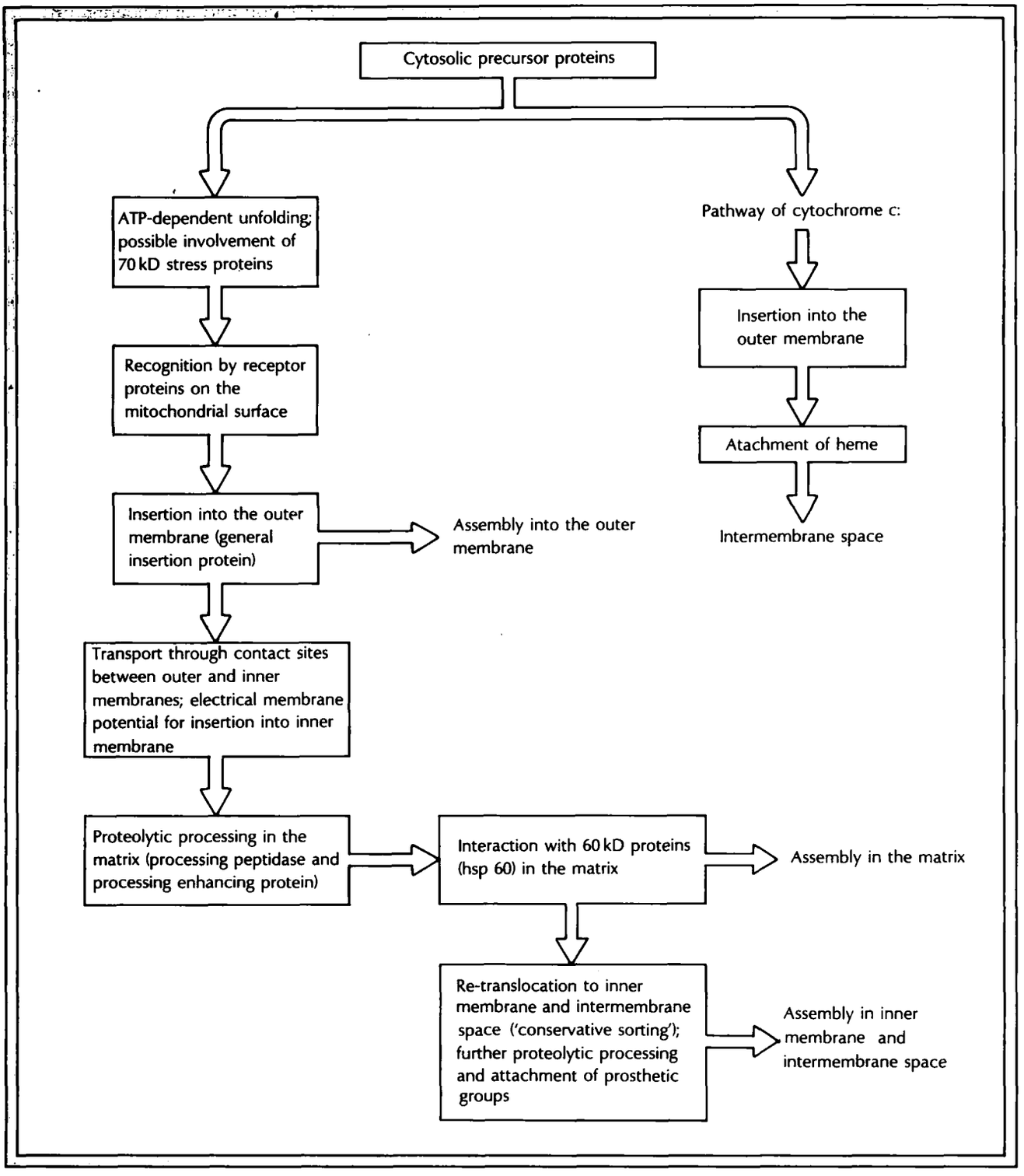

Fig. 2. Mechanisms and steps of mitochondrial protein import.

tein denaturants such as urea or at alkaline pH [16]. The contact sites can be saturated by membrane-spanning precursors [17]. In summary, proteinaceous components seem to be of great importance for both the structure and the function of contact sites.

Processing and folding of imported precursors

The positively charged targeting sequence (presequence) is proteolytically cleaved off by the processing peptidase in the mitochondrial matrix. The processing pep-

tidase was purified and was found to be a soluble protein of about 57 kD [18]. A protein of about 52 kD which is mainly associated with the inner mitochondrial membrane greatly enhances the processing activity; it is termed the 'processing enhancing protein' (PEP) [18]. However, the processing peptidase and PEP do not form a detectable complex. PEP may bind precursor proteins protruding into the matrix space and then present them to the processing peptidase. Two previously described yeast mutants which are defective in maturation of mitochondrial precursor proteins were found to be deficient in processing peptidase and PEP, respectively (Yaffe and Schatz, *Proc Natl Acad Sci USA* 1984, 81:4819–4823) [19,20]. The biochemical purification and characterization of these two components of the mitochondrial import machinery thus allowed the determination of the functional defect in the two mutants. Processing peptidase and PEP have significant homology in their primary sequences, suggesting that they may have originated from a common ancestor [20,21]. Several precursor proteins undergo a second proteolytic cleavage step either in the mitochondrial matrix or in the intermembrane space [1]. The proteases which perform these processing steps have not yet been identified.

Recently, a new procedure for the selection of yeast mutants affected in mitochondrial protein import was worked out by testing the functional assembly of a nuclear-encoded matrix protein [20]. Several new mutants were found in addition to the two mutants described above. One of the mutants is defective in a constitutively expressed stress protein ('heat-shock protein') of about 60 kD (hsp60) [22]. Hsp60 is homologous to the chloroplast ribulose 1,5-bisphosphate carboxylase (Rubisco) subunit-binding protein and the *Escherichia coli* heat-shock protein groEL [23] (Hemmingsen *et al., Nature* 1988, 333:330–334). These three proteins are named 'chaperonins', since they apparently ensure proper protein interaction during the assembly of oligomeric protein complexes. Hsp60 interacts with precursor proteins that are transported into the mitochondrial matrix; this step is a prerequisite for assembly of the precursors into protein complexes and for the further transport of precursors into, or across, the inner mitochondrial membrane (see below). Hsp60 may act by conferring a conformation on precursors which is required for assembly or intramitochondrial sorting [22]. The action of a stress protein in the mitochondrial matrix may represent a second ATP-dependent step in mitochondrial protein import, in addition to the ATP-dependent unfolding of cytosolic precursors.

Intramitochondrial sorting of precursors

Precursor proteins destined for the outer mitochondrial membrane or the mitochondrial matrix follow relatively simple sorting pathways (Fig. 1, 2). Outer membrane proteins interact with a specific receptor on the mitochondrial surface, insert into the membrane, with the help of GIP, and then become assembled in the outer membrane. Matrix proteins also interact with specific receptors and with GIP and are then translocated through contact sites into the matrix [1].

Precursor proteins which are destined for the intermembrane space or the inner membrane follow more elaborate sorting pathways (Figs. 1, 2). Most of them also use surface receptors, GIP and contact sites for transport into the matrix. After proteolytic processing and interaction with hsp60, the precursors are redirected into or across the inner membrane (Hartl *et al., Cell* 1986, 47:939–951) [24]. The mechanisms of retranslocation from the matrix space into or across the inner mitochondrial membrane resemble the transport mechanisms in prokaryotes, the ancestors of mitochondria. This led to the following model of intramitochondrial sorting ('conservative sorting') [24]. The mitochondrial genes that originally encoded mitochondrial proteins were transferred to the nucleus and acquired a segment coding for a positively charged presequence. The presequence directs the precursors through contact sites into the mitochondrial matrix. After the first proteolytic cleavage, which results in the removal of the positively charged targeting sequence, the precursor is released into its ancestral folding and assembly pathway. Several precursors carry a second targeting signal in the C-terminal half of the presequence. This relatively hydrophobic signal strongly resembles prokaryotic leader sequences which direct the export of prokaryotic proteins [1]. In fact, the second targeting signal seems to direct translocation of precursors across the inner mitochondrial membrane, and is cleaved off at the intermembrane space side of the membrane. Originally, it was proposed that the second (hydrophobic) signal sequence acts as a 'stop-transfer sequence' by preventing the further translocation of a precursor protein into the matrix (van Loon and Schatz, *EMBO J* 1987, 6:2441–2448); the precursor protein would then reach its location in the inner membrane or the intermembrane space by lateral diffusion in the inner membrane. As discussed above, this model does not apply to the sorting pathways of precursor proteins studied so far. However, the possibility that some precursors of integral inner membrane proteins may follow such a pathway cannot be excluded.

Cytochrome *c*, a protein of the intermembrane space, uses an import pathway which is quite distinct from the pathways of the other mitochondrial precursor proteins. The precursor protein, apocytochrome *c*, can insert into the outer mitochondrial membrane without the aid of surface receptors or GIP [6,25]. Cytochrome *c* heme lyase, a protein of the intermembrane space which is responsible for the covalent addition of heme to the apoprotein, seems to have a crucial role in the translocation of cytochrome *c* into the intermembrane space. Mutants which are defective in cytochrome *c* heme lyase are deficient in binding and import of cytochrome *c* [26] (Dumont *et al., EMBO J* 1987, 6:235–241). Apocytochrome *c* may spontaneously insert into the outer membrane and binds with high affinity, forming a complex which includes heme lyase. After addition of heme, the holoprotein is released into the intermembrane space [25]. C-terminal portions of the precursor protein are ap-

parently important for targeting of apocytochrome *c* to mitochondria (Stuart *et al.*, *EMBO J* 1987, 6:2131–2137).

Why are several (often very hydrophobic) proteins synthesized within mitochondria, although most mitochondrial proteins are encoded by nuclear genes? One possible explanation is that such hydrophobic proteins cannot be translocated through the cytosol and the two mitochondrial membranes, or that they would not be able to assume the correct orientation and conformation for assembly when imported from outside the organelle. Nagley *et al.* [27] excluded these possibilities using an elegant approach. They replaced the mitochondrial gene for a membrane protein by an artificial nuclear gene that also contained a segment encoding a presequence. The protein was successfully imported into mitochondria and functionally assembled into a multi-subunit complex. Hence, the most likely reason for the lack of transfer of the remaining mitochondrial genes to the nucleus appears to be the divergence of the genetic code.

Conclusions

Rapid progress in the field of mitochondrial protein import led to the identification of several components of the import machinery by biochemical and genetic approaches. The generation and characterization of defined translocation intermediates on the import pathways of precursor proteins opens the way for the identification of more components. The conformation of precursor proteins is important for membrane translocation and assembly of proteins. Elucidation of the functional and structural properties of mitochondrial precursor proteins and of the transport components is not only essential for the understanding of mitochondrial biogenesis, but is also important for the unravelling of intracellular protein transport and biogenesis of cell organelles in general.

Acknowledgements

We thank R.A. Stuart for critical reading of the manuscript.

Annotated references and recommended reading

- • Of interest
- •• Of outstanding interest

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A net positive charge is essential for the function of this presequence, as is the case for most mitochondrial targeting sequences. With this precursor protein, the midportion of the presequence is sufficient for targeting, whereas in many other presequences the N-terminal half carries sufficient targeting information.

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18. •• HAWLITSCHEK G, SCHNEIDER H, SCHMIDT B, TROPSCHUG M, HARTL F-U, NEUPERT W: **Mitochondrial protein import: identification of processing peptidase and of PEP, a processing enhancing protein.** *Cell* 1988, 53:795–806.
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19. • YANG M, JENSEN RE, YAFFE MP, OPPLIGER W, SCHATZ G: **Import of proteins into yeast mitochondria: the purified matrix processing protease contains two subunits which are encoded by the nuclear *MAS1* and *MAS2* genes.** *EMBO J* 1988, 7:3857–3862.
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