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Minireview

The Mitochondrial Protein Import Pathway: Are Precursors Imported through Membrane Channels?

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Mitochondrial biogenesis requires the import of hundreds of different proteins from the cytosol. Protein import into mitochondria is a multistep pathway that includes recognition of precursor proteins by machinery both in the cytoplasm and on the mitochondrial surface, translocation of the precursor across one or both mitochondrial membranes, and folding of the protein after its import into the organelle. Over the past several years, many components of the import machinery have been identified using both biochemical and genetic methods. Recently, significant progress has been made determining the function of some of these import proteins. One purpose of this minireview is to summarize our current understanding of the import pathway, and to introduce the topics of the minireviews that will follow. The other goal of this minireview is to discuss recent findings suggesting that proteins are translocated across both the mitochondrial inner and outer membranes through aqueous channels.

KEY WORDS: Mitochondria; MCC; PSC; import pathway; membrane channels.

INTRODUCTION

In this minireview series, our aim is to summarize current views about the mitochondrial protein import pathway. Over the past ten to fifteen years, more than 25 different proteins have been identified that play specific roles in import. Some of these proteins are critical for the recognition and targeting of cytoplasmically-synthesized precursor polypeptides. Other proteins mediate the translocation of precursors across either the mitochondrial inner or outer membranes. Still others orchestrate the folding and assembly of proteins after their import into the organelle. Since much of the import machinery (but by no means all) has been identified, the current focus of the field is to determine the mechanism of each step in the import pathway.

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In this introductory article our purpose is threefold. First, we will briefly outline the different steps in the import pathway and point out the specific proteins that mediate these steps. Second, we will introduce the topics of the following minireviews. Third, we will summarize recent evidence suggesting that proteins are translocated across the mitochondrial membranes through aqueous channels.

THE MITOCHONDRIAL OUTER MEMBRANE CONTAINS A MULTI-SUBUNIT IMPORT COMPLEX

Virtually all mitochondrial proteins are encoded on nuclear genes, translated in the cytosol, and then imported into the organelle. The targeting information for most of these imported mitochondrial proteins is carried on amino-terminal presequences (Horwich *et al.*, 1985; Hurt *et al.*, 1984, 1985). Although the primary amino acid sequence varies, presequences are thought to share a common three-dimensional structure: a positively-charged, amphipathic structure (Endo *et al.*, 1989; Roise *et al.*, 1986, 1988). It has been

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proposed that presequences are recognized by receptor proteins on the mitochondrial surface. In the yeast *Saccharomyces cerevisiae,* the putative import receptor is composed of at least four outer membrane (OM) proteins called Tom20p, Tom22p, Tom37p, and Tom70p (see Fig. 1; Gratzer *et al.,* 1995; Hase *et al.,* 1983; Hines *et al.,* 1990; Lithgow *et al.,* 1994; Moczko *et al.,* 1993, 1994; Ramage *et al.,* 1993). Similar proteins have been identified in *Neurospora crassa* (Kiebler *et al.,* 1993; Söllner *et al.,* 1989, 1990), and also recently in humans (Goping *et al.,* 1995; Hanson *et al.,* 1996; Seki *et al.,* 1995). As discussed in the minireview by V. Haucke and T. Lithgow, part of the interaction between the presequence and the receptor is thought to be electrostatic. In particular, Tom20p and Tom22p contain domains facing the cytosol that are rich in acidic amino acid residues, ideal to interact with the positively-charged presequence (Bolliger *et al.,* 1995; Haake *et al.,* 1995; Haucke *et al.,* 1995). It is important to note, however, that convincing binding studies using purified precursors (or presequence peptides) and the putative receptors are presently lacking. Hence alternative functions for the OM import machinery are discussed in the minireview by D. Roise.

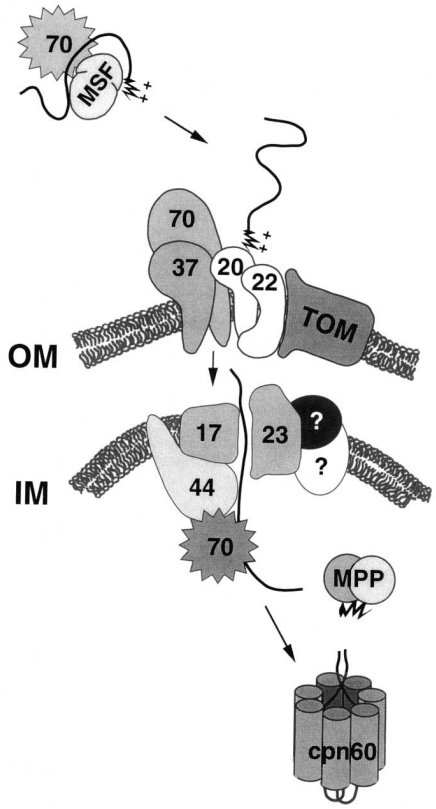

Fig. 1. Model of the mitochondrial protein import machinery. Most imported mitochondrial proteins carry positively-charged presequence at the amino terminus. Cytosolic chaperones, such as the Hsp70 or the MSF proteins, prevent misfolding of the precursor prior to import. The precursor interacts with a multi-subunit receptor on the surface of the outer membrane, which includes the Tom20, Tom22, Tom37, and Tom70 proteins (*T*ranslocase of *o*uter *m*embrane proteins of 20, 22, 37, and 70 kDa, respectively). Import across the mitochondrial outer membrane is mediated by a TOM complex consisting of at least five proteins (Tom40, Tom30, Tom8, Tom7, and Tom6). Translocation across the inner membrane is mediated by the Tim44, Tim23, and Tim17 proteins (*T*ranslocase of *i*nner *m*embrane proteins of 44, 23, and 17 kDa, repectively), with the help of inner membrane proteins not yet identified (indicated as '?'). The mitochondrial matrix Hsp70 protein interacts with Tim44p (and other chaperones, not shown) to "pull" precursors through the inner and outer translocation machinery. The mitochondrial presequence is cleaved off by a two-subunit processing protease (MPP). Folding of the imported protein is mediated by chaperonin 60 (cpn60), with the help of additional chaperonins (not shown). OM, outer membrane; IM, inner membrane.

Studies using isolated mitochondria have shown that import can occur after the complete synthesis of the precursor protein. As diagrammed in Fig. 1, two classes of cytoplasmic chaperones maintain newly-synthesized precursor proteins in a loosely-folded and import-competent configuration (Komiya *et al.,* 1996): members of the cytosolic Hsp70 family of heat-shock proteins (Deshaies *et al.,* 1988; H. Murakami *et al.,* 1988; K. Murakami *et al.,* 1988), and the recently-discovered mitochondrial stimulation factor (MSF; Alam *et al.,* 1994; Hachiya *et al.,* 1994; Komiya *et al.,* 1994). MSF in particular has been shown to have both a presequence and an ATP-dependent unfolding activity. Furthermore, MSF targets the bound precursor to specific proteins (Tom37p and Tom70p) on the mitochondrial surface. Chaperones also play important roles at later steps in the import pathway (see Fig. 1). For example, a matrix-localized Hsp70 protein (mt-Hsp70) is proposed to drive the translocation of precursors across the mitochondrial membranes (see the minireview by D. Cyr; Kronidou *et al.,* 1994; Kubrich *et al.,* 1994; Rassow *et al.,* 1994; Schneider *et al.,* 1994; Ungermann *et al.,* 1996). In addition, the role of mt-Hsp70 and other chaperones, such as cpn60, in the folding and assembly of proteins after their import is discussed in the minireview by J. Martin.

A multi-subunit complex of proteins in the outer membrane, called the TOM complex (Fig. 1), has been identified by genetics, chemical crosslinking, and by coimmunoprecipitation (Baker *et al.,* 1990; Kassenbrock *et al.,* 1993; Kiebler *et al.,* 1990, 1993; Moczko *et al.,* 1992; Söllner *et al.,* 1992; Vestweber *et al.,* 1989). The TOM complex contains at least five pro-

teins (Tom40p, Tom30p, Tom8p, Tom7p, and Tom6p) and is proposed to mediate the translocation of precursors across the outer membrane (see the minireview by V. Haucke and T. Lithgow). The Tom20 and Tom22 proteins are tightly associated with the TOM complex, whereas Tom37p and Tom70p appear to be only be weakly associated (Gratzer *et al.*, 1995; Honlinger *et al.*, 1995; Kiebler *et al.*, 1990, 1993; Moczko *et al.*, 1992; Ramage *et al.*, 1993). Recently, translocation across the mitochondrial outer membrane has been reconstituted using purified OM vesicles (Mayer *et al.*, 1995). The presequence appears to interact sequentially with two sites on the OM vesicles in an ATP-independent manner: a *cis* site, which is sensitive to protease digestion, and a *trans* site on the inner face of the OM. It is thought that the cytosolic domains of the Tom20 and Tom22 proteins constitute the *cis* site. The composition of the *trans* site is not yet clear (Bolliger *et al.*, 1995; Nakai *et al.*, 1995).

THE INNER MEMBRANE CARRIES A COMPLETE IMPORT MACHINE

If the outer membrane is removed from mitochondria using detergents or by osmotic shock, the resulting mitoplasts remain competent for import (Hwang *et al.*, 1989; Ohba and Schatz, 1987; Schwaiger *et al.*, 1987). Therefore, the inner membrane (IM) must have machinery to both recognize precursor proteins and to translocate them across the IM. Three inner membrane proteins have been identified and shown to be essential for import (see Fig. 1): Tim44p (Blom *et al.*, 1993; Maarse *et al.*, 1992; Scherer *et al.*, 1992), Tim23p (Dekker *et al.*, 1993; Emtage and Jensen, 1993), and Tim17p (Maarse *et al.*, 1994; Ryan and Jensen, 1994). All three proteins were first identified as yeast mutants defective in import, and all were later shown to be essential for cell viability. Tim44p (Kubrich *et al.*, 1994; Scherer *et al.*, 1992), Tim23p (Kubrich *et al.*, 1994; Ryan and Jensen, 1993), and Tim17p (Kubrich *et al.*, 1994) can each be chemically crosslinked to a precursor arrested in transit across the IM, indicating that all three proteins are in close proximity to the translocating polypeptide chain. Tim23p has a hydrophilic amino-terminal region followed by four putative membrane spans. Recent studies have shown that the N-terminal domain faces the intermembrane space and is essential for Tim23p function (Emtage and Jensen, submitted; Ryan *et al.*, submitted). Since this domain contains several clusters of acidic amino acids, the N-terminus has been proposed to function as a presequence receptor on the IM surface (Emtage and Jensen, 1993). Tim17p lacks the N-terminal "head" domain of Tim23p, but has a homologous set of four transmembrane spans. Based on their homology and hydrophobicity, Tim23p and Tim17p have also been proposed to function as subunits of a protein-translocating channel (Kubrich *et al.*, 1994; Ryan and Jensen, 1994).

Tim44p is a peripheral membrane protein and has been shown to interact with the mitochondrial Hsp70 protein, mt-Hsp70p. As discussed in the minireview by D. Cyr, mt-Hsp70 and the inner membrane potential drive the translocation of the precursor into the matrix (Blom *et al.*, 1995; Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994; Ungermann *et al.*, 1994, 1996). Two models have been proposed to explain how mt-Hsp70 mediates translocation (Glick, 1995). First, multiple binding of mt-Hsp70p to a precursor entering the matrix may prevent "backwards" diffusion. Alternatively, mt-Hsp70 may actively "pull" the precursor into the matrix by binding to the precursor and to the membrane-bound Tim44 protein.

Both genetic and biochemical evidence suggest that the inner membrane import components form functional complexes. For example, overexpression of Tim17p rescues a temperature-sensitive lethal *tim23* mutant (Ryan and Jensen, 1994), and *tim17*, *tim23*, and *tim44* mutants display synthetic lethality in genetic crosses (Blom *et al.*, 1995). Furthermore, Tim23p and Tim17p cofractionate after detergent solubilization of the mitochondrial inner membrane (Berthold *et al.*, 1995; Blom *et al.*, 1995; Ryan *et al.*, submitted), and can be chemically crosslinked to each other (Ryan *et al.*, submitted). Coimmunoprecipitation studies have also identified potential new members of the inner membrane import machinery. Using antibodies to Tim23p, one study coprecipitated proteins of 30 kDa and 14 kDa (Berthold *et al.*, 1995), whereas two other studies identified a 55-kDa and a 20-kDa protein (Blom *et al.*, 1995; Ryan *et al.*, submitted). The exact composition of the IM import machinery awaits further analysis. It appears, however, that a single large translocation complex forms during import across the mitochondrial inner membrane. In particular, when a mitochondrial precursor was arrested spanning the inner membrane, a large complex containing Tim44p, Tim23p, Tim17p, and mt-Hsp70 (and presumably other proteins) coimmunoprecipitated with the precursor (Berthold *et al.*, 1995).

BOTH MITOCHONDRIAL MEMBRANES HAVE CHANNEL ACTIVITIES CORRELATED WITH PROTEIN IMPORT

A critical step in sorting proteins to specific cellular compartments is the translocation of the polypeptide across the organelle membrane. To circumvent the problems associated with inserting charged residues into the hydrophobic environment of the lipid bilayer, it has been proposed that proteins are transported across organelle membranes through an aqueous pore or channel (Blobel and Dobberstein, 1975). Over the last several years studies using the tools of biophysics and electrophysiology have indicated that proteins are translocated across the endoplasmic reticulum through aqueous channels or pores. For example, fluorescent probes have been incorporated into the signal sequence of a protein during its synthesis (Crowley *et al.*, 1993, 1994). When the nascent chain was arrested during its translocation into the endoplasmic reticulum, measurements of the fluorescence lifetimes of the nascent chain–ribosome–membrane complex revealed that the probes were in an aqueous environment rather than buried in the nonpolar environment of the membrane. Using electrophysiological techniques, Simon *et al.* (Simon and Blobel, 1991) fused rough microsomes to one side of a planar lipid bilayer separating two aqueous compartments. Addition of puromycin to one compartment caused a large increase in membrane conductance and channels of 220 picosiemens (pS) were observed. Puromycin apparently induced the clearance of nascent protein chains from the lumen of protein-conducting channels in the lipid bilayer. Similar work also indicates that proteins are secreted from bacteria via aqueous channels (Simon and Blobel, 1992).

Both mitochondrial membranes contain a number of channel activities. The activities of two of these channels, one in the inner membrane, MCC, and one in the outer membrane, PSC, have been implicated in mitochondrial protein import. MCC (multiple conductance channel or mitochondrial megachannel) is a channel with large conductance (>1000 pS) found in the mitochondrial inner membrane of both mammals (Kinnally *et al.*, 1989, 1995, 1996; Zoratti and Szabó, 1995) and yeast (Lohret and Kinnally, 1995a). In patch-clamp studies the activity of MCC was shown to be affected by mitochondrial presequence peptides (Fig. 2; Lohret and Kinnally, 1995b). Presequence peptides cause a momentary blockage of conductance through MCC (a flicker blockade) that is reversible and dose-dependent. The flickering between open and substate conformations seen in MCC upon addition of the peptide is presumed to be the transient occlusion of the channel during the translocation of the presequence peptide. Recently, we have found that antibodies to the inner membrane import protein, Tim23p, completely block MCC activity (Lohret *et al.*, submitted). Neither peptides nor ions are conducted through MCC in the presence of Tim23p antibodies. Furthermore, we found that normal activity of MCC prepared from the temperature-sensitive *tim23-1* mutant is dramatically altered: MCC no longer responded to the presequence peptide, but ion conductance was normal (Lohret *et al.*, submitted). It appears that the *tim23-1* mutation (a glycine-to-aspartate change in the third putative transmembrane segment of Tim23p) specifically blocks the translocation of the peptide through the MCC channel. Our results indicate that Tim23p is required for MCC activity, and suggest that proteins are translocated across the mitochondrial inner membrane through the MCC channel.

MCC has several properties consistent with its action as an import channel in the mitochondrial inner membrane. For example, MCC has a pore size estimated to be 2.8 nm (Kinnally *et al.*, 1996), which is sufficiently large to allow the passage of precursor proteins. In addition, MCC appears to translocate peptides in a potential-dependent direction: from positive to negative environments (Lohret and Kinnally, 1995b). Since the mitochondrial matrix is electronegative, an early step in the translocation of full-length precursor proteins across the inner membrane may be the passage of the amino-terminal presequence through MCC.

In the outer membrane, a channel activity called PSC (peptide sensitive channel) has also been implicated in mitochondrial protein import (Henry *et al.*, 1996; Juin *et al.*, 1995; Thieffry *et al.*, 1988, 1992). Like MCC, PSC has a large conductance (>1000 pS) and this conductance is transiently blocked by presequence peptides. In preliminary experiments, PSC can be removed from detergent-solubilized outer membrane preparations using antibodies to the outer membrane preparations using antibodies to the outer membrane import protein, Tom40p (discussed in Henry *et al.*, 1996). Thus, like MCC in the inner membrane, PSC may mediate the translocation of precursors across the outer membrane.

The above electrophysiology studies linking membrane channels to mitochondrial protein import are tantalizing, but nonetheless preliminary. However, at least three additional observations support the view that precursors cross the mitochondrial membranes

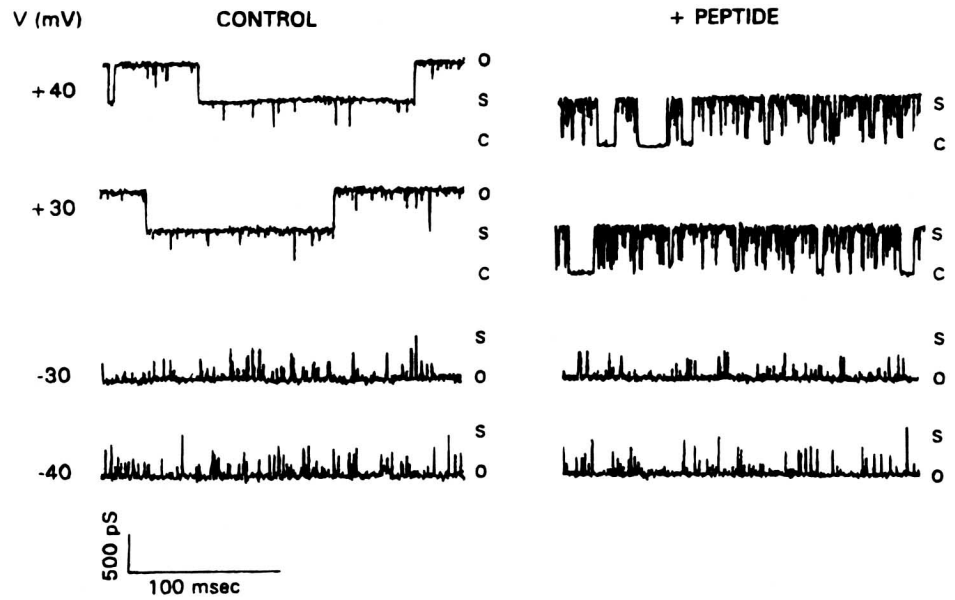

Fig. 2. MCC, a channel activity in the mitochondrial inner membrane channel, may be part of the protein import machinery. Presequence peptides transiently block the conductance through MCC. A typical current trace of MCC activity from patches excised from proteoliposomes prepared from mitochondrial inner membrane proteins as described (Lohret *et al.*, 1995) either in the absence (left panel) or presence (right panel) of 50 μM peptide based on the first 13 amino acids of the cytochrome oxidase subunit IV precursor. O, S, and C correspond to the open, substate, and closed conductance levels.

through protein-translocating channels. First, imported proteins arrested in transit across the mitochondrial membranes can be extracted from the mitochondrial import machinery by alkaline pH and urea (Pfanner *et al.*, 1987). Aqueous perturbants would not be expected to remove precursors embedded directly in the lipid bilayer. Second, the translocation of precursors into isolated OM vesicles is reversible (Mayer *et al.*, 1995). In particular, cleavage of the presequence that was translocated across the OM and bound to the *trans* site (by a processing protease enclosed in the vesicle) allowed the mature domain to slide out of the OM vesicle and into the supernatant. Third, inhibition of mt-hsp70 function blocks the unidirectional translocation of precursors into the matrix, allowing the precursor to backslide out of the IM import machinery (Ungermann *et al.*, 1994). Thus, translocation of the precursor across the IM (and the OM) is reversible and appears to occur through passive channels (Fig. 3).

FUTURE DIRECTIONS

While channels may solve many of the thermodynamic problems with protein translocation across the mitochondrial membranes, protein-translocating channels raise other problems. For example, since the mitochondrial inner membrane must maintain an electrochemical gradient for the synthesis of ATP, how does an import channel permit the passage of proteins without causing a leak in the membrane potential? In preliminary experiments, we find that MCC may be regulated by the presequence (Campo *et al.*, 1996). MCC is quiescent at membrane potentials found in active mitochondria (e.g., 150 mV), but MCC activity is induced at these voltages by presequence peptides. Therefore, MCC would not dissipate the electrochemical potential of the inner membrane in the absence of protein import. How MCC is regulated at the molecular level is not known. Future studies will reveal whether MCC is a gated channel, or whether the import machinery normally assembles around the precursor during import, thus forming the channel *de novo*.

How do mitochondrial channels sort membrane proteins? Recent studies indicate that the same translocation machinery in the endoplasmic reticulum is used by both secreted and membrane proteins (Do *et al.*, 1996; High *et al.*, 1993). It is proposed that a hydrophobic transmembrane segment causes the channel subunits to separate and thus allow lateral diffusion of

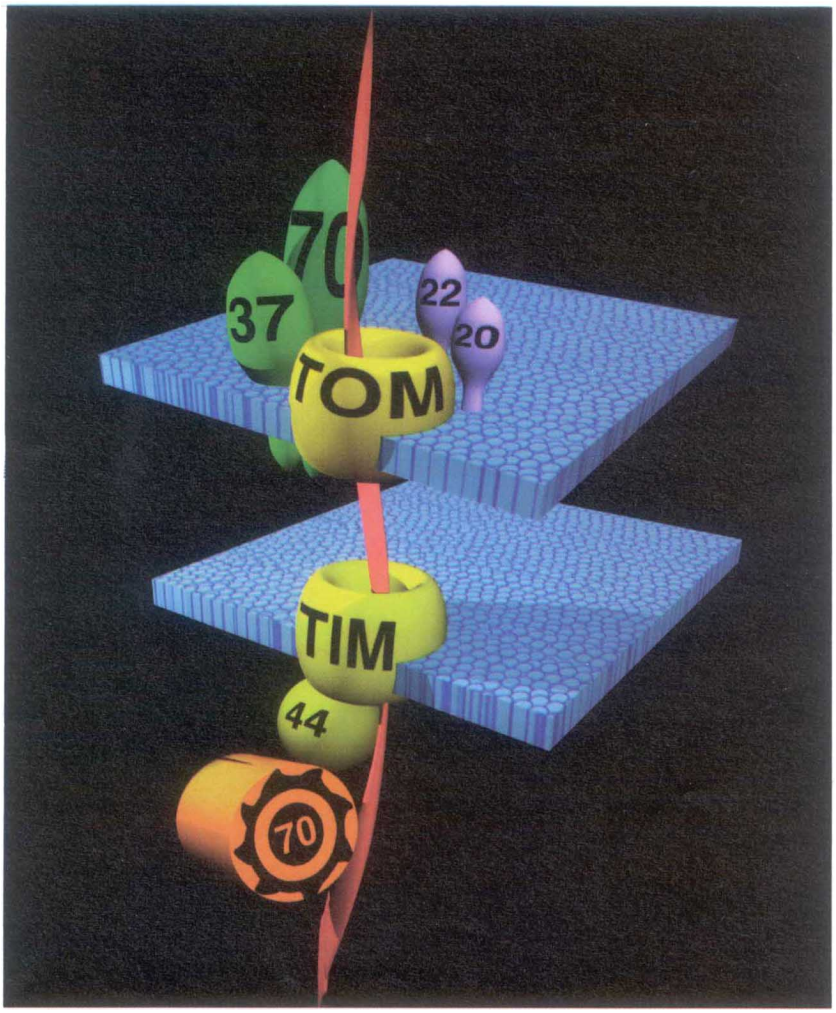

Fig. 3. Are precursors translocated across the mitochondrial membranes through aqueous channels? This diagram summarizes recent electrophysiology studies that suggest that channel activities in both the inner and outer membrane may participate in mitochondrial protein import. Multi-subunit complexes of proteins in the outer membrane (TOM) and in the inner membrane (TIM) are proposed to form aqueous channels that allow passage of the precursor (shown in red) into the matrix. In this model, the outer membrane Tom20, Tom22, Tom37, and Tom70 proteins function as receptors for the cytoplasmically-synthesized precursor protein, and direct the precursor into the TOM channel. The Tim44 and mt-Hsp70 proteins then "pull" the precursor through both channels into the matrix.

the polypeptide into the bilayer. It is possible that mitochondrial channels will similarly discriminate between proteins destined for the matrix or for the inner and outer membranes. However, we have recently found that several polytopic IM proteins do not require the inner membrane Tim23 protein for their import (Emtage and Jensen, submitted). If Tim23p is a subunit of the inner membrane channel, then our results raise the possibility that some proteins do not use this channel for their import. Some proteins destined for the IM may have a different import pathway (and use different machinery) than that used by proteins imported into the matrix.

While the above observations raise the exciting possibility that precursors are translocated across both mitochondrial membranes through aqueous channels (see Fig. 3), final proof awaits future studies. For example, by engineering fluorescent groups onto specific

residues of a precursor protein, it will be possible to probe the physical environment surrounding the precursor as it crosses either the outer or inner membranes. Such experiments are currently underway in several laboratories. Ultimately, determining the mechanisms by which proteins are imported into mitochondria awaits the reconstitution of the import machinery using purified components.

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