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MINI-REVIEW

Mitochondrial Protein Import

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Abstract

Most polypeptides of mitochondria are imported from the cytosol. Precursor proteins contain targeting and sorting information, often in the form of amino-terminal presequences. Precursors first bind to receptors in the outer membrane. Two putative import receptors have been identified: a 19-kilodalton protein (MOM19) in *Neurospora* mitochondria, and a 70-kilodalton protein (MAS70) in yeast. Some precursors integrate directly into the outer membrane, but the majority are translocated through one or both membranes. This process requires an electrochemical potential across the inner membrane. Import appears to occur through a hydrophilic pore, although the inner and outer membranes may contain functionally separate translocation machineries. In yeast, a 42-kilodalton protein (ISP42) probably forms part of the outer membrane channel. After import, precursors interact with "chaperonin" ATPases in the matrix. Presequences then are removed by the matrix protease. Finally, some proteins are retranslocated across the inner membrane to the intermembrane space.

Key Words: Mitochondria; protein import; translocation; contact sites; intracellular sorting.

Introduction

A mitochondrion contains two membranes and two aqueous compartments, each of which has a unique polypeptide composition (Fig. 1). Almost all proteins of this organelle are synthesized on cytoplasmic ribosomes. Precursor proteins bind to receptors in the outer membrane, and then are translocated into mitochondria in an energy-dependent manner. The information for sorting to mitochondrial subcompartments is located in

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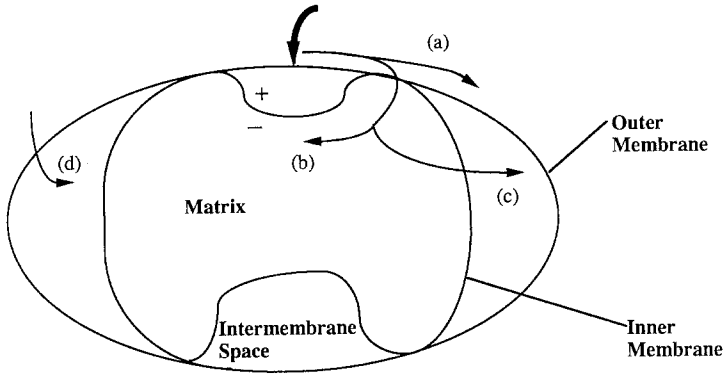

Fig. 1. Known pathways of protein import into mitochondria. A mitochondrion contains inner and outer membranes, an internal matrix space, and an intermembrane space. An electrochemical potential normally is present across the inner membrane. Import pathways for nuclear-encoded mitochondrial proteins are indicated by arrows. The first step in this process is the binding of precursor proteins to the surface of the organelle (heavy arrow). Some polypeptides are retained in the outer membrane (a). Most precursors are translocated through contact sites between the two membranes in a potential dependent manner. The majority of these proteins remain in the matrix (b). Some precursors then are retranslocated across the inner membrane to the intermembrane space (c). Cytochrome *c*, which is not discussed in this review, reaches the intermembrane space by a more direct route (d). In addition, some proteins may become arrested during translocation across the inner membrane, and diffuse laterally from contact sites (not shown).

linear amino acid sequences, usually near the amino terminus of the precursor. In most cases these "presequences" are removed by one or more specific proteolytic cleavages. Most mitochondrial precursors seem to be imported along a common pathway, indicated by arrows in Fig. 1, with the final location of a particular protein determined by how much of this pathway it traverses. While the signals that target proteins to these compartments are well characterized, the molecular mechanisms of translocation are only beginning to be understood.

Since mitochondrial biogenesis is the subject of recent and comprehensive reviews (Nicholson and Neupert, 1988; Hartl *et al.*, 1989; Attardi and Schatz, 1988; Pfanner and Neupert, 1987a), we have tried to emphasize questions that are of current interest in the field. As a result, the length of a given section is not proportional to the information available about that topic. We have restricted the discussion to mitochondria; comparisons with other protein translocation systems have been summarized by Verner and Schatz (1988). For simplicity, we have omitted references to the following items, all of which are covered in the reviews cited above:

(1) The intermembrane space protein cytochrome *c* is imported by a mechanism that is unique among mitochondrial proteins so far examined (see also Stuart and Neupert (1990)).

(2) A small number of proteins are synthesized inside the matrix on mitochondrial ribosomes. Most of these polypeptides are translocated into the inner membrane.
(3) Many mitochondrial proteins are assembled into oligomeric complexes.
(4) After being imported, some proteins are modified by the addition of prosthetic groups such as heme.

Whenever appropriate, we have described relevant experiments in more or less chronological order, in an effort to show how current ideas have evolved.

Binding of Precursors to Mitochondria

The first step in import is the binding of precursors to receptors on the mitochondrial surface. Treatment of mitochondria with proteases inhibits translocation without disrupting the structural integrity of the organelle, implying that proteinaceous components of the outer membrane are involved (Gasser *et al.*, 1982a; Zwinzinski *et al.*, 1984). A more detailed analysis is made possible by arresting translocation at the level of binding to the outer membrane. Precursors can be trapped by lowering the temperature (Freitag *et al.*, 1982) or by deenergizing the inner membrane (Zwizinski *et al.*, 1983). In either case the association is productive: after release of the translocation arrest, the bound precursor is imported to its correct final location. Different precursors vary with regard to the amount of specific binding, the affinity of the interaction, and the sensitivity of binding to a potential across the inner membrane (Hartl *et al.*, 1989). In contrast to the unprocessed precursor, intermediate and mature forms of cytochrome b_2 do not bind to the outer membrane, suggesting that the matrix-targeting sequence is the major determinant of receptor recognition (Riezman *et al.*, 1983c). Hydrophobic domains in some precursor proteins may also influence their binding affinities (Pfanner *et al.*, 1987c).

The outer membrane contains more than one type of import receptor. Insertion of porin is blocked by elastase, whereas translocation of the precursor to the F_1-ATPase ß subunit (F_1ß) is resistant to this treatment (Zwizinski *et al.*, 1984). A purified water-soluble form of porin (ws-porin) competitively inhibits the initial binding of F_1ß and other precursors, but not that of the ADP/ATP carrier (Pfaller *et al.*, 1985; Pfaller and Neupert, 1987; Pfaller *et al.*, 1988). These data suggested the existence of three classes of receptors, although the different experiments give contradictory results about the relationship between binding of porin and F_1ß. Ws-porin was used also

to characterize a subsequent step in the import process. The initial interaction of the ADP/ATP carrier with mitochondria can be resolved into two stages (Pfanner and Neupert, 1987b), and it was shown that ws-porin interferes only with the later one. This intermediate represents a point beyond the binding of precursors to their receptors, but before matrix-targeted precursors have undergone potential-dependent insertion into the inner membrane. Since different precursors all seem to use the same translocation machinery at this stage, Pfaller *et al.* (1988) proposed the term general insertion protein, or GIP.

Although the nature of this hypothetical GIP is not known, considerable progress has been made in identifying receptor molecules. Antisera directed against specific proteins of the outer membrane were tested for their ability to inhibit import. Such experiments are made easier by the relatively simple polypeptide composition of this membrane (Riezman *et al.*, 1983b). Ohba and Schatz (1987a) first used this method to show that an antiserium raised against a 45-kD band of yeast outer membrane proteins reduces import of several precursors. The inhibitory activity of this serum was found later to correlate with the presence of contaminating IgG's against a 42-kD outer membrane protein, ISP42 (see below). Antibodies against ISP42 block formation of a translocation intermediate that appears under conditions of NTP depletion (Eilers *et al.*, 1988; V. Hines, unpublished observations). A possible involvement of ISP42 in earlier binding steps is under investigation. More recently, Söllner *et al.* (1989) identified a 19-kD outer membrane protein of *Neurospora* (MOM19) as a probable import receptor. Antibodies against MOM19 inhibit the binding and import of precursors to porin and $F_1\beta$, but not the ADP/ATP carrier. Low concentrations of elastase generate a 17-kD fragment of MOM19, which supports import of the $F_1\beta$ precursor but not of porin. It seems that MOM19 contains more than one domain involved in precursor recognition. This finding resolves the inconsistency in the earlier studies (see above). The present model thus calls for two receptors, MOM19 and a distinct receptor for the ADP/ATP carrier. It is striking that the ADP/ATP carrier is the only precursor tested that contains internal rather than amino-terminal targeting sequences (Pfanner *et al.*, 1987b; Smagula and Douglas, 1988). This distinction may be the basis for the existence of two receptor types.

Another strategy for identifying receptor proteins was used by Gillespie *et al.* (1985). They showed that a chemically synthesized matrix-targeting peptide inhibits the import of several precursor proteins into rat heart mitochondria. This peptide can be crosslinked to a protease-sensitive outer membrane component of 30 kD (Gillespie, 1987). The relationship, if any, between this protein and ISP42 or MOM19 is unknown.

The possible presence of targeting sequence receptors in the inner membrane has not been tested directly. However, translocation into mitoplasts, in which the outer membrane has been ruptured, shows a similar presequence requirement as import into whole mitochondria (Hwang *et al.*, 1989; see below). If there are specific binding sites in the inner membrane, it will be interesting to find out if more than one class exists, whether the membrane potential influences precursor binding affinities, and what role these receptors play during import into intact mitochondria.

Lipids also may influence how precursors interact with mitochondria. Synthetic matrix-targeting peptides can form amphiphilic helices that perturb natural and artificial phospholipid bilayers (Roise *et al.*, 1986; von Heijne, 1986); amphiphilicity of a presequence correlates with its import efficiency (Roise *et al.*, 1988; Roise and Schatz, 1988; Lemire and Schatz, 1989). In the case of an artificial fusion construct containing a mitochondrial presequence attached to dihydrofolate reductase (DHFR), an intereaction with outer membrane lipids accompanies partial unfolding of the protein (Endo *et al.*, 1989). It has not been determined whether this mechanism also operates with natural precursors.

Sorting of Proteins to Mitochondrial Subcompartments

As there is relatively little new information in this area, we have provided only a general outline of sorting signals and mechanisms. For a more detailed treatment the reader should consult other recent reviews (Hurt and van Loon, 1986; Schatz, 1987; Hartl *et al.*, 1989).

Information for the sorting of mitochondrial proteins resides primarily in targeting sequences, usually located near the amino terminus of the precursor. Polypeptides that follow a complex import pathway may have more than one such domain. These sequences have been defined by two approaches: (1) In gene fusion experiments, "passenger" proteins can be directed to various mitochondrial subcompartments by attaching the appropriate targeting sequence. (2) Deletion or mutation of particular sequences can alter the sorting of a mitochondrial precursor. Both of these strategies have limitations. For instance, the efficiency of a matrix-targeting sequence can depend upon the passenger protein (van Steeg *et al.*, 1986; Verner and Lemire, 1989), and deletion of certain sequences may alter the physical properties of a precursor (Chen and Douglas, 1987b).

Matrix Proteins

Cytoplasmically synthesized proteins are directed to the matrix by amino-terminal targeting sequences. In most but not all cases these peptides

are removed by a specific protease (see below). Hurt *et al.* (1984) showed that the presequence of yeast cytochrome oxidase subunit IV can direct DHFR, normally a cytosolic protein, into the matrix. Similar results were obtained with other presequences and passenger proteins (Horwich *et al.*, 1985; Emr *et al.*, 1986; van Loon *et al.*, 1986). Shortened versions of many presequences retain matrix-targeting function (Hurt *et al.*, 1985; Keng *et al.*, 1986). The targeting sequence is not always located at the extreme amino-terminus of the precursor (Horwich *et al.*, 1986, 1987), and some precursors contain redundant matrix-targeting information (Bedwell *et al.*, 1987; Ellis *et al.*, 1987).

Matrix-targeting signals exhibit no obvious sequence homologies. However, they generally contain few if any acidic residues, and are rich in basic and hydroxylated amino acids. Computer analysis and biophysical studies indicate that many presequence peptides can form amphiphilic α-helices (von Heijne, 1986; Roise *et al.*, 1986). It seems that α-helix formation is not as critical as amphiphilicity and positive charge for presequence function (Allison and Schatz, 1986; Horwich *et al.*, 1987; Roise *et al.*, 1988; Lemire *et al.*, 1989).

The criteria of basic charge and amphiphilicity are quite general, and in fact matrix-targeting function has been found for sequences not normally involved in mitochondrial sorting, including random and artificial presequences (Hurt *et al.*, 1986; Allison and Schatz, 1986; Hurt and Schatz, 1987; Baker and Schatz, 1987; Vassarotti *et al.*, 1987; Banroques *et al.*, 1987; Bibus *et al.*, 1988; Bedwell *et al.*, 1989; Lemire *et al.*, 1989). Import of these constructs is usually (but not always) inefficient, and it has been suggested that they can bypass certain components of the import machinery, such as receptors in the outer membrane (Pfanner *et al.*, 1988b; Söllner *et al.*, 1989). These artificial precursors may be useful for determining whether a given component is essential for translocation, or whether it acts to increase the specificity and rate of mitochondrial protein import.

Inner Membrane Proteins

For many polypeptides that are associated with the inner membrane, the sorting mechanism appears to be the same as for soluble proteins of the matrix or intermembrane space. The ADP/ATP carrier is unusual: it has no cleavable presequence, but contains internal targeting information (Pfanner *et al.*, 1987b; Smagula and Douglas, 1988). Moreover, this protein may bind to an outer membrane receptor distinct from those used by many other precursors (Söllner *et al.*, 1990). On the other hand, import of the ADP/ATP carrier requires both a membrane potential and a hypothetical "general insertion protein" (see above), and the internal targeting domains resemble typical

matrix presequences. It seems that import of the ADP/ATP carrier uses at least part of the pathway followed by other precursors.

Proteins of the Intermembrane Space

The import of several proteins into the intermembrane space requires a potential across the inner membrane. These precursors contain an amino-terminal matrix-targeting signal, often followed by a second sorting sequence that includes a stretch of hydrophobic amino acids (Hurt and van Loon, 1986). It was proposed that hydrophobic sequences can act as "stop-transfer" domains for the inner membrane (Hurt and van Loon, 1986; van Loon and Schatz, 1987; Nguyen and Shore, 1987; Nguyen *et al.*, 1988). Such a model becomes more credible if translocation contact sites are considered to be dynamic structures (see below). While this mechanism cannot be ruled out in all cases, an alternative pathway has been demonstrated for several intermembrane space proteins. In this "conservative sorting," precursors first are translocated completely across the inner membrane, and then are redirected to the intermembrane space (Hartl *et al.*, 1986, 1987). This second translocation presumably uses an export machinery retained from the prokaryotic ancestor of mitochondria.

Nakai *et al.* (1989a) replaced the presequence of cytochrome c_1, including both matrix- and intermembrane space-targeting domains, with a different matrix-targeting sequence. Surprisingly, this construct delivered functional cytochrome c_1 to the intermembrane space. The import pathway for this fusion protein is not known.

Outer Membrane Proteins

Outer membrane precursors lack cleavable presequences and, at least in the case of mitochondrial porin, can be imported in the absence of a potential across the inner membrane (Mihara *et al.*, 1982; Freitag *et al.*, 1982; Gasser and Schatz, 1983). However, assembly of porin into the outer membrane involves receptors and a "general insertion protein" that are also used by matrix precursors (Pfaller *et al.*, 1988; Söllner *et al.*, 1989). It is unclear whether or not inner membrane components are involved in porin import (Gasser and Schatz, 1983; Ono and Tuboi, 1987; Pfaller *et al.*, 1988). For the MAS70 protein of yeast, sorting information is located within the amino-terminal 41 residues of the polypeptide (Riezman *et al.*, 1983a; Hase *et al.*, 1984, 1986). The first 12 amino acids can direct a passenger protein into the matrix (Hurt *et al.*, 1985; Nakai *et al.*, 1989b). Residues 10–37 are uncharged, and may constitute a stop-transfer domain that anchors the MAS70 in the outer membrane. In support of this model, a hydrophobic sequence inserted near the amino terminus of a matrix precursor redirects this protein

to the outer membrane (Nguyen *et al.*, 1988). On the other hand, cytochrome *c* peroxidase is imported to the intermembrane space, even though it contains a stretch of uncharged residues immediately after the matrix-targeting sequence (Kaput *et al.*, 1982; Reid *et al.*, 1982). A similar behavior is seen for fusion constructs with shortened cytochrome c_1 presequences (van Loon *et al.*, 1987). Why some proteins are retained in the outer membrane whereas others are not remains an open question.

Proteolytic Processing of Mitochondrial Precursors

The Matrix Processing Protease

As it was the first component of the mitochondrial import machinery to be identified (Böhni *et al.*, 1980), the matrix protease has been intensively studied. This enzyme specifically cleaves the amino-terminal presequences of proteins that are translocated across the inner membrane. Cleavage is not essential for translocation (Zwizinski *et al.*, 1983; Hurt *et al.*, 1985; Yaffe *et al.*, 1985). A precursor processing activity was first described in yeast (Böhni *et al.*, 1980; McAda and Douglas, 1982), and later in several other eukaryotic species (Miura *et al.*, 1982; Conboy *et al.*, 1982; Schmidt *et al.*, 1984). The matrix protease is a soluble protein; its activity is blocked by the metal chelators EDTA and *o*-phenanthroline, and can be restored by the subsequent addition of divalent cations such as Co^{2+} and Zn^{2+}; cleavage activity is unaffected by inhibitors of serine proteases. The enzyme has been shown to be an endopeptidase (Ou *et al.*, 1989). The matrix protease has been purified to homogeneity from *Neurospora* (Hawlitschek *et al.*, 1988), yeast (Yang *et al.*, 1988), and rat liver mitochondria (Ou *et al.*, 1989). In all cases the purified enzyme consists of two nonidentical subunits of similar molecular weight. For the *Neurospora* enzyme these polypeptides are readily dissociated. The larger (57-kD) subunit by itself shows slow cleavage of mitochondrial presequences, and has been designated MPP for matrix processing peptidase (Hawlitschek *et al.*, 1988). The smaller (52-kD) subunit, which stimulates proteolytic activity, is termed PEP for processing enhancing protein. PEP was estimated to be 15-fold more abundant in mitochondria than MPP, and 75% of the PEP is attached to the inner membrane (Hawlitschek *et al.*, 1988). PEP is now known to be identical to subunit I of the ubiquinone–cytochrome *c* reductase complex of *Neurospora*, and therefore appears to be a multifunctional protein (Schulte *et al.*, 1989). This finding explains both the relative excess of PEP over MPP and its membrane localization.

The situation in yeast is different: both subunits are completely soluble in the matrix, where they are tightly associated and present in approximately equimolar amounts (Witte *et al.*, 1988; Jensen and Yaffe, 1988; Yang *et al.*, 1988; Yang *et al.*, in preparation). Yaffe and Schatz (1984) identified the genes encoding these polypeptides by screening temperature-sensitive mutants for accumulation of mitochondrial precursors. Two complementation groups were found and designated *mas1* and *mas2*. These mutants are deficient in processing of precursors (Yaffe *et al.*, 1985; Jensen and Yaffe, 1988), although there is also an effect on translocation *in vivo* (Yaffe and Schatz, 1984). The wild-type alleles of MAS1 (also known as MIF1) and MAS2 (or MIF2) have been cloned and sequenced, and the gene products were shown to be identical to the 48 kD and 51 kD subunits of the yeast protease, respectively (Witte *et al.*, 1988; Jensen and Jaffe, 1988; Pollock *et al.*, 1988; Yang *et al.*, 1988). PEP is homologous to MAS1 and MPP to MAS2; however, in contrast to the *Neurospora* PEP, the MAS1 protein is distinct from (although homologous to) subunit I of yeast ubiquinone–cytochrome *c* reductase (Schulte *et al.*, 1989). The MAS1 and MAS2 genes are very similar, with an overall identity of about 30%; they probably arose from a common ancestor (Jensen and Yaffe, 1988; Pollock *et al.*, 1988).

The MAS protease cleaves most precursors at a unique position, but the factors that determine this specificity are poorly understood. There is often an arginine residue at position-2 relative to the cleavage site (Miura *et al.*, 1986; Kalousek *et al.*, 1988; Hendrick *et al.*, 1989). Processing efficiency may depend upon regions both in the amino-terminal portion of the presequence (Hurt *et al.*, 1987; Kraus *et al.*, 1988) and in the mature protein (Vassarotti *et al.*, 1987).

Since the MAS genes code for a processing protease, it is surprising that precursors accumulate outside the mitochondria in mutant cells (Yaffe and Schatz, 1984). Perhaps this enzyme also play a part in translocation. To address these and other questions, the two MAS genes were placed under control of a galactose-inducible promoter, which allows for either overproduction or depletion of the corresponding proteins (Geli *et al.*, 1990). Mitochondria isolated from yeast cells depleted of either subunit contain unprocessed precursors inside the inner membrane. When incubated with *in vitro*-synthesized precursors, these mitochondria are severely defective in presequence cleavage, but still import efficiently. These results suggest that the accumulation of precursors in the cytosol of the mutant cells might be an indirect effect, and not caused by a change in the translocation machinery. In the same study, it was observed that growth arrest of yeast depleted of MAS proteins is prevented by spreading the cells on nutrient-poor media, where they divide more slowly than on rich media. Apparently yeast can tolerate a reduced rate of mitochondrial biogenesis if growth is not too rapid. This

observation is important for future genetic studies of mitochondrial protein import. For instance, any number of mutations that reduce the growth rate might suppress a temperature-sensitive defect in one of the MAS genes.

Additional Proteolytic Processing Steps

Several proteins of the intermembrane space are proteolytically processed in two steps, first by the matrix protease and then by an additional enzyme (Daum *et al.*, 1982; Gasser *et al.*, 1982b; Ohashi *et al.*, 1982; Reid *et al.*, 1982; Teintze *et al.*, 1982; Sidhu and Beattie, 1983). The second cleavage event presumably occurs in the intermembrane space. Pratje and Guiard (1987) and Pratje *et al.* (1983) isolated a yeast mutant that is defective in maturation of cytochrome b_2 and subunit II of cytochrome oxidase, but not of cytochrome *c* peroxidase, suggesting that at least two different enzymes carry out these later processing steps. Mitochondria from mutant cells contain an intermediate form of cytochrome b_2, and thus provide an assay for purification of one of these proteases (A. Schneider, unpublished observations).

Some precursors undergo two consecutive cleavages in the matrix (Sztul *et al.*, 1987, 1988). The first of these is carried out by the MAS protease, and the second by a different enzyme, which removes an additional eight or nine amino acids to generate the mature protein (Kalousek *et al.*, 1988). These intermediate prepeptides contain a conserved three-amino acid motif (Hendrick *et al.*, 1989; von Heijne *et al.*, 1989).

Energy Requirements for Import

The first suggestion that energy might be required for protein import into mitochondria was the observation that the uncoupler CCCP (carbonyl-cyanide *m*-chlorophenylhydrazone) blocks translocation of precursors in intact cells (Hallermayer and Neupert, 1976; Harmey *et al.*, 1976). However, the import of cytochrome *c* was inhibited by CCCP in these experiments, whereas later studies gave the opposite result (Zimmermann *et al.*, 1981). Nelson and Schatz (1979) found that a variety of conditions which are expected to reduce ATP levels in the matrix inhibit precursor import. The interpretation of these data was complicated because both ATP concentrations and the electrochemical potential across the inner membrane were affected by these treatments. Subsequent *in vitro* experiments demonstrated that import across the inner membrane requires both a membrane potential (Gasser *et al.*, 1982a; Schleyer *et al.*, 1982; Kolanski *et al.*, 1982) and nucleoside triphosphates (NTP) (Pfanner and Neupert, 1986; Eilers *et al.*, 1987; Chen and Douglas, 1987a).

Role of the Membrane Potential

The assembly of outer membrane porin is unaffected by eliminating the electrochemical potential across the inner membrane (Mihara *et al.*, 1982; Freitag *et al.*, 1982; Gasser and Schatz, 1983). By contrast, the import of proteins into the matrix and inner membrane is blocked by uncouplers or valinomycin plus K^+. Import efficiency appears to depend upon an electrochemical potential $\Delta\psi$ rather than the total protonmotive force Δp (Pfanner and Neupert, 1985). A potential is also required for the import of intermembrane space proteins, since these precursors are partially or completely translocated across the inner membrane (Daum *et al.*, Reid *et al.*, 1982; Gasser *et al.*, 1982b; Teintze *et al.*, 1982). It is unclear whether the membrane potential plays a role in export of proteins from the matrix to the intermembrane space (Hartl *et al.*, 1987; Clarkson and Poyton, 1989).

When the mitochondrial membrane potential in intact cells is dissipated by uncouplers, precursor proteins accumulate in unprocessed form in the cytosol (Reid and Schatz, 1982a; Jaussi *et al.*, 1982). Current evidence suggests that the potential facilitates an early interaction of matrix-targeting sequences with the inner membrane. A potential is needed only for insertion of the amino-terminal portion of a precursor protein into the inner membrane; subsequent translocation of the rest of the polypeptide is potential-independent (Schleyer and Neupert, 1985; Eilers *et al.*, 1988). The import of the ADP/ATP translocator, which appears to contain internal rather than amino-terminal sorting signals, is also potential-dependent (Pfanner *et al.*, 1987b). Bedwell *et al.* (1987) examined the import efficiency of mutant forms of the F_1-ATPase ß subunit (F_1ß) in wild-type and respiratory-deficient ρ^- yeast. As ρ^- cells lack both an electron transport chain and a functional proton-translocating ATPase, they should maintain only a weak potential (Hay *et al.*, 1984). The authors found that certain alterations in the amino-terminal region of F_1ß prevent import in ρ^- but not in wild-type yeast. They concluded that these suboptimal presequences function only when there is a strong potential across the inner membrane.

All of these results are consistent with the idea that positively charged matrix-targeting sequences are "electrophoresed" across the inner membrane as an early step in translocation (Pfanner and Neupert, 1985; Roise *et al.*, 1986). If this interpretation is correct, it represents a mechanism unique to the mitochondrial inner membrane. The only other protein translocation system known to be affected by a membrane potential is the bacterial plasma membrane, where the orientation of the potential relative to translocation is opposite to that found in mitochondria (Chen and Tai, 1985; Geller *et al.*, 1986). It seems equally likely that the structural motif of a basic, amphiphilic presequence is recognized by a specific receptor protein, in analogy to the

docking protein and signal sequence receptor that bind precursors targeted to the endoplasmic reticulum (Krieg *et al.*, 1986; Kurzchalia *et al.*, 1986; Wiedmann *et al.*, 1987). Reduction of the membrane potential might alter the conformation of such a receptor, thereby lowering its affinity for targeting sequences and inhibiting the initiation of translocation into the matrix. In such a model the energy stored in the electrochemical gradient would not necessarily provide the driving force for movement of presequences across the membrane.

Nucleoside Triphosphates

The role of NTP in mitochondrial protein import is not fully understood. Nonhydrolyzable analogues of ATP do not support translocation, indicating that cleavage of the phosphodiester bond is necessary (Pfanner and Neupert, 1986; Eilers *et al.*, 1987; Chen and Douglas, 1987a). One fundamental question is whether NTP functions in the matrix, outside the inner membrane, or both. (The outer membrane of mitochondria is permeable to nucleotides and other small molecules.) Import of at least some precursors requires members of the hsp70 class of heat-shock proteins, both *in vivo* (Deshaies *et al.*, 1988) and *in vitro* (Murakami *et al.*, 1988). These proteins, which presumably use cytosolic ATP, function to prevent tight folding of precursors. Eilers and Schatz (1986) found that stabilizing the tertiary structure of a protein prevents its translocation into mitochondria. They used a fusion construct between the presequence of cytochrome oxidase subunit IV and the cytosolic enzyme DHFR (Hurt *et al.*, 1984). Addition of methotrexate, a substrate analogue that binds to DHFR, abolished import of the fusion constuct, implying that the DHFR moiety must be at least partially denatured for translocation to occur. A similar behavior was seen with a construct containing metallothionein fused to a mitochondrial presequence: import was blocked in the presence of copper ions (Chen and Douglas, 1987c). Conversely, the rate of import is increased by destabilizing the tertiary structure of a precursor, either by urea treatment (Eilers *et al.*, 1988) or by mutagenesis (Vestweber and Schatz, 1988c). The requirement for NTP thus may reflect a need for "chaperone" proteins to maintain precursors in an import-competent state (Pfanner *et al.*, 1987d). This hypothesis explains why import of loosely-folded nascent chains of a matrix protein needs little or no NTP (Verner and Schatz, 1987). Similarly, reducing NTP levels does not prevent either insertion of a denatured form of porin into the outer membrane (Pfanner *et al.*, 1988a) or import of a mutated $F_1\beta$ precursor (Chen and Douglas, 1988).

One observation is inconsistent with this simple model: NTP is required for maximal import rates of a native as well as a urea-denatured precursor,

although the denatured form imports many times faster (Eilers *et al.*, 1988). This result points to another role for NTP in addition to its postulated involvement in cytosolic hsp70 function. Hwang and Schatz (1989) demonstrated that NTP inside the matrix is required for translocation across the inner membrane. When NTP levels outside the mitochondria are severely reduced, import is unaffected as long as NTP is present in the matrix. However, depletion of NTP in the matrix blocks import regardless of the external concentration. In contrast, the assembly of *in vitro*-synthesized porin into the outer membrane required NTP only outside the inner membrane. The exact site of NTP utilization in the matrix is not known, but a reasonable guess is that NTP hydrolysis is somehow used to "pull" a precursor through the inner membrane.

Once a polypeptide reaches the matrix, it can do one of three things: (1) it can remain as a monomer, either soluble or associated with the inner face of the inner membrane. (2) It can integrate into a multimeric complex in the matrix (Hurt *et al.*, 1985; van Loon and Young, 1986; Pfanner and Neupert, 1987a). (3) It can be retranslocated across the inner membrane into the intermembrane space (Hartl *et al.*, 1986, 1987), and once again form a monomeric protein or part of a complex. Many proteins fold and assemble spontaneously *in vitro*. However, stress proteins are probably needed *in vivo* to enable newly synthesized polypeptides to attain their final conformation (Pelham, 1986; Rothman, 1989). Similarly, proteins of the hsp60 class seem to interact with precursors soon after their import into the matrix. Hsp60 is related to the *E. coli* heat-shock protein GroEL and the Rubisco subunit-binding protein of chloroplasts (Hemmingsen *et al.*, 1988; McMullin and Hallberg, 1988). A mutation in the yeast *MIF4* gene, which codes for hsp60, prevents the assembly and complete processing of several mitochondrial proteins (Cheng *et al.*, 1989). Ostermann *et al.* (1989) showed that folding of newly translocated precursors takes place on the surface of an hsp60 "scaffold." ATP hydrolysis is required for this folding reaction and the subsequent release of the polypeptide. It is not known whether ATP is used by the hsp60 molecule itself or by an associated factor. Since import into the matrix and proteolytic processing can take place in the absence of functional hsp60 (Cheng *et al.*, 1989), this ATP requirement appears to be distinct from the involvement of NTP in translocation into the matrix (Hwang and Schatz, 1989). In the case of proteins that are reexported to the intermembrane space, hsp60 probably functions to maintain the intermediates in a translocation-competent state, in a similar manner as hsp70 proteins in the cytosol (see above). A possible additional role of NTP in reexport across the inner membrane has not been investigated. Whether the folding and assembly of proteins in the intermembrane space involves chaperones is unknown.

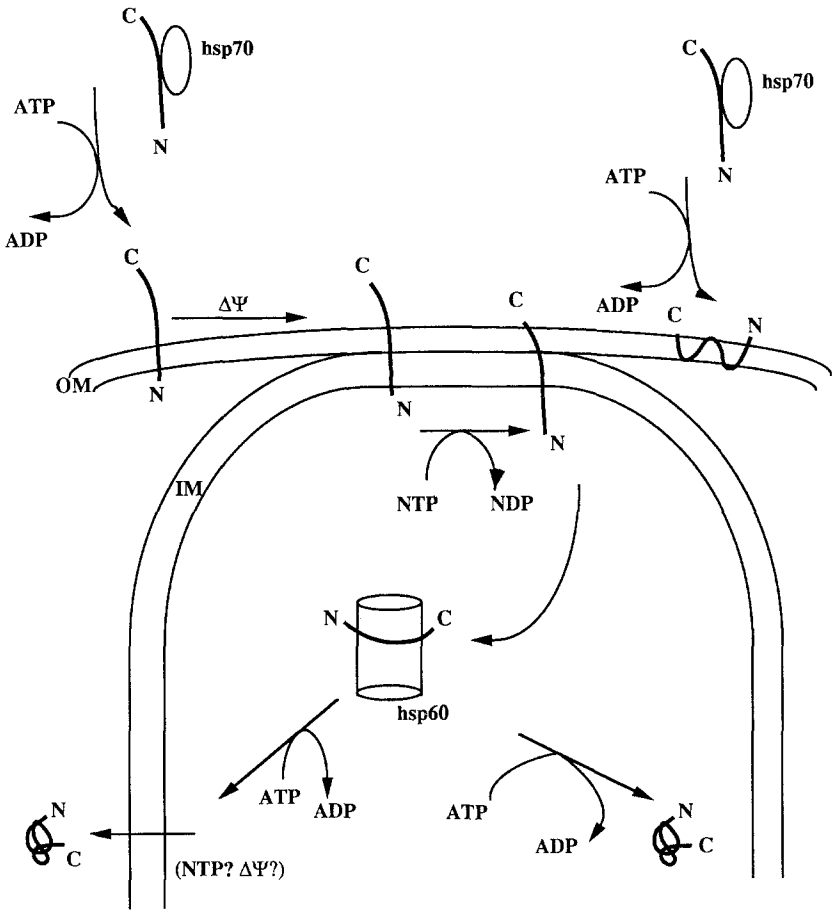

Fig. 2. Energy-dependent steps in import. This diagram summarizes current interpretations of how energy is used at various stages of import. Newly synthesized precursors remain in a translocation-competent state by associating with cytosolic hsp70 stress proteins. Transfer of precursors to the import machinery is thought to require ATP hydrolysis. Some proteins then are integrated directly into the outer membrane. For all other precursors, an electrochemical potential ($\Delta\psi$) is needed for an early interaction with the inner membrane. Subsequent translocation across the inner membrane requires nucleoside triphosphates (NTP) in the matrix, but not $\Delta\psi$. An hsp60 complex uses ATP to assist folding and assembly of newly imported proteins in the matrix. Intermembrane space-targeted proteins are transferred from hsp60 to a second translocation machinery in the inner membrane. The energetics of this reexport process are unknown. OM: outer membrane. IM inner membrane. N,C: amino and carboyx termini of imported proteins.

A protein of the hsp70 family is also present in the mitochondrial matrix (Craig *et al.*, 1989). This protein has been purified to homogeneity (Leustek *et al.*, 1989; S. Hwang, unpublished data). Although the corresponding gene is essential for growth of the cell (Craig *et al.*, 1987), an interaction with mitochondrial precursors has yet to be described.

The various stages at which energy is utilized in the import pathway are summarized in Fig. 2.

Translocation through Mitochondrial Membranes

Are Translocation Contact Sites Stable or Dynamic Structures?

Hackenbrock (1968) identified sites of close apposition between inner and outer membranes in rat liver mitochondria. Inner and outer membranes remain associated in mitoplasts, in which the continuity of the outer membrane has been disrupted with digitonin (Hackenbrock and Miller, 1975). When mitochondria are prepared from cycloheximide-treated yeast, cytoplasmic ribosomes are specifically bound to the outer membrane in regions where the two membranes are close together (Kellems *et al.*, 1974, 1975). These ribosomes, which are enriched in mRNA's for imported mitochondrial proteins, apparently have been arrested during cotranslational translocation of polypeptide chains (Ades and Butow, 1980; Suissa and Schatz, 1982). However, posttranslational import of mitochondrial precursors occurs readily *in vivo* (Hallermeyer *et al.*, 1977; Reid and Schatz, 1982b) and *in vitro* (Gasser *et al.*, 1982a), and it remained open whether this process also involves contact sites.

Schleyer and Neupert (1985) used low temperature and the prebinding of precursor proteins to antibodies to trap an intermediate in the import pathway. Under these conditions the amino terminus of the precursor crosses the inner membrane and is processed by the matrix protease, whereas the majority of the polypeptide chain remains outside the outer membrane. This trapped intermediate was later localized to contact regions between the two membranes by immunoelectron microscopy (Schwaiger *et al.*, 1987). These results suggested that posttranslational import in mitochondria also can occur at stable contact sites. Vestweber and Schatz (1988a) created a chimeric protein consisting of bovine trypsin inhibitor crosslinked to the carboxy-terminus of an artificial mitochondrial precursor protein. This construct become stuck during translocation, with its amino terminus in the matrix and the trypsin inhibitor moiety exposed on the mitochondrial surface (Fig. 4). The chimeric protein (which can be prepared in 100 μg amounts) is able to saturate the translocation machinery, as judged by inhibition of import of

other precursors. Quantitation of this inhibition yielded an estimate of between 100 and 1000 "import sites" per isolated yeast mitochondrion. An average of 4200 import sites per mitochondrion in *Neurospora* was calculated using a different partially-translocated intermediate, consisting of the amino-terminal 167 amino acids of cytochrome b_2 fused to DHFR (Rassow *et al.*, 1989). The presence of methotrexate blocks translocation of the DHFR domain (Eilers and Schatz, 1986), but not of the cytochrome b_2 sequence.

Such trapped intermediates can serve as biochemical markers for import sites. After sonication of mitochondria and separation of the vesicles on a sucrose gradient, an antibody-precursor complex is enriched at a position intermediate in density between inner and outer membranes (Schwaiger *et al.*, 1987). Pon *et al.* (1989) used a similar procedure to subfractionate mitochondria into three distinct vesicle populations: a dense inner membrane fraction, a light outer membrane fraction, and an intermediate band containing polypeptides from both membranes. When import sites are tagged, either with an irreversibly stuck chimeric protein (Vestweber and Schatz, 1988a) or with a reversibly bound intermediate that accumulates under conditions of NTP depletion (Eilers *et al.*, 1988), these markers cofractionate with the intermediate-density band. Upon addition of ATP, the latter intermediate is chased into a protease-protected location, implying that the isolated vesicles are still competent for translocation. The intermediate-density fraction contains inner and outer membrane vesicles attached by pointlike contacts. This fraction is also enriched in at least two polypeptides, which reside in the inner membrane but are concentrated in areas of apposition between the two membranes in intact mitochondria. Current efforts are directed at biochemical and molecular genetic analysis of these proteins.

The function of stable contact zones as unique sites of protein import was put into question by the observation that mitoplasts, in which the outer membrane has been ruptured by osmotic shock, can import precursors directly through their inner membrane (Ohba and Schatz, 1987b). In these experiments the mitochondria first are rendered import-incompetent by treatment with trypsin, which selectively inactivates components exposed on the cytoplasmic surface of the outer membrane. Subsequent osmotic shock restores efficient import and processing of precursors. Antibodies against outer membrane proteins inhibit import in mitochondria but not in mitoplasts, further indicating that residual outer membrane elements are not needed for translocation in mitoplasts. This process requires a membrane potential and unfolding of precursor proteins, as in whole mitochondria. These findings were confirmed and extended by Hwang *et al.* (1989) who showed that precursor import into mitoplasts needs NTP, and that the requirement for a matrix-targeting sequence is similar in mitoplasts and whole mitochondria. Moreover, purified inner membrane vesicles essentially

free of outer membranes can import precursors. In summary, the mitochondrial inner membrane contains a protein translocation activity with many similarities to that seen in intact mitochondria.

These observations are of practical importance, as they will allow the translocation machineries in the inner and outer membranes to be studied independently of one another. They also raise interesting questions about the mechanism of import through the two membranes. One weakness of these studies is that they use disrupted mitochondria as a means to gain direct access to the inner membrane. It can be argued that translocation contact sites are ruptured during osmotic shock, thereby exposing cryptic import channels. We have devised a strategy to address this question by taking advantage of a fusion construct used previously in our laboratory. The presequence of cytochrome c_1 can direct an attached DHFR moiety into mitochondria (van Loon *et al.*, 1986). Upon cleavage of the presequence, DHFR is released in soluble form in the intermembrane space. We modified this construct so that a second matrix-targeting sequence is present on the soluble DHFR moiety. If functional import sites exist in the inner membrane of intact mitochondria, this protein then should be imported from the intermembrane space into the matrix. Preliminary results suggest that this reaction does occur *in vivo* (B. Glick and V. Geli, unpublished observations). Since normal precursors necessarily enter mitochondria from outside the outer membrane, what is the role of these free inner membrane translocation sites? One possibility is that they have no function in normal import, although we consider this unlikely. It may be that import through both membranes can take place only at stable contact junctions, but that components of the translocation apparatus are in equilibirum with free pools in the inner and outer membranes. Such a model is depicted in Fig. 3A.

On the other hand, we may need to reexamine the evidence that import always occurs where the two membranes are closely apposed. Mitochondria do appear to contain zones of contact between the inner and outer membranes (Knoll and Brdickza, 1983), but in the absence of arrested translocation intermediates, it is not yet possible to distinguish a functional import site from a mere juxtaposition of the membranes. Whenever an intermediate that spans both membranes has been identified, there has been something in the carboxy-terminal portion of the precursor that prevented complete translocation. These obstructions include ribosomes (Kellems *et al.*, 1975), antibodies (Schleyer and Neupert, 1985), a disulfide-bonded polypeptide (Vestweber and Schatz, 1988a), and stabilization of the tertiary structure of the protein by a tightly bound ligand (Rassow *et al.*, 1989). Consider the model shown in Fig. 3B. In this scheme a precursor first passes partially though the outer membrane, and then is engaged by a second translocation machinery in the inner membrane. The two translocation processes could

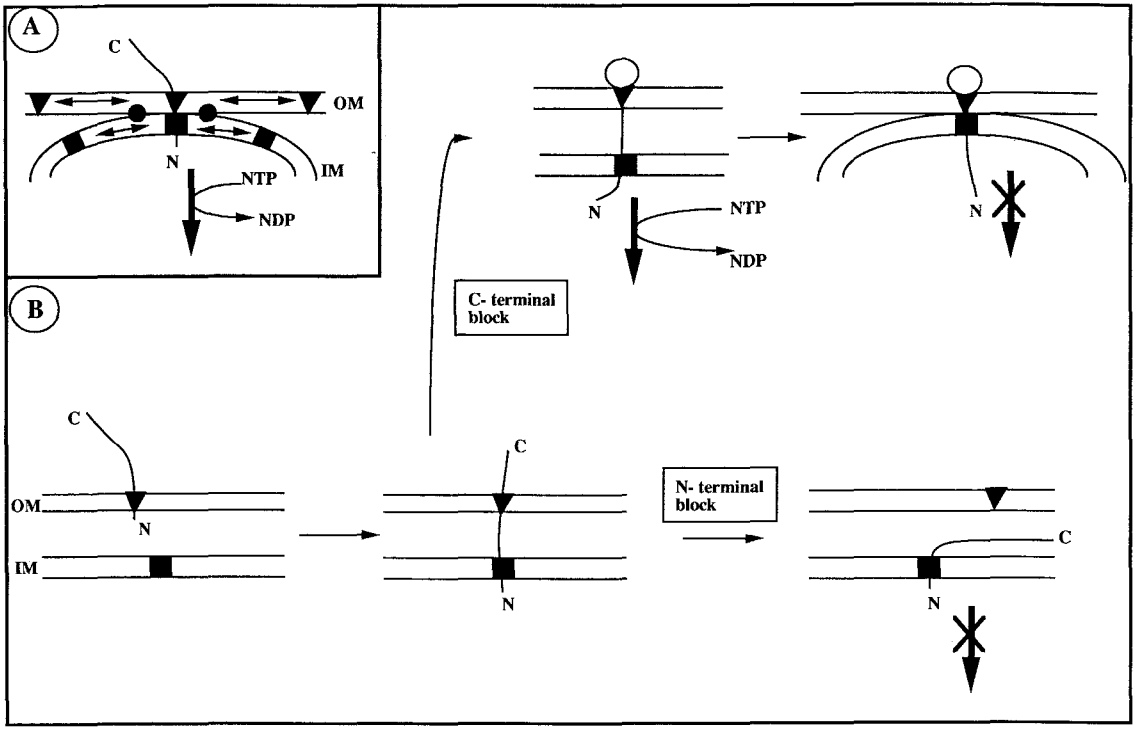

Fig. 3. Two alternative views of translocation contact sites. For simplicity, precursors are shown as extended polypeptide chains. (A) Inner and outer membranes are held in close proximity by attachment proteins. Import always occurs at these junctions, but the translocation-machinery components can diffuse laterally in the membranes (double-headed arrows). Nucleoside triphosophate (NTP) hydrolysis is used to pull the precursor into the matrix (heavy arrow). (B) Import can take place where the two membranes are not tightly associated. A precursor first passes partially through the outer membrane, and then is recognized by the inner membrane machinery. Translocation through the inner membrane is NTP-dependent. If the carboxy-terminal domain of the precursor contains an obstruction (indicated by a circle in the diagram), only the inner membrane will continue translocation, thereby pulling the two membranes into close contact. In this case the geometry is identical to that expected from model A. On the other hand, if translocation of the amino-terminal domain is blocked—for example, by reducing matrix NTP levels—the precursor will be trapped in the intermembrane space. The existence of such an intermediate is not predicted by model A. OM: outer membrane. IM: inner membrane. N, C: amino and carboxy termini of precursor proteins. ■: inner membrane import machinery. ▼: ISP42, and possibly other components of outer membrane machinery. ●: hypothetical attachment proteins between inner and outer membranes.

occur simultaneously, but without requiring that the membranes be tightly joined. If an obstruction is present that blocks passage of the carboxy terminus across the outer membrane, the inner membrane machinery will continue to translocate the precursor until further movement is impossible. The result will be that the two membranes are pulled into close apposition. One way to test this model would be to block translocation at the amino-terminal rather than the carboxy-terminal domain. Under conditions of NTP depletion, a translocation intermediate forms in which the amino terminal portion of a precursor interacts with the inner membrane, but cannot pass completely in the matrix (Eilers *et al.*, 1988). Recent experiments indicate that a fraction of this intermediate is protease-resistant in whole mitochondria, but not in mitoplasts (S. Hwang and C. Wachter, unpublished observations). It appears that in this case the precursor has passed completely through the outer membrane into the intermembrane space, but has not yet reached the matrix. Upon addition of ATP the precursor is chased across the inner membrane. These data seem incompatible with stable contact regions functioning as the sole sites of import; instead, they support the model shown in Fig. 3B. Further experiments are needed to distinguish conclusively between these two schemes.

The Import Channel

What is the molecular environment through which a protein crosses the two mitochondrial membranes? Are the membranes fused at these points? Do the precursors interact directly with lipids, or is there a proteinaceous pore? If such a channel exists, what are the protein components? How large a domain can be translocated? The answers to these questions are beginning to emerge. Apparent fusions between inner and outer membranes have been reported in freeze-fracture studies (van Venetie and Verkleij, 1982). However, the existence of these structures would be hard to reconcile with the observed different lipid compositions of the two membranes (Parsons *et al.*, 1967). Thin-section electron microscopy has yielded no evidence for such fusions (Rassow *et al.*, 1989). Using improved staining techniques, we see distinct inner and outer membranes in yeast mitochondria, even at points where cytoplasmic ribosomes are bound (B. Glick and L. Pon, unpublished observations). It seem likely that precursors must pass through two distinct membranes.

Pfanner *et al.* (1987a) showed that partially translocated precursors can be released from the membranes by alkaline pH and urea, suggesting that import occurs in a hydrophilic, presumably proteinaceous environment. Attachment of a charged molecule to the carboxy terminus of a precursor protein does not prevent import, further indicating that precursors pass

through a hydrophilic channel (Vestweber and Schatz, 1988b). This channel does not allow passage of some tightly folded proteins (see above), but it does translocate a precursor linked to holocytochrome *c*, which contains a covalently bound heme group (Vestweber and Schatz, 1988b). While precursors normally may cross the membranes in an extended form (Rassow *et al.*, 1989), they could in some cases retain domains of secondary or even tertiary structure (Bychkova *et al.*, 1988). Mitochondria can also import constructs containing stretches of single- or double-stranded DNA attached to a precursor protein (Vestweber and Schatz, 1989). These findings suggest that once the targeting sequences have been recognized, a flexible translocation pore may accept any structure up to a certain size. The molecular architecture of this channel is still a matter of speculation.

Vestweber *et al.* (1989) have identified the first known protein component of the translocation machinery in mitochondrial membranes. They used a chimeric construct with bovine trypsin inhibitor crosslinked to the carboxy terminus of a precursor protein (see above). The trypsin inhibitor moiety remains stuck outside the outer membrane, while the amino-terminal domain is accessible to the matrix processing protease. The crosslinker then should be located in or near the import channel in the outer membrane (Fig. 4). By using a trifunctional, photoactivatable crosslinker, the authors found that a single polypeptide becomes covalently attached to the stuck precursor. This polypeptide is recognized by antisera directed primarily against 45-kD components of the outer membrane. Earlier work had shown that these antisera inhibit the import of several precursors into mitochondria (Ohba and Schatz, 1987a). This inhibitory activity was traced to the presence of contaminating antibodies against a 42-kD outer membrane protein. The anti-42-kD IgG's are also responsible for recognition of the crosslinked adduct. Thus, by two independent approaches, this protein—called ISP42, for 42-kD import site protein—has been implicated in translocation. Since it is associated with the chimeric precursor at a late stage of import, ISP42 may form part of a channel in the outer membrane. When submitochondrial vesicles are separated on a density gradient, ISP42 is associated primarily with outer membrane markers rather than with an intermediate-density "contact site" fraction (L. Pon and D. Vestweber, unpublished observations). It may be that ISP42 diffuses in and out of stable contact sites. In the alternative model shown in Fig. 3B, ISP42 would be simply a component of a separate outer membrane translocation apparatus. As the gene for ISP42 has been cloned (K. Baker, unpublished data), sequence and genetic information should help clarify the function of this protein. A stuck precursor containing a trifunctional crosslinker is now being used with inner membrane vesicles, where a different protein of lower molecular weight has been identified (T. Jascur, unpublished observations).

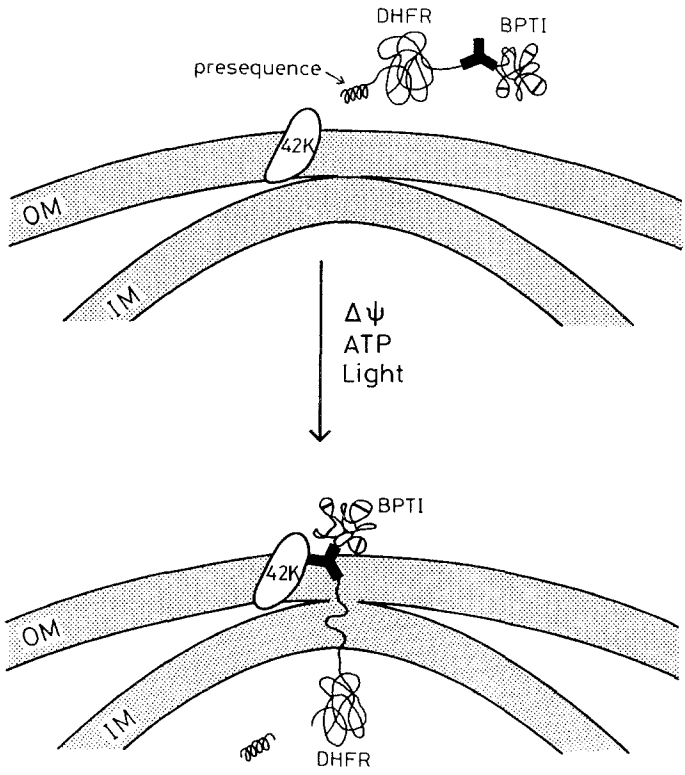

Fig. 4. Crosslinking of a 42-kD outer membrane protein to a chimeric precursor that is trapped in translocation contact sites. An artificial precursor containing a mitochondrial presequence fused to dihydrofolate reductase (DHFR) was derivatized at its carboxy terminus by a trifunctional crosslinker. Bovine pancreatic trypsin inhibitor (BPTI) then was attached to the second arm of the crosslinker. When this construct was imported in the presence of ATP and a membrane potential ($\Delta\psi$), an arrested translocation intermediate was formed, with the amino terminus of the DHFR domain in the matrix and the BPTI moiety still outside the outer membrane. Activation of the third arm of the crosslinker by light results in the covalent attachment of a 42-kD protein of the outer membrane. OM: outer membrane. IM: inner membrane.

Summary and Prospects

Binding of Precursors to Mitochondria

Mitchondrial precursors associate with specific receptors in the outer membrane. One apparent receptor protein (MOM19) has been identified, and others are waiting to be discovered. The biochemistry of these binding interactions is only partially understood. It will be interesting to learn how

precursors are transferred to the translocation machinery, and whether import into the matrix requires a second set of receptors in the inner membrane.

Sorting and Proteolytic Processing of Mitochondrial Precursors

After binding to the mitochondrial surface, some proteins are retained in the outer membrane. The majority, however, are translocated into the inner membrane or the matrix. A few polypeptides then are reexported across the inner membrane to the intermembrane space. These stages define a common pathway for the import of mitochondrial proteins (Fig. 1). Different precursors all contain a general mitochondrial targeting signal ("matrix targeting sequence"), usually near the amino terminus. In the absence of additional sorting information, proteins follow a default pathway into the matrix. While matrix-targeting sequences have been extensively studied, less is known about the signals that control sorting to the other compartments. For most proteins that cross the inner membrane, amino-terminal presequences are removed by specific processing enzymes. One of these, the matrix protease, has been characterized genetically and biochemically.

Energy Requirements for Import

Nucleoside triphosphates (NTP) and a membrane potential both play a part in translocation (Fig. 2). ATP is probably used by chaperone proteins in the cytosol and the matrix to modulate folding of precursors. In addition, NTP is required in the matrix for import across the inner membrane, perhaps reflecting the action of an energy-dependent "translocase." The role of the membrane potential is not yet understood. One possibility is that positively charged matrix-targeting sequences are electrophoresed across the inner membrane. Alternatively, the potential could influence the function of some component of the import machinery.

Translocation through Mitochondrial Membranes

Mitochondrial precursors can be trapped in a conformation that spans both inner and outer membranes, suggesting that import takes place at contact sites between the membranes (Fig. 3A). On the other hand, since precursors can be imported directly through the inner membrane, it may be that mitochondria contain two separate translocation machineries that act in tandem (Fig. 3B). The reexport of proteins from the matrix to the intermembrane space is not well characterized, but might involve a separate mechanism related to bacterial secretion. It seems that import into the matrix occurs through a flexible hydrophilic pore. A recently identified outer membrane protein (ISP42) might form part of this channel (Fig. 4). As the

function of this and other components are elucidated, we will begin to understand how polypeptides can cross a lipid bilayer.

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