Journal of Bioenergetics and Biomembranes, Vol. 29, No. 1, 1997

Minireview

Recognition and Binding of Mitochondrial Presequences during the Import of Proteins into Mitochondria

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Received May 30, 1996; accepted October 29, 1996

Nuclear-encoded mitochondrial proteins are imported into mitochondria due to the presence of a targeting sequence, the presequence, on their amino termini. Presequences, which are typically proteolyzed after a protein has been imported into a mitochondrion, lack any strictly conserved primary structure but are positively charged and are predicted to form amphiphilic α-helices. Studies with synthetic peptides corresponding to various presequences argue that presequences can partition nonspecifically into the mitochondrial outer membrane and that the specificity of translocation of precursors into mitochondria may depend on interactions of the presequence with the electrical potential of the inner membrane. Although proteins of the outer membrane that are necessary for the translocation of precursor proteins have been proposed to function as receptors for presequences, the binding of presequences to these proteins has not been demonstrated directly. Proteins of the mitochondrial outer membrane may not be responsible for the specificity of translocation of precursors but may instead function, together with cytosolic molecular chaperones, to maintain precursor proteins in conformations that are competent for translocation as the precursors associate with the mitochondrial surface.

KEY WORDS: Protein transport; mitochondrial presequences; electrical potential; receptors; molecular chaperones; amphiphilic helix.

INTRODUCTION[2]

The last two decades have seen a dramatic increase in the qualitative understanding of the process by which proteins are translocated across biological membranes. In most cases, translocation can now be studied using *in vitro* systems, and in many cases, components responsible for effecting translocation—both within the membranes themselves, and within the aqueous spaces on either side of the membranes—have been identified and at least partly characterized (Schatz and Dobberstein, 1996). Despite the identification of these components, however, a precise physical understanding of the mechanism by which proteins are recognized by and translocated across membranes has not yet been achieved. Indeed, many of the current models for protein translocation overinterpret the available experimental evidence and suggest a stronger physical understanding of the problem than actually exists. It is my hope in this short review to reexamine the published biochemical and biophysical studies on the mechanism of protein import into mitochondria, to identify weaknesses in the analysis of these studies, and to propose a somewhat more realistic physical model for the function of mitochondrial presequences in the translocation process.

HOW DID WE GET HERE?

Most of the current knowledge in the field of mitochondrial protein import has resulted from the

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[2] Although it has been said that a picture is worth a thousand words, I have chosen not to include any figures in this review. It is my feeling that the representation of a biological system in a schematic model often generates the false sense that the system is better understood than it actually is. In order to limit the bias caused by the presentation of a model for the recognition of presequences, I have chosen not to do so.

0145-479X/97/0400-019$12.50/0

combined use of genetic, biochemical, and cell biological techniques. Once the conditions had been established to follow the translocation of proteins into purified mitochondria *in vitro* (Korb and Neupert, 1978; Maccecchini *et al.*, 1979; Zimmermann and Neupert, 1980; Gasser *et al.*, 1982), it was possible to begin the search for components of the protein translocation apparatus and to develop models for the physical mechanism by which proteins cross the mitochondrial membranes. Early experiments used proteases to degrade exposed proteins on the surface of isolated mitochondria and to show that the loss of the surface proteins correlates with a decrease in the binding of precursor proteins to the mitochondria (Riezman *et al.*, 1983; Zwizinski *et al.*, 1984). The treated mitochondria also showed a corresponding decrease in the ability to import precursor proteins. The surface proteins involved in the uptake of the precursors were subsequently identified through the generation and use of specific antibodies against these proteins (Ohba and Schatz, 1987; Söllner *et al.*, 1989, 1990; Hines *et al.*, 1990; Pain *et al.*, 1990). For example, antibodies specific for a mitochondrial outer membrane protein from *N. crassa*, Tom20,[3] inhibited the import of a subset of precursor proteins into isolated mitochondria and decreased the binding of two precursors to the mitochondria (Söllner *et al.*, 1989). Treatment of the mitochondria with elastase caused a progressive inhibition of the import of two different precursor proteins, and the effects correlated with the generation of partially cleaved forms of Tom20 by the protease. Similar biochemical approaches were used to characterize another outer membrane protein, Tom70, that is involved in early steps of the import process for some precursor proteins (Söllner *et al.*, 1990). Both proteins have homologues in yeast that appear to have similar functions (Hines *et al.*, 1990; Ramage *et al.*, 1993; Moczko *et al.*, 1994). More recently, biochemical and genetic studies have identified two other proteins of the outer membrane, Tom22 and Tom37, that are also involved in the import of precursor proteins into mitochondria (Kiebler *et al.*, 1990, 1993; Lithgow *et al.*, 1994a; Nakai and Endo, 1995; Gratzer *et al.*, 1995). Although the physical properties and functions of the Tom proteins are not yet well understood, it has been suggested that the proteins form a heterooligomeric complex in the outer mitochondrial membrane and that they function as receptors for precursor proteins synthesized in the cytosol (Kiebler *et al.*, 1990; Söllner *et al.*, 1992; Haucke *et al.*, 1996; see also Haucke and Lithgow, this volume).

Because the ultimate goal in studying the import of proteins into mitochondria is to understand the physical mechanism by which the proteins pass through the membranes, it is not surprising that the terminology of biophysical chemistry has been adopted to describe the import process. The recognition of precursor proteins in the cytoplasm is thought to be mediated by receptor proteins on the surface of the mitochondria, in much the same way that polypeptide hormones are recognized by receptors on the surface of the plasma membrane. Likewise, the passage of polypeptides through the mitochondrial membranes is thought to be mediated by aqueous channels, just as the movement of ions across membranes is effected by the well-studied channel proteins. The appeal of using receptors and channels as archetypes for the components of a mitochondrial import apparatus is not hard to understand: the basic properties of receptors and channels are taught in almost every introductory biochemistry course, and it takes little effort to apply the framework of these well-characterized systems to the problem of protein translocation. Unfortunately, in the case of mitochondrial protein import, the models are based on relatively weak experimental evidence and ignore the physical properties of mitochondrial targeting sequences that have been established in well-defined experimental systems.

WHEN IS A RECEPTOR REALLY A RECEPTOR?

There is a long experimental history in the study of the receptors of the plasma membrane that can be applied to the analysis of the problem of mitochondrial protein translocation (Cuatrecasas and Hollenberg, 1976). In the study of receptor function, it is generally considered important to determine the quantitative relationship between the binding of a ligand to a receptor and the ultimate biological event that the binding causes. In order to establish this relationship, a number of experimental observations should be considered. These include: (1) the binding affinity of the ligand for the receptor; (2) the saturability of the binding; (3) the reversibility of the binding; (4) the ability of ana-

[3] Proteins involved in translocation are known either as Tom (for translocase of the outer membrane) or Tim (for translocase of the inner membrane), with a number that corresponds to the approximate size of the protein in kDa. This consensus nomenclature was recently described (Pfanner *et al.*, 1996).

logs and/or antagonists of the ligand to interact with the receptor; (5) the tissue distribution—or, in the case of receptors for mitochondrial precursors, the subcellular distribution—of the binding activity; and (6) the relationship between the fractional occupancy of the receptor and the observed biological effect. In the case of mitochondrial protein translocation, the biological effect is simply the movement of the precursor protein across the mitochondrial membranes.

With well-characterized receptors and ligand-gated channels, the analysis of the binding interactions using well-defined binding and signal transduction assays and the above criteria has provided satisfying descriptions of the physical mechanisms of these proteins (Cuatrecasas and Hollenberg, 1976; Montal *et al.*, 1986; Cerione, 1991). When the same criteria are applied to the interactions between precursor proteins and isolated mitochondria, however, it is clear that there is very little experimental support for the designation of any of the outer membrane proteins as *receptors* in the traditional sense. In most cases, the functions of the Tom proteins are defined not by direct measurement of the binding affinity of a Tom protein for a mitochondrial precursor, but rather by showing that the destruction of the Tom protein results in the inhibition of translocation activity. While these studies clearly demonstrate that the Tom proteins are involved in some way in the process of protein import into mitochondria, and that they are likely to interact with mitochondrial precursors during translocation, they do not provide strong evidence that these proteins specifically recognize the precursors.

WHY IS IT SO HARD TO MEASURE BINDING?

There are a number of reasons why the binding properties of the Tom proteins have been difficult to characterize. First, unlike binding studies with hormone receptors, which typically make use of highly purified and well-characterized radiolabeled ligands, the characterization of mitochondrial receptors is generally performed with precursor proteins that have been expressed in crude reticulocyte lysates. The possible involvement of factors from the lysate makes it difficult to interpret binding experiments, and the inexact concentration of precursors in the lysate makes it impossible to measure the dependence of the binding on the concentration of the precursor. Without a concentration dependence, the binding affinities cannot be calculated. Second, even when precursors have been expressed in bacteria and purified, they are often relatively unstable and are particularly sensitive to aggregation and other nonspecific effects (Eilers and Schatz, 1986; Jaussi *et al.*, 1987; Murakami *et al.*, 1988; Sheffield *et al.*, 1990; Iwahashi *et al.*, 1992; Becker *et al.*, 1992; Hajek and Bedwell, 1994). These properties make the pure precursors difficult to use in equilibrium binding experiments because the populations of free and bound precursors are not well defined and because they vary over the time course of an experiment. The instability may also explain why only a relatively small fraction of any given precursor is typically competent for productive binding and import. Although experimental data are typically scaled relative to the maximum observed binding or import to gloss over this problem, the actual efficiency of binding and import is generally quite low. It is doubtful that the results of binding studies with hormone receptors would be considered significant if only 10–20% of the ligands used in an assay were found to be competent for binding.

The redundancy of the Tom proteins also complicates how any binding data with wild-type mitochondria are interpreted. Three of the surface proteins, Tom20, Tom37, and Tom70, can be disrupted individually with relatively minor effect (Ramage *et al.*, 1993; Moczko *et al.*, 1994; Gratzer *et al.*, 1995), but Tom22 is essential for cell growth and protein import under all conditions (Lithgow *et al.*, 1994a; Nakai and Endo, 1995; Hönlinger *et al.*, 1995). Surprisingly, cells disrupted in any two of the Tom20, Tom37, or Tom70 proteins can compensate for the defects and grow normally by increased production of Tom22 (Lithgow *et al.*, 1994b). The autonomous behavior of the individual Tom proteins makes it difficult to assign mechanistic roles for these proteins, although a few recent studies have begun to address these questions (Haucke *et al.*, 1995; Mayer *et al.*, 1995a; Bolliger *et al.*, 1995).

WHAT SHOULD A RECEPTOR RECOGNIZE?

Because there are still some uncertainties about the function of the Tom proteins, it is worth thinking in general about how a receptor for a mitochondrial precursor would be expected to behave. In order for a mitochondrial surface protein to function as a receptor, the protein must recognize and bind to the segment of a precursor that is responsible for targeting the

precursor to the mitochondria. For most mitochondrial precursors, this segment corresponds to the amino-terminal presequence (Hurt and Van Loon, 1986; Hartl *et al.*, 1989). Mitochondrial presequences lack a common primary structure and instead share a common physical property—the ability to form a positively-charged, amphiphilic helix (Roise and Schatz, 1988). Experiments with artificial presequences (Allison and Schatz, 1986), with randomly-generated presequences (Baker and Schatz, 1987; Lemire *et al.*, 1989), and with presequences modified by site-directed mutagenesis (Bedwell *et al.*, 1989) have confirmed that the primary structure of a functional presequence is unimportant as long as positive charge and a predicted amphiphilic structure are maintained. The lack of a common primary structure among presequences makes it difficult to understand how the mitochondrial receptors could bind specifically to a precursor protein. In the case of the well-characterized major histocompatability proteins, which also bind peptides with a wide variety of primary sequences, there are still specific residues that must be present as anchor residues in peptides having high binding affinity for these proteins (Matsumura *et al.*, 1992; Madden *et al.*, 1992; Stern *et al.*, 1994). Molecular chaperones represent another class of proteins that recognize a wide variety of primary sequence. These proteins have a somewhat lower requirement for specific primary structure (Flynn *et al.*, 1991; Blond-Elguindi *et al.*, 1993; Gragerov *et al.*, 1994; Endo *et al.*, 1996) and may provide a possible model for how presequences are recognized. Nevertheless, high-affinity binding interactions are thought to depend on a precise fit between a ligand and its receptor, and it is difficult to understand how a precise fit could be maintained with such a wide variety of primary structures.

Even if a presequence can bind specifically to a mitochondrial surface protein, it still faces the subsequent problem of being transferred productively through the mitochondrial membranes. In all other receptor systems, the binding step is a dead end: once a ligand binds to its receptor, it can proceed no farther. In contrast, a receptor for presequences would need to interact with its ligand in a transient, vectorial fashion, and contact with other sites in the precursor would presumably need to be maintained in order to avoid the disassociation of the precursor from the mitochondrial surface and the release of the precursor into the cytoplasm. Although such vectorial transfers are not unprecedented in biology, they usually require highly specific and stable interactions to some part of the transferred molecule. Good examples include the contacts between the ribosome and the transfer RNA during the elongation of polypeptides or those between a nucleic acid primer sequence and its DNA template during replication of DNA or transcription of RNA. For the transfer of a precursor protein from the site of binding to the site of translocation, it is not clear how such interactions would be mediated. Recent results indicate that proper vectorial transfer of a precursor across the mitochondrial outer membrane depends on an attached presequence; cleavage of the presequence on the trans side of the outer membrane allows the mature portion of the precursor to slip, nonproductively, out of the translocation pore (Mayer *et al.*, 1995b). These results indicate that the presequence functions as the handle through which the precursor protein must be held, not just for the initial association of the precursor with the surface of the mitochondria, but also as the precursor is passed through the membranes.

HOW ELSE COULD A PRESEQUENCE FUNCTION?

Because presequences are relatively short polypeptides, and because they typically function independently of the protein to which they are attached, it has been useful to use synthetic presequences as probes for the interactions with mitochondria. Model studies with synthetic presequences have confirmed that presequences indeed form amphiphilic helices (Roise *et al.*, 1986; Tamm, 1986; Epand *et al.*, 1986; Aoyagi *et al.*, 1987; Karslake *et al.*, 1990; Hoyt *et al.*, 1991; Bruch and Hoyt, 1992; Hammen *et al.*, 1994). These sequences can associate with natural and artificial lipid bilayers and, at sufficiently high concentrations, can disrupt the membranes to which they are bound. The interactions of the synthetic presequences with membranes are dependent both on hydrophobic and electrostatic interactions (Swanson and Roise, 1992; Wang and Weiner, 1994). In some cases the interactions can be altered by a transmembrane electrical potential (Roise *et al.*, 1986; de Kroon *et al.*, 1991; Maduke and Roise, 1993), although this effect may not be universal (Skerjanc *et al.*, 1987).

Synthetic presequences have also been used in import experiments with isolated mitochondria. They can inhibit the import of radiolabeled precursor proteins (Gillespie *et al.*, 1985; Furuya *et al.*, 1987; Cyr and Douglas, 1991), and their import has also been

examined directly (Pak and Weiner, 1990; Furuya *et al.*, 1991; Roise, 1992. The import of presequences into mitochondria depends on the electrical potential across the inner mitochondrial membrane, a result consistent with the dependence of precursor translocation of the potential (Schleyer and Neupert, 1985; Martin *et al.*, 1991). Surprisingly, however, the import is not affected by treatment of mitochondria with trypsin to remove the Tom proteins, nor is it dependent on any cytosolic cofactors (Pak and Weiner, 1990; Furuya *et al.*, 1991). In quantitative binding and translocation assays, it was found that the yeast cytochrome oxidase subunit IV presequence binds directly to the lipid bilayer of the mitochondrial outer membrane (Roise, 1992). The subsequent import of this bound presequence displayed kinetics that were strictly first-order with respect to the concentration of the bound presequence. This result indicated that the presequence does not saturate its sites of translocation, even at nearly micromolar concentrations.

The results of the binding and import experiments with synthetic presequences argue that the interactions of presequences with the mitochondrial surface do not depend on Tom proteins. Because the presequence represents the only portion of a precursor protein that is necessary for the specific uptake of the precursor into mitochondria *in vivo* or *in vitro*, it is difficult to conceive of a mechanism by which the Tom proteins could function as receptors for mitochondrial precursors in the traditional sense. At the same time, because the partitioning of a presequence into a phospholipid bilayer is a nonspecific event, there must be another mechanism by which the specificity of the translocation process is conferred. One possibility is that the presequence, after partitioning nonspecifically into the outer membrane, engages a membrane-embedded receptor from within the lipid phase of the membrane. A candidate for this receptor is Tom40, a protease-resistant protein of the outer membrane that is essential for cell growth (Baker *et al.*, 1990) and that can be crosslinked to a precursor protein trapped in the process of translocation (Vestweber *et al.*, 1989). A second mechanism by which the specificity of translocation may be conferred involves the interaction of the presequence with cardiolipin after the presequence has partitioned nonspecifically into the outer membrane. There is some evidence that this lipid, which is present almost exclusively in the mitochondrial inner membrane, can interact in a specific manner with presequences (Ou *et al.*, 1988; Leenhouts *et al.*, 1993; Török *et al.*, 1994; Snel *et al.*, 1995). These interactions could only take place, however, after the presequence had passed through the outer membrane. In either of these mechanisms, the relatively small fraction of the total presequence that is bound specifically would be overwhelmed by the large amount of nonspecific binding and would not necessarily be observed in quantitative binding assays.

Another intriguing possibility is that the membrane potential of the inner membrane plays a role in conferring specificity on the translocation process. The potential, which is a unique feature of the inner membrane, provides a huge thermodynamic driving force for the uptake of cationic molecules from the cytoplasm into the mitochondrial matrix space. In order to accumulate in the matrix, however, a molecule must be able to pass through the mitochondrial inner membrane. Some molecules, such as rhodamine 123, a specific mitochondrial stain, can diffuse directly across lipid bilayers and do not require the presence of a transporter in the inner membrane (Chen, 1988). Others, such as the polyamines, require a specific transporter in the inner membrane to facilitate their uptake (Toninello *et al.*, 1992). The potential appears to be specific to mitochondria even in plant cells, which do not accumulate rhodamine 123 into their chloroplasts (Wu, 1987).

Because the translocation of presequences across the inner membrane depends on the electrical potential (Schleyer and Neupert, 1985; Martin *et al.*, 1991), and because the potential is a unique feature of the mitochondrial inner membrane, it is tempting to speculate that the potential may be responsible for the specificity of translocation of precursors into mitochondria. We have shown that a synthetic presequence can be translocated across protein-free lipid bilayers in a potential-dependent manner (Maduke and Roise, 1993). This result indicates that presequences may behave in a manner similar to that of rhodamine 123 and may not require a transporter. It does not, however, exclude the involvement of one or more transporters in the process. Although any mechanism that includes a role for the electrical potential in determining the specificity of import of the precursor requires that the presequence be able to interact with the potential, there is recent evidence that presequences can passively cross the outer membrane in a reversible manner (Mayer *et al.*, 1995b). This transfer may place the presequence in a position where it can be attracted by the membrane potential.

IF THE RECEPTORS AREN'T RECEPTORS FOR PRESEQUENCES, WHAT ARE THEY?

The results of quantitative binding and import experiments with synthetic mitochondrial presequences indicate a lack of saturable binding and translocation sites on the surface of mitochondria. These findings argue against the view that the Tom proteins serve as receptors for presequences. Since the Tom proteins are clearly involved in the translocation of full-length precursor proteins into mitochondria, what other possible roles could these proteins play in the process? One possibility is that the Tom proteins may be involved in modulating the interactions between molecular chaperones and precursor proteins. Cytosolic molecular chaperones are essential for the transport of proteins into various subcellular organelles in living cells (Deshaies *et al.*, 1988; Gething and Sambrook, 1992) and are thought to function by maintaining newly-synthesized proteins in conformations that are competent for translocation. Although it has generally been assumed that cytosolic molecular chaperones are not involved in the specific targeting of precursor proteins to membranes, two recent reports suggest that at least one chaperone may help to transfer precursor proteins to the surface of mitochondria via interactions with Tom proteins (Hachiya *et al.*, 1995, Komiya *et al.*, 1996). The chaperone, which is termed the mitochondrial import stimulation factor (MSF), was purified as a heterodimer from rat liver extracts (Hachiya *et al.*, 1993). It stabilizes precursor proteins synthesized in wheat germ lysate that would otherwise lose the ability to be imported into isolated mitochondria. Unlike reticulocyte lysate, which appears to contain one or more proteins with similar activities (Ono and Tuboi, 1988; Murakami and Mori, 1990), wheat germ lysate does not appear to contain factors that stabilize precursor proteins. Precursors synthesized in wheat germ lysate, therefore, rapidly lose the ability to be imported into isolated mitochondria. Like many other chaperones, MSF has ATPase activity, and the activity is stimulated by the presence of some precursor proteins and synthetic mitochondrial presequences (Hachiya *et al.*, 1994; Komiya *et al.*, 1994). The ATPase activity was inhibited by isolated mitochondrial outer membranes, but not by membranes isolated from mitochondria that had been treated with trypsin. In addition, a complex formed between MSF and a purified, urea-denatured, precursor form of adrenodoxin could bind to outer membranes, and the binding has recently been shown to be dependent on Tom37 and Tom 70 (Hachiya *et al.*, 1995). It has also recently been shown that cytosolic hsp70, a more general molecular chaperone than MSF, can also stimulate the import of the adrenodoxin precursor into mitochondria under some conditions (Komiya *et al.*, 1996). The dependence of this activity on surface components of the mitochondria appears to be slightly different than that of MSF; when both MSF and hsp70 are present together, the MSF-dependent pathway appears to dominate.

The recent results with cytosolic molecular chaperones suggest that a function of at least some of the Tom proteins may be to interact with the chaperones. It is not yet clear, however, exactly how the Tom proteins perform these functions. Although MSF is said to recognize precursor proteins specifically, the specificity is not precise: the ATPase activity of MSF is stimulated by apocytochrome *c*, a mitochondrial protein that follows an import pathway independent of the Tom proteins (Mayer *et al.*, 1995c), and is not stimulated by rhodanese, a mitochondrial protein with a noncleavable presequence (Hachiya *et al.*, 1994). The presence of a synthetic mitochondrial presequence increases the affinity of MSF for purified outer membranes (Komiya *et al.*, 1996). This result, however, is not by itself a strong indication that the function of the chaperone is to recognize mitochondrial precursor proteins. We have recently found that the affinity of yeast cytosolic hsp70 for synthetic presequences correlated exactly with the ability of the presequences to import attached proteins into mitochondria (Endo *et al.*, 1996), yet there is no evidence that hsp70 is directly involved in the targeting of precursor proteins to mitochondria. The affinity of MSF and hsp70 for amphiphilic presequences may simply reflect the importance of protecting the cell from this type of sequence, which if left free in the cytosol might promote protein aggregation or inappropriate interactions of proteins containing these sequences with cellular membranes. That the binding of MSF to outer membranes increases in the presence of a presequence may or may not be related to the mechanism of targeting.

Although it is premature to make any conclusions about the generality of the interactions between molecular chaperones and Tom proteins, the new results may suggest an alternative view of the function of the Tom proteins. Molecular chaperones are involved in every step in the transport of a mitochondrial protein, from the synthesis of the precursor in the cytoplasm to the folding of the mature protein in the matrix space. Most

of the chaperones involved in mitochondrial protein import that have been identified so far function in the soluble spaces of the cell, either in the cytoplasm or in the mitochondrial matrix (Gething and Sambrook, 1992; Stuart *et al.*, 1994; Martinus *et al.*, 1995). It would not be unexpected, however, that chaperones would also be necessary to protect a protein as it passes through the mitochondrial membranes. Instead of thinking of the components of the outer and inner membranes as receptors and channels, perhaps it would be more appropriate to consider their roles as integral membrane chaperones. A protein extended across a lipid bilayer may be as sensitive to the aggregation of hydrophilic amino acid segments as an unfolded protein is sensitive to the aggregation of hydrophobic residues in aqueous solution. The Tom and Tim proteins may inhibit undesirable interactions in the membrane by forming a flexible interface between the lipids, the translocating proteins, and themselves. Under some conditions, the Tom proteins could appear to promote binding interactions in a receptor-like manner, but these interactions may not be strictly dependent on presequences nor would they necessarily be responsible for the specificity of targeting. As membrane chaperones, it would also not be surprising that the Tom proteins would be involved in the transfer of precursors from cytosolic chaperones, although this function again may not necessarily be dependent on presequences. It is worth noting here that the internal hsp70 of the mitochondrial matrix space displays functionally important interactions with translocation components of the mitochondrial inner membrane (Rassow *et al.*, 1995). Perhaps these interactions are analogous to those that occur on the outer surface of the mitochondria.

There are several appealing aspects to the idea that the Tom proteins may function as membrane chaperones. First, it may explain the redundancy of the Tom proteins. Just like the cytosolic hsp70s, which can be disrupted individually without causing great distress to the cell (Deshaies *et al.*, 1988), the individual Tom20, Tom37, and Tom70 proteins are not essential. Although Tom22 is an essential component of the translocation apparatus, this may reflect its unique membrane-spanning topology. There are indications that the segment of Tom22 exposed to the intermembrane space plays an essential role in translocation (Bolliger *et al.*, 1995), although this possibility remains disputed (Lill and Neupert, 1996). The presence of at least one membrane chaperone with segments in the intermembrane space may be critical for the mitochondria. The function of Tom proteins as membrane chaperones may also explain the presence of bypass pathways into mitochondria (Pfaller *et al.*, 1989). These pathways, by which precursors continue to be imported into mitochondria lacking surface proteins, are difficult to explain if the surface proteins function as traditional receptors. This contradiction is not an issue, however, if the surface components function as chaperones. Just as denatured proteins are often able to fold under laboratory conditions in the absence of soluble chaperones, precursor proteins may be able to be translocated into mitochondria in the absence of membrane chaperones. Under more demanding conditions, however, the chaperones control the process and make it more efficient. Short peptides, such as the synthetic presequences, may not depend on membrane chaperones under any conditions.

SUMMARY

Although a great deal of effort has been directed at determining the physical mechanism by which proteins are translocated into mitochondria, there is still not enough detailed experimental evidence about the process for it to be understood at the molecular level. Many components necessary for translocation have been identified, both in the cytosol, in the mitochondrial membranes, and in the matrix space, but the exact functions played by these components remain to be elucidated. Of critical importance as the components of the translocation system are characterized will be the application of rigorous biophysical methods to study the problem. Only if the components are analyzed under well-defined conditions can a true mechanism for the process be determined.

We have studied the biophysical properties of synthetic presequences and found that the presequences do not bind to saturable binding sites on the mitochondrial surface, nor is their rate of translocation into mitochondria saturable. Experiments in model membrane systems suggest that presequences may behave as cationic, membrane-permeant molecules and may not be recognized by mitochondrial receptors as is conventionally believed. Instead, the recognition may take place after presequences have partitioned into the outer membrane, either by components of the outer or inner membrane, or by the electrical potential of the inner membrane. Because the interactions between precursor proteins and surface components of the mitochondria have been defined only by indirect means, it

is impossible to conclude that these components are able to bind presequences specifically. Instead, the components may serve to mediate interactions between precursors, cytosolic chaperones, and the mitochondrial surface or they may provide chaperone-like functions in the mitochondrial membranes. Until the components of the outer membrane have been purified, reconstituted, and analyzed by rigorous physical methods, it is premature to conclude that that these components are receptors for presequences.

ACKNOWLEDGMENT

I thank Dr. Toshiya Endo for helpful discussions.

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