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How Do Polypeptides Cross the Mitochondrial Membranes?

Minireview

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Many newly synthesized proteins must cross intracellular membranes to reach their functional locations in the eukaryotic cell. In recent years, considerable information has accumulated about the initiation of this process and steps following the transfer across membranes of the endoplasmic reticulum (ER), mitochondria, chloroplasts, and peroxisomes. However, the translocation process per se, i.e., precisely how a polypeptide chain crosses a membrane, is largely obscure. We discuss this problem using protein translocation into mitochondria as an example (for reviews, see Eilers and Schatz, 1988; Pfanner and Neupert, 1990; Hartl and Neupert, 1990).

A molecular description of this process should be able to answer the following basic questions. What are the conformational states of a polypeptide chain during movement across the membranes? What is the environment of a polypeptide chain in transit? How is the membrane permeability barrier maintained during polypeptide movement? The inner membrane of mitochondria remains impermeable to protons and other ions despite a massive influx of polypeptides into mitochondria (~50–150 polypeptides per second are translocated into each mitochondrion of a rapidly growing fungal cell). What are the energetics of this transport process? Proteins have to be unfolded to be translocated and are refolded after translocation. What provides the energy for unfolding, and does the free energy of refolding help drive the translocation process?

We present a summary of the transfer of proteins across the two mitochondrial membranes (from the cytosol into the mitochondrial matrix) and propose a hypothesis about the physical pathway and energetics by which polypeptide chains cross the membranes.

Folding Reactions during Translocation

Polypeptide chain unfolding is a prerequisite for membrane translocation (Schleyer and Neupert, 1985; Eilers and Schatz, 1986). Cytosolic factors such as heat shock proteins of 70 kd (hsp70s) may help keep precursor proteins in a loosely folded ("unfolded") conformation by multiple cycles of precursor binding and ATP-dependent release (Deshaies et al., 1988; Murakami et al., 1988; Pfanner et al., 1990). This role is in agreement with their proposed function as molecular chaperones or polypeptide chain binding proteins (for reviews, see Ellis and Hemmingsen, 1989; Rothman, 1989; Pelham, 1988). However, translocation may require complete loss of tertiary structure (see below). The polypeptide chain is still unfolded when it has completed transmembrane transfer; in the case of mitochondria, this was found to be true for precursor proteins interacting with heat shock proteins (mitochondrial hsp70 and hsp60) in the mitochondrial matrix (Ostermann et al., 1989; Kang et al., 1990). Acquisition of stable tertiary structure only occurs after association with hsp60 and probably other protein factors as well.

Polypeptide Chains in Transit

By preventing the unfolding of a polypeptide domain on the cytosolic side, translocation intermediates that span both mitochondrial membranes can be accumulated (Schleyer and Neupert, 1985; Chen and Douglas, 1987; Rassow et al., 1989). Their amino terminus is in the mitochondrial matrix, whereas the carboxyl terminus faces the cytosol. What is the state of the membrane-spanning portion of these molecules? About 50 amino acid residues are found to be sufficient to span both mitochondrial membranes (reviewed in Hartl and Neupert, 1990). The polypeptide should span at least across two lipid bilayers (having a total thickness of 10–12 nm). The distance across the two protein-rich mitochondrial membranes is predicted to be 15–20 nm, which is in agreement with the distance actually measured by electron microscopy. The structure of the membrane-spanning portion of the polypeptide is probably not α-helical, because in this conformation 50 amino acid residues would be 7.5 nm in length. More likely, it is either in a β sheet (17.5 nm) or very extended (18.1 nm) conformation such that the polypeptide backbone is exposed.

Spanning intermediates possess intriguing properties. Only limited amounts of them can be accumulated (about 1000–5000 molecules per mitochondrion; Vestweber and Schatz, 1988; Rassow et al., 1989). They are not spontaneously released from the membranes, even when only a few amino acid residues are exposed on the matrix side and a completely folded domain (e.g., derived from dihydrofolate reductase) is on the outside. Accumulation of spanning intermediates in vitro or in vivo does not cause de-energization of mitochondria. Thus, the spanning intermediates do not cause leakage of protons or potassium ions across the inner mitochondrial membrane. How can the membrane remain sealed when the amino acid side chains in transit vary greatly with respect to size, hydrophobicity, and charge? This question is particularly challenging in light of the fact that a very few proteinaceous pores (formed by colicins) can dissipate a membrane potential in E. coli.

The exact environment of polypeptide chains in transit is unknown. Precursor proteins spanning both membranes can be extracted with aqueous perturbants, similar to the case of precursor proteins spanning the ER membrane. Although the significance of this approach is clearly limited, it provides a preliminary indication that the intermediates are in a hydrophilic (probably proteinaceous) environment. Furthermore, a number of imported mitochondrial proteins (particularly those translocated

into the intermembrane space or inner membrane) contain stretches of more than 20 uncharged amino acid residues. These polypeptides do not become arrested upon passage through the mitochondrial membranes into the matrix, demonstrating that the hydrophobic sequences do not act as stop-transfer sequences (Hartl et al., 1987). In several cases, the hydrophobic stretches appear to serve as signals for reexport to the intermembrane space (e.g., cytochrome c_1) or for reinsertion into the inner membrane (e.g., subunit 9 of F_0–ATPase) (Hartl and Neupert, 1990). One may thus argue that the precursor proteins pass through an environment, such as a hydrophilic pore, that shields them from the lipid bilayer.

Energy Requirements for Membrane Passage

Why is metabolic energy needed to translocate a protein from one side of the membrane to the other? Practically all precursor proteins differ from their mature counterparts in conformation or aggregation state, and most precursors carry additional sequences that are cleaved off during or after translocation (Eilers and Schatz, 1988; Pfanner and Neupert, 1990). Thus, in principle, the differences between the free energies of folding each protein in the cytosol versus the matrix could be sufficient to drive import. Nevertheless, distinct requirements for external energy sources are observed.

Hsp70s, in cooperation with ATP (and other protein factors), are required in the cytosol to prevent aggregation or malfolding of precursor proteins (Deshaies et al., 1988; Murakami et al., 1988; Pfanner et al., 1990). Although rather important for keeping various precursors in a translocation-competent state, this requirement is probably not immediately relevant for membrane translocation (see below). The electrical potential across the inner mitochondrial membrane ($\Delta\psi$) is essential for the translocation of the presequence of precursors. Further translocation does not require $\Delta\psi$. Hence, $\Delta\psi$ is an energy source to initiate or trigger the movement.

Surprisingly, the actual translocation process appears not to require energy in the form of free ATP. A hybrid precursor protein containing the complete dihydrofolate reductase at the carboxyl terminus was accumulated in a membrane-spanning fashion such that dihydrofolate reductase was outside the outer membrane and the processed amino terminus was in the matrix space. Drastic reduction of the levels of ATP did not reduce the efficiency of unfolding of the precursor polypeptide or its transfer into the matrix (Pfanner et al., 1990). Similarly, addition of ATP was not required when precursors synthesized in vitro were unfolded with 7 M urea and then imported into mitochondria (Ostermann et al., 1989). In contrast, other precursor proteins, such as that of the β subunit of F_1–ATPase ($F_1\beta$), directly imported from reticulocyte lysate had a distinct ATP requirement.

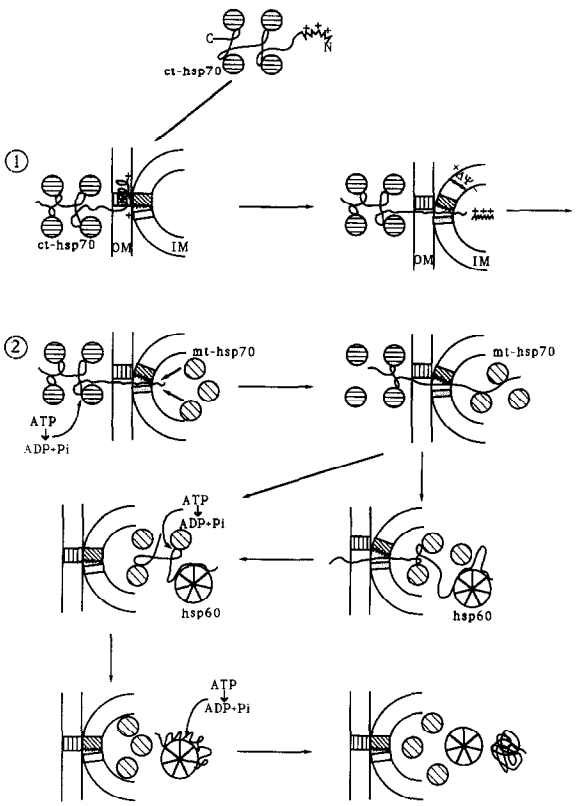
How can these seemingly contradictory findings be explained? A satisfying explanation is that some precursors (such as pre-$F_1\beta$) require ATP to release bound cytosolic hsp70. If cytosolic hsp70 is not associated with precursors (because it has been removed by urea treatment, or because a protein such as dihydrofolate reductase assumes a native conformation without exposing binding sites for hsp70), ATP is not required in the cytosol. Thus once the translocation has been initiated, it appears a polypeptide chain can cross the mitochondrial membranes without any requirement for added ATP.

What then provides the energy for the vectorial movement of precursors? Upon reaching the matrix, precursors bind components in the mitochondrial matrix (in particular mitochondrial hsp70 and hsp60) and remain in a rather unfolded state. In fact, mitochondrial hsp70 is necessary for protein import into mitochondria (Kang et al., 1990). Transfer of precursors into mitochondria is defective in yeast *ssc1* cells, which have an altered mitochondrial hsp70. Isolated mitochondria also showed a defect in import. Precursors were found to be arrested in contact sites between both membranes. The mutant phenotype could be overcome in vitro when urea-denatured precursors were imported. Precursors imported into the matrix under such conditions remained in an unfolded state in association with mutant mitochondrial hsp70. These findings suggest that mitochondrial hsp70 has a dual role: it is required both for translocation of polypeptide chains after their insertion into translocation contact sites and folding of newly imported polypeptides in the matrix (Kang et al., 1990).

Intermediates associated with mitochondrial hsp70 appear to be transferred to hsp60, where they undergo ATP-dependent folding (Ostermann et al., 1989). The energy for the movement through the membrane may be derived from binding to mitochondrial hsp70 and perhaps additional matrix components rather than from folding to the final conformation. Thus, ATP hydrolysis is not directly required for import. However, indirectly it is necessary to allow new rounds of transport since the release of mitochondrial hsp70 from the incoming precursor most likely requires ATP hydrolysis. Import of chemical amounts of precursors indeed requires ATP hydrolysis in the matrix (Hwang and Schatz, 1989). It should be noted that, in addition, part of the energy requirement may be derived from a partial refolding: going from an extremely unfolded conformation to a conformation with a small amount of secondary structure.

Hypothesis

We propose that the overall process is divided into two major steps (see figure). The first step is the triggering of translocation. During synthesis, many mitochondrial precursor proteins interact with cytosolic hsp70s to prevent aggregation and misfolding. A transport-competent conformation of precursors is thereby stabilized. Moreover, in certain cases, binding to cytosolic hsp70s may prevent folding to a stable mature form and keep the presequences exposed. With the help of receptors (MOM19 and MOM72) and other components in the outer membrane such as the general insertion protein, the presequences are then inserted and translocated across the outer membrane (Pfanner and Neupert, 1990). At contact sites, the positively charged presequences respond to the electrical membrane potential and are translocated across the inner membrane. In this way, the precursors become inserted into the proteinaceous translocation machinery and are unfolded so that the polypeptide backbone becomes ex-



Hypothetical Translocation Pathway of Mitochondrial Precursor Proteins from the Cytosol into the Matrix

(1) Triggering step: The amino-terminal presequence of a precursor protein is inserted into the mitochondrial outer membrane (OM) and, by responding to the membrane potential $\Delta\psi$, gets translocated across the inner membrane (IM), leading to extensive unfolding of the polypeptide segment spanning the membranes. By binding cytosolic (ct) hsp70s, some precursor proteins avoid misfolding.

(2) Translocation and folding step: Mitochondrial (mt) hsp70s bind the extended polypeptide chain emerging on the matrix side, pull the precursor across, and thereby facilitate unfolding of the domains on the cytosolic side. In the cytosol, ATP hydrolysis is required for those precursors that interact with cytosolic hsp70s to release the hsps. During or after membrane translocation, the polypeptide is transferred from mitochondrial hsp70 to hsp60, where it becomes folded. ATP hydrolysis is needed for release of the polypeptide from mitochondrial hsp70, as well as folding on and release from hsp60.

posed. In the matrix, the presequences then interact with the processing-enhancing protein and are cleaved by the mitochondrial processing peptidase (Hartl and Neupert, 1990).

The second step is the translocation of the major part of the polypeptide chain. This translocation is not driven by $\Delta\psi$, but by the binding of mitochondrial hsp70s to the unfolded polypeptide chain emerging on the inner face of the inner membrane. Because hsp70s preferentially interact with unfolded polypeptides (Rothman, 1989), mitochondrial hsp70 may exhibit a very high affinity for the extended precursor polypeptide chains, while cytosolic hsp70s may bind with a lower affinity to the partially folded portions of the precursor on the cytosolic side. The protein has to unfold in order to progress through the contact sites. Protein components assisting in this reaction may be present on the mitochondrial surface. This unfolding occurs in a stepwise fashion as translocation proceeds, rather than by complete unfolding of the entire precursor. Minor local unfolding events allow the polypeptide chain to move into and across the membrane, with binding of mitochondrial hsp70s on the other side "pulling" the polypeptide chain further in. Translocation thus is driven by the high affinity binding of mitochondrial hsp70s to the unfolded precursors.

It should be borne in mind that the energy for complete unfolding of precursors (as a prerequisite for translocation) is as low as 5–15 kcal/mol. ATP is required for the release of polypeptide chains from mitochondrial hsp70s, setting mitochondrial hsp70s free for new rounds of transport. ATP is also needed in the cytosol to release hsp70s from those precursors that interact with cytosolic hsp70s. In a series of successive steps requiring ATP hydrolysis, the incoming polypeptide chain then interacts with hsp60 and thereby becomes folded and eventually released.

An important part of this hypothesis is that the polypeptide chain has to be extended while crossing the membranes. This implies certain properties of the components of the putative translocation machinery. One may speculate that the inner membrane contains a component that faces the polypeptide chain backbone. The particular shape and motion of components that interact with the amino acid side chains could then be a means to avoid leakage of protons or other ions. Furthermore, complete unfolding would explain why there is no apparent specificity for defined sequences or certain conformations in precursor proteins once the triggering step has been overcome. This lack of specificity is quite striking, in view of the generally observed requirement for distinct sequences or three-dimensional structures in practically all biological reactions.

Relevance for Other Membranes

In the ER, a number of components involved in translocation have been identified by genetic and biochemical means. However, a distinct role could not be assigned to any of these components. Triggering of translocation in the ER and mitochondria may be quite different: the signal sequences are different in structure, and a requirement for a membrane potential has not been found for the ER. There is, however, some evidence in favor of a rather extended structure of polypeptide chains in transit (Bergman and Kuehl, 1979). Furthermore, cytosolic hsp70s have a distinct role in transport, perhaps very similar to that in mitochondrial translocation (summarized by Pelham, 1988). Strikingly, BiP, the hsp70 homolog in the ER lumen, is essential for protein transfer into the ER (Vogel et al., 1990). However, a direct role of BiP in polypeptide chain movement across the membrane has not been demonstrated. BiP almost certainly has a variety of additional functions in posttranslocational processes. In summary, all the essential elements of the above hypothesis, hsp70s on both sides of the membrane and extensive unfolding, appear to be present in the ER system. Since translocation in the ER is mainly cotranslational, the role of cytosolic hsp70s may not be as important as in mitochondria, where translocation is mainly posttranslational.

Prokaryotic protein export seems to be considerably

different with regard to the role of hsp70s. Many secreted proteins depend on cytosolic components, such as SecB and SecA, that appear to have a role in folding and unfolding but in a manner different from hsp70 function. Whereas SecB has no requirement for ATP, SecA is believed to generate unfolded regions of presecretory proteins in a stepwise and ATP-dependent manner on the inner face of the bacterial plasma membrane (Wickner, 1989). A role for DnaK, the hsp70 homolog in the cytosol of prokaryotes, has been reported for secretion of LacZ fusion proteins (Phillips and Silhavy, 1990). However, a homolog of hsp70 has not been found in the periplasmic space, and a role for ATP hydrolysis in the periplasmic space is not likely.

The transport of proteins into chloroplasts may well share some critical features, such as dependence on hsp70s and hsp60, with mitochondrial protein import, especially in view of the putative evolutionary relationship, although a $\Delta\psi$ is not needed (Keegstra, 1989). However, current knowledge about the transport apparatus of chloroplasts does not allow detailed conclusions about the mechanisms involved. Similarly, it is difficult to make predictions about protein import into microbodies/peroxisomes; but the presence of signal sequences in the extreme carboxyl terminus of precursors (Lazarow, 1989) may imply the existence of a distinct mechanism.

Perspectives and Limitations

Translocation of precursors into mitochondria does not follow a uniform process. Precursors destined for the outer membrane (e.g., porin) and some of those destined for the intermembrane space (e.g., apocytochrome c which does not pass through the matrix) use a different pathway that may not involve complete unfolding. Such precursors, in particular those destined for the outer membrane, apparently share initial steps and components; however, they have to insert into or cross only the "leaky" outer mitochondrial membrane (Hartl and Neupert, 1990; Pfanner and Neupert, 1990).

Future studies will have to characterize additional components of the mitochondrial import apparatus, such as components in the cytosol, membrane contact sites, and the matrix. The reconstitution of unfolding, translocation, and folding reactions with purified components should allow a test of the predictions made in this hypothesis.

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