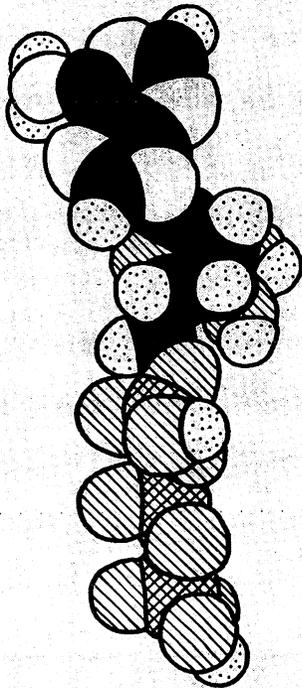


BIOENERGETICS

**STRUCTURE and FUNCTION
of ENERGY TRANSDUCING SYSTEMS**

Edited by
Takayuki OZAWA and Sergio PAPA



With 126 Figures and 25 Tables

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Intracellular and Intramitochondrial Sorting of Mitochondrial Precursor Proteins

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Upon division, any eucaryotic cell must regenerate its original content of the various organelles. As membranes cannot be synthesized *de novo* the organelles must multiply by growth and division. Lipids seem to become inserted into the growing membrane with the help of "transfer" or "exchange" proteins. Newly synthesized proteins, as far as we understand, are incorporated into membranes by two different mechanisms. One is the membrane flow mechanism in which proteins are first inserted into the membrane or lumen of the rough endoplasmic reticulum (r-ER) and are then delivered to the Golgi apparatus, the lysosome, the plasma membrane and the other organelles of the endomembrane system, or are secreted into the extracellular space. This mechanism requires fusion and segregation of membrane vesicles. The initial insertion of the protein into the r-ER appears to be by a cotranslational reaction in the majority of cases (*i.e.*, by ribosomes attached to the ER membrane). The second mechanism involves the synthesis of precursor proteins on free ribosomes in the cytoplasm. The proteins are then post-translationally inserted into the respective organelles, such as mito-

chondria, chloroplasts, glyoxysomes, and peroxisomes (1). A huge number of proteins are subject to this targeting process from the cytoplasm to their specific sites of function. Therefore, a highly elaborate system for sorting in the cell must exist. This includes targeting signals on the precursor proteins and recognition and translocation machinery of the respective organellae.

In this article we want to focus on the import of mitochondrial proteins. On the one hand, we will discuss the characteristics of the precursor proteins and the signals that they contain which direct the proteins to the organelle and to different subcompartments. On the other hand, factors in the cytoplasm and structures and components of the mitochondrion that function as import receptors, translocation contact sites and maturation enzymes will be described. Finally, the energy requirement of these processes will be discussed.

INTRACELLULAR SORTING

The synthesis of all cellular proteins, with the exception of those that are made by the protein synthetic apparatus of mitochondria and chloroplasts, starts on free cytoplasmic ribosomes. The majority of proteins are synthesized to completion on the free ribosomes and discharged into the cytoplasm. These proteins then have to be sorted post-translationally. Synthesis of proteins that enter the secretory pathway also starts on free ribosomes. As soon as the signal sequence emerges from the ribosome, however, it interacts with a ribonucleo-protein, the signal recognition particle (SRP). SRP then directs the ribosome to the r-ER by binding to its receptor, the docking protein (or SRP receptor) on the surface of the r-ER. Protein synthesis then continues in a vectorial fashion across the membrane into the r-ER lumen (for more details on cotranslational translocation of proteins see ref. 2).

All proteins that function in a compartment other than the cytoplasm must have targeting signals which direct them to their unique organelle. These are very often contained within N-terminal sequences, but are also found within the polypeptide chain in some cases. The N-terminal peptides can either be transient or permanent structures. Consensus or minimal sequences have been identified for targeting to a

variety of organelles (An up-to-date overview of the common features of targeting signals to the various organelles is given in ref. 3). Signals for the import of proteins into mitochondria are probably the most thoroughly investigated targeting sequences. They occur as either cleavable N-terminal extensions as in most of the matrix and inner membrane proteins, or they may be non-cleavable signals as in proteins destined for the outer membrane and some of the intermembrane space and inner membrane proteins (1).

SIGNALS IN MITOCHONDRIAL PRECURSOR PROTEINS

Recently, a number of laboratories have begun exploiting recombinant DNA techniques to dissect mitochondrial precursor proteins in order to define the minimal structures that are necessary for mitochondrial targeting and import. Most of these studies were carried out *in vitro* but occasionally *in vivo* studies were performed to confirm the significance of the *in vitro* findings.

1. Mitochondrial Targeting Signals

Hybrid proteins were constructed in several laboratories that contained the cleavable N-terminal sequences of mitochondrial proteins fused to the complete sequence of the cytoplasmic protein dihydrofolate reductase (DHFR). These fusion proteins could be imported into mitochondria *in vitro* and *in vivo*. They were also processed (4, 5). A truncated mitochondrial protein where the cleavable N-terminal sequence had been removed was no longer targeted to mitochondria (4). Two proteins without cleavable presequence have been probed for a targeting signal so far. One is the yeast 70 kD outer membrane protein and the other the ADP/ATP carrier from the inner membrane of yeast. The first 41 amino acids of the 70 kD protein fused to β -galactosidase (β -gal) inserted the fusion protein into the outer membrane of isolated yeast mitochondria in the correct orientation of the 70 kD protein (6). In the case of the ADP/ATP carrier, the first 115 amino acids were sufficient to target β -gal to mitochondria *in vivo* (7). From the data it is concluded that either the cleavable presequences or non-cleavable N-terminal sequences or yet undefined sequences within the mitochondrial

protein are sufficient to direct a protein to the mitochondrion. The common theme of all mitochondrial targeting signals looked at so far is that they are rich in positively charged and hydroxylated amino acids, they contain hydrophobic amino acids and can form amphipathic helices (8).

2. *Intramitochondrial Sorting Signals*

So far we have been dealing with targeting. The mitochondrion, however, is a complex organelle with two membranes, thus giving rise to four subcompartments: the outer membrane, intermembrane space, inner membrane and the matrix. Therefore, beside targeting signals there must be additional signals for intramitochondrial sorting.

The 70 kD outer membrane protein of yeast is synthesized as a mature size protein. The first 41 amino acids of the 70 kD protein were shown to anchor the protein in the outer membrane, with the major part of the protein facing the cytoplasmic side of the membrane (6). The N-terminal 21 amino acids fused to a passenger protein, however, directed this hybrid protein into the mitochondrial matrix (9). Similar results were obtained when the cleavable presequences of the intermembrane space protein cytochrome c_1 was put in front of a "passenger" protein (26). A short stretch of 10–20 amino acids enriched in basic residues was able to direct such a fusion protein into the mitochondrial matrix, whereas a fusion with the entire presequence could be found in the intermembrane space. This stretch resembles the cleavable signals of mitochondrial matrix proteins. It was therefore proposed that all these sequences are "matrix targeting" sequences, and that targeting to the mitochondrion means targeting to the mitochondrial matrix (8). According to this hypothesis sorting into the other mitochondrial compartments requires hydrophobic stretches which are thought to stop the transport across the outer or the inner membrane, thereby defining outer or inner membrane proteins; they were called "stop transfer" sequences (8). With intermembrane space proteins, like cytochrome c_1 , the "stop transfer" sequence is cleaved off at the outer surface of the inner membrane to release the hydrophilic portion of the protein into the intermembrane space. Convincing experimental support for this model is, however, not available. One possible drawback of the model is that it

cannot readily explain why some proteins are trapped by their "stop transfer" sequences in the outer membrane whereas others pass through the outer membrane and are stopped at the inner membrane only.

PROPERTIES OF THE SORTING SYSTEM

The various signals in the mitochondrial precursor proteins discussed so far must be read and decoded by some sorting machinery. Sorting acts on multiple levels and the mitochondrial components involved are not well characterized at a molecular level.

As far as we understand the order of events, we can ask the following questions: how do the precursor proteins travel through the cytoplasm? Why do they specifically insert into mitochondrial membranes and not into others? Why does the import of most mitochondrial proteins depend on the membrane potential? All inner membrane and matrix proteins must obviously cross the outer membrane to get to the inner membrane or go through it. Does this occur in two steps with a soluble intermediate in the intermembrane space, or are the two membranes traversed in a single step? Is this translocation mediated by proteins? Why do newly imported proteins not readily leave the mitochondrion again?

1. Cytosolic Factors Involved in the Import of Mitochondrial Proteins

Most mitochondrial precursor proteins are synthesized on free ribosomes. Thus a small but detectable pool of precursors is found in the cytoplasm *in vivo*. How do precursor proteins "travel" from the ribosome to the mitochondrial surface? *In vitro* studies indicated that the ADP/ATP carrier and ATPase SU9 precursors synthesized in the reticulocyte lysate form oligomers (1). Other proteins like cytochrome *c* or porin behave like monomers or dimers. Whether the oligomers are homo-oligomers, or whether they are hetero-oligomers associated with "helper" proteins is not known. Several laboratories have reported that proteinaceous and RNA containing cytoplasmic factors may be involved in import of mitochondrial proteins (8). The molecular mechanism of the cofactor function, however, remains to be elucidated.

There have been reports that cytoplasmic ribosomes can be found

associated with the mitochondrial outer membrane, and in one study it was shown that cytosolic mRNA coding for mitochondrial proteins is enriched in the mitochondrial fraction (1). This may indicate that in some cases import starts before the nascent chain is detached from the ribosome.

2. Mitochondrial Import Receptors

The first step in the import of mitochondrial proteins is high affinity binding to saturable sites on the mitochondria. This could be shown by Scatchard plot analysis for a variety of proteins including the outer membrane protein porin (Pfaller and Neupert, manuscript in preparation), the cytochrome *c* of the intermembrane space (10) and the ADP/ATP carrier of the inner membrane (11). The affinities and number of binding sites determined are summarized in Table I.

Scatchard plot analysis requires large amounts of precursor proteins so that usually it cannot be done with newly synthesized precursors from *in vitro* protein synthetic systems. This has only been possible in the case of the ADP/ATP carrier. In the case of porin, however, it could be shown that the mature protein isolated from the outer membrane of *Neurospora crassa* regained properties of the precursor (*e.g.*, protease sensitivity) upon denaturation, extraction of lipids and resolubilization in a detergent free buffer. This water-soluble porin could bind specifically to saturable sites at the outer mitochondrial membrane since it prevented the import of newly synthesized porin precursor (12). The specific binding of ¹⁴C-labelled water-soluble porin to mitochondria at 0°C could compete with an excess of unlabelled water-soluble porin. The chemically prepared porin precursor appears to compete with the import of

TABLE I
Comparison of the Affinities and Numbers of Binding Sites for Mitochondrial Precursor Proteins

	Affinity (K_a) (M^{-1})	Number of binding sites (pmol/mg)	Reference
Apocytochrome <i>c</i>	2.2×10^7	90	10
Porin	0.7×10^9	5-7	(Pfaller and Neupert in preparation)
ADP/ATP carrier	1.1×10^9	2-5	11

ATPase SU9 and the ADP/ATP carrier at low temperature but not with ATPase SU2 (Pfaller and Neupert, manuscript in preparation). This can be taken as indication that porin, the ADP/ATP carrier and SU9 use the same import receptor, whereas the receptor for SU2 is a different protein.

Several authors showed that import of mitochondrial precursor proteins is abolished by pretreating mitochondria with proteases (1). This implies that the binding sites are proteinaceous import receptors. Treatment of mitochondria with different proteases provided evidence that distinct receptors exist (13). Elastase treatment of mitochondria, for example, abolished import of the ADP/ATP carrier and porin, while it did not affect the import of ATPase SU2. While the receptors for most proteins are highly susceptible to proteases (0.5–1.0 μg of trypsin per mg of mitochondria per ml reduced import of ATPase SU2 by 75%) (13), import of cytochrome *c* is much less protease sensitive (40 μg of trypsin under the same conditions is required (Köhler *et al.*, manuscript in preparation)). Thus several receptor-like components of the import machinery have been defined which are necessary for import of different proteins. It is not known how many receptors mediate the import of the mitochondrial proteins. However, as the outer membrane contains only a limited set of proteins one must conclude that groups of proteins use common receptors.

Recently, a protein was isolated from *N. crassa* mitochondria which could bind apo-cytochrome *c* with a high affinity, thus exhibiting properties of a receptor. Surprisingly, the protein turned out to be an inter-membrane space component (Köhler *et al.*, manuscript in preparation). This could explain the high protease resistance of the apo-cytochrome *c* receptor. It has been shown that apo-cytochrome *c* can spontaneously insert into artificial membranes (14). If it is inserted into the outer mitochondrial membrane, it might then interact with the binding protein at the inner face of the outer membrane. This interaction could be responsible for the high affinity binding observed. Apo-cytochrome *c* was prepared from holo-cytochrome *c* by chemical removal of the heme group. This chemically prepared apo-cytochrome *c* was imported into mitochondria and converted to holo-cytochrome *c* by heme addition. Therefore it could be used for binding studies. Apo-cytochrome *c* pre-

pared in this way does not compete with the import of any other protein investigated so far, and does not require a membrane potential for import. Apo-cytochrome *c* from different species, however, can compete with each other for binding to mitochondria. This, together with the observation that the binding protein has an unusual location suggests that apo-cytochrome *c* uses a unique import pathway (10).

In a number of studies the effect of chemically synthesized peptides (with the sequence of known prepieces) on the import of proteins was investigated. Usually they inhibited import, but since all of these peptides are membrane active, the primary effect in most cases may well have been on dissipating the membrane potential and not a specific receptor interaction. There exists only one report where the authors claim that the effect of the peptide could be overcome by increasing the amount of precursor added, so that in this case real competition was observed (15). All these findings taken together, namely, protease sensitivity, competition, high affinities, and limited numbers of binding sites, are clear indications of proteinaceous import receptors.

3. *Energy Requirement*

Import of most mitochondrial proteins is inhibited by uncouplers of oxidative phosphorylation (16). That means that some sort of energy is required for the import of these proteins. By a series of experiments it was shown that the membrane potential and not the pH gradient or the total proton motive force, is required for the translocation of proteins across the inner membrane (17). Import of outer membrane and some intermembrane space proteins does not depend on the membrane potential (16).

From recent work of our group it is known that the membrane potential is needed only for the translocation of an N-terminal part of the precursor protein across the inner membrane. Once this part of the protein has been translocated, import continues independently of the membrane potential (18). This observation has been extended to a number of proteins, including the ADP/ATP carrier which does not contain a cleavable N-terminal extension.

As the mitochondrion is the only organelle with the membrane potential negative inside, and as all known presequences carry a net

positive charge (the ADP/ATP carrier also contains clusters of positive charges, though the targeting signal has not been defined yet), it has been argued that the charged presequences could be affected by the membrane potential even outside the mitochondrion and that it is the membrane potential that is the prerequisite for the specific interaction of the mitochondrial precursors with the organelle (8). This view, however, does not take into account the finding that some proteins can bind to mitochondria in the absence of the membrane potential in such a way that upon restoration of the potential they can be imported from their binding sites. This indicates that even in the absence of the membrane potential the proteins bind to specific receptors from which they can be imported. We speculate that the role of the membrane potential might be to facilitate import by an electrophoretic effect in a step following the initial recognition event (17). Other effects of the membrane potential, such as perturbation of the membrane lipid structure, however, cannot be excluded.

4. *Translocation Contact Sites*

The mitochondrion is an organelle with two membranes. Therefore, proteins that have to be translocated into the matrix must traverse two lipid bilayers. This could happen in two steps with an intermediate in the intermembrane space, or in a single step across both membranes. We have presented evidence that translocation across the two membranes indeed occurs in one step. When the precursors of ATPase SU2 or cytochrome c_1 , synthesized in the reticulocyte lysate, were imported into mitochondria *in vitro* at a temperature below 12°C, it was observed that the proteins were correctly processed at the N-terminus by the processing peptidase, but were still sensitive to externally added proteases. Raising the temperature to 25°C for a short time rendered the proteins protease resistant indicating that this particular form of the protein was on the correct import pathway. The shift to the protease resistant localization was independent of the membrane potential. It also occurred if uncouplers were added before the temperature shift, whereas no import could be observed if the membrane potential had been dissipated prior to import. These observations were interpreted in terms of a translocation intermediate spanning both inner and outer

membranes simultaneously (18). The low temperature translocation intermediate is not stable in the membrane. Even at 0°C it moves to a protease resistant location with a halftime of 30 min (Schwaiger and Neupert, unpublished data). This could mean that the rigidity of the membrane controls the rate of import under these circumstances.

A translocation intermediate with the same properties could also be generated by a different approach. The precursors of ATPase SU2 or cytochrome c_1 synthesized in the reticulocyte lysate were reacted with a specific antibody. When the precursor-antibody complex was allowed to interact with mitochondria a substantial amount of the complex associated with the mitochondria in a way that the N-terminus was processed while the C-terminal part associated with the antibody was still protease sensitive (18).

Since ATPase SU2 does not bind to mitochondria nonspecifically (1) and since the antibody-SU2 complex is the only protein A binding component present on the mitochondrial surface, immunocytochemistry with the protein A-gold technique could be applied to locate the translocation intermediates on the mitochondrial surface. Mitochondria containing the antibody-ATPase SU2 complex were isolated from the reticulocyte lysate, washed and then incubated with protein A-gold. After a second washing step the mitochondria were fixed in glutaraldehyde and sectioned. Gold grains (visualized by electron microscopy) were found associated with the mitochondria only in places where the two membranes came close together forming translocation contact sites (Schwaiger *et al.*, manuscript in preparation). They resembled the sites of contact previously seen by Hackenbrock (19), the function of which is not known. The translocation contact sites seem to be stable structures because they survived shearing forces or digitonin treatment which removed part of the outer membrane. The contact sites persisted regardless of whether a translocation intermediate had been generated before or not. In addition, following digitonin treatment which removed 60 to 80% of the outer membrane (as judged by the removal of porin), the formation of the translocation intermediate was virtually unaffected. Control experiments where the mitochondria had been shaved with trypsin, to remove the receptors before the digitonin treatment, demonstrated that no additional receptors or import sites exist on the now

exposed inner membrane (Schwaiger and Neupert, manuscript in preparation). It seems possible that all proteins which are imported in a membrane potential dependent fashion are imported *via* translocation contact sites. This was directly shown for ATPase SU2, the cytochrome c_1 (18), the FeS of complex III (20), the ADP/ATP carrier (Schwaiger *et al.*, unpublished data) and the ATPase SU9 (Pfanner and Neupert, unpublished data).

When import of a fusion protein containing the cytochrome oxidase SU IV prepiece and the complete sequence of DHFR was studied in the presence or absence of methotrexate, a drug which binds to the active site of DHFR stabilizing the folded conformation, it was observed that in the presence of methotrexate import was no longer possible (21). We performed experiments where we reacted precursor proteins with Fab fragments instead of complete antibody molecules and asked whether this complex could be imported. The precursor Fab complex, however, formed the translocation intermediate as did the precursor IgG complex (Schwaiger and Neupert, unpublished data). These experiments suggest that the precursors must at least partially unfold to be imported.

5. *Sorting of Proteins into Mitochondrial Compartments*

We have studied the import of the iron sulphur protein (Rieske FeS protein) of the complex III, a protein whose functional hydrophilic portion is localized on the outer surface of the inner membrane. The cytoplasmically synthesized precursor of the FeS protein (p-FeS) contains a 32 amino acid long N-terminal extension which is processed in two steps. Import *in vitro* was found to be receptor mediated and to depend on the membrane potential. The occurrence of a translocation intermediate of the protein could also be demonstrated. The first processing step which yields the intermediate size form (i-FeS) is performed by the matrix-located processing peptidase, as shown by incubation of the precursor (synthesized in the reticulocyte lysate) with the partially purified processing peptidase. If the processing peptidase in intact mitochondria was inhibited by EDTA and *o*-phenanthroline, protease resistant precursor could be found associated with the mitochondria. This p-FeS protein was readily extracted from the mitochondria by sonication in low salt. Upon digitonin fractionation it behaved like fumarase,

a matrix protein. Under conditions where the processing peptidase was not inhibited, but only the second processing step was, i-FeS accumulated. This species was also located in the matrix according to its extractability in buffers with low salt and by digitonin fractionation. The assembled mature FeS protein fractionated with the inner membrane and was extracted only by 0.1 M sodium carbonate. Under appropriate conditions accumulated p-FeS or i-FeS could be chased to the mature sized form, which behaved essentially like the mature protein *in vivo*; it was protease sensitive under low digitonin concentrations that opened the outer membrane and released intermembrane space markers but left the inner membrane intact. The imported precursor was protease resistant under the same conditions (20).

On the basis of these experimental data the following model for the import of the FeS protein is proposed. p-FeS interacts with mitochondrial import receptors. It is imported completely into the matrix *via* translocation contact sites, where the cleavage of the matrix targeting sequence occurs. The so generated i-FeS is then translocated back across the inner membrane. Thereby a second processing takes place and the Fe/S cluster is formed, to yield the mature protein that assembles into complex III. Exactly where the second processing step takes place is currently being investigated together with the question as to whether the second cleavage is a prerequisite for retranslocation, or whether the second cleavage is a prerequisite for retranslocation, or whether the second prepeptide is a targeting sequence for the retranslocation across the inner membrane and is cleaved after transfer. In any case, the octapeptide cleaved in the second processing step cannot be a "stop transfer" sequence as proposed for the equivalent sequence of the precursor of cytochrome c_1 (8).

The model of FeS protein sorting appears unexpectedly complicated at a first glance; however, it fits well into the endosymbiotic hypothesis of mitochondrial and chloroplast origin. All essential functions of what is now the mitochondrion must originally have been synthesized within the organelle in the early stages of endosymbiosis. Proteins of the outer surface of the endosymbiont membrane had to be translocated across this membrane. Their mitochondrial equivalents are proteins such as subunit II of cytochrome oxidase which is encoded by

the mitochondrial genome and which has a large hydrophilic domain at the outer surface of the inner membrane (22). Other proteins for which the genes were transferred to the host cell nucleus developed import mechanisms. Thus, part of the import pathway of the FeS protein may reflect an evolutionary conserved mechanism. Two "inventions" were necessary so that most genes coding for mitochondrial proteins could move from the mitochondrial genome to the nucleus: mitochondrial matrix targeting sequences on the precursor proteins, and import receptors in connection with translocation contact sites in the mitochondrion. Thus, proteins from the endosymbiont (*e.g.*, inner membrane and matrix proteins) could be sorted to their functional location in the mitochondrion by first bringing them to the compartment where they had originally been synthesized and then routing them back through their ancestral sorting pathway.

The FeS protein of the bc_1 complex from *Rhodospseudomonas sphaeroides* is structurally and functionally homologous to the equivalent protein from *N. crassa*. The sequence of the gene and the biosynthesis of the protein have been investigated (23). It is made as a precursor 1 kD larger than the mature protein. To assemble into the functional complex the precursor must cross the photosynthetic membrane. The processing of the precursor could occur at the stage of translocation. These reactions are equivalent to the second processing of the mitochondrial enzyme.

6. *Maturation of Precursor Proteins*

Mature mitochondrial proteins differ from their precursors. In most cases, cleavage of the N-terminal targeting sequence is the first maturation step (1). The processing peptidase that performs this cleavage has recently been purified about 5,000-fold from *N. crassa* and a specific antibody has been raised. The peptidase appears to be a monomeric protein with an apparent molecular weight of 57,000 (Hawlitshchek *et al.*, manuscript in preparation). The proteolytic activities that are responsible for the second maturation steps of intermembrane space proteins have not yet been characterized.

A number of proteins are not proteolytically processed. In these cases conformational changes such as the dimerization of the ADP/ATP

carrier, or addition of cofactors like heme to cytochrome *c* may start maturation. Finally, many proteins are assembled into complexes to become functional.

CONCLUSIONS

From the experimental findings described, the following requirements for import into the different mitochondrial subcompartments can be deduced. Mitochondrial precursor proteins contain targeting signals. The common feature they share may be the ability to form amphipathic helices enriched in basic and hydroxylated amino acids. These characteristics seem to be sufficient to direct the protein into the mitochondrial matrix. Additional cleavable or not cleavable sequences may have sorting functions. Whether sorting works by a "stop transfer" function of these sequences must be verified however, since no direct experimental support is yet available.

A number of mitochondrial components are required for import. All proteins examined so far use protease sensitive import sites. These are the only structures known to be involved in the import of outer membrane proteins whose targeting sequences are not cleaved (Fig. 1A).

Matrix proteins are imported in an energy-dependent manner using receptors and translocation contact sites (Fig. 1B). They are synthesized with N-terminal extensions, but exceptions may exist where no proteolytic processing occurs.

Inner membrane proteins contain either a cleavable matrix targeting sequence or a non-cleavable signal within the protein, as is the case for the ADP/ATP carrier (Fig. 1C). Import of inner membrane proteins requires import receptors and depends on the membrane potential. Translocation contact sites are also used. It is currently being investigated whether inner membrane proteins are laterally sorted from the contact sites or whether they also form soluble intermediates in the matrix which are then reinserted into the membrane.

Import of intermembrane space proteins seems to follow at least two different pathways. Some proteins contain N-terminal extensions that are processed twice. They have a matrix targeting and a presumed "stop transfer" or retranslocation signal. The import of these proteins

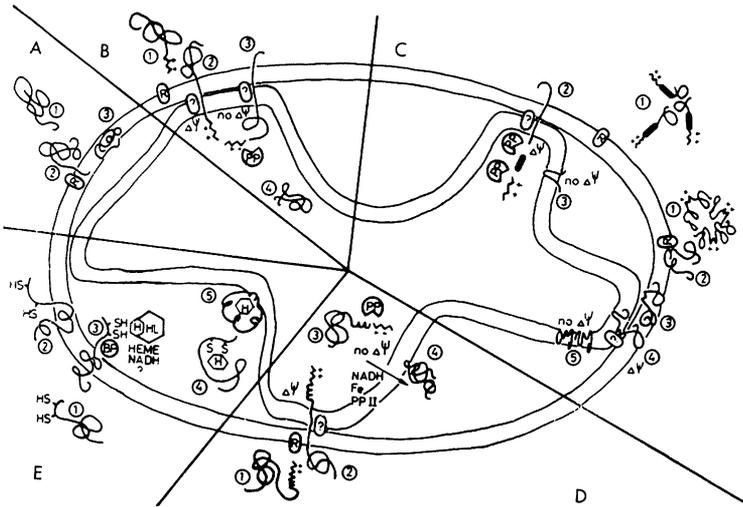


Fig. 1. Models for the import of proteins into the four mitochondrial subcompartments. A, porin (outer membrane protein); B, ATPase SU2 (matrix protein); C, ATPase SU9, ADP/ATP carrier (inner membrane proteins); D, FeS protein (inner membrane/intermembrane space protein); E, cytochrome *c* (intermembrane space protein). R, receptor; ?, hypothetical proteinaceous component of the translocation contact site; H, heme; HL, heme lyase; BP, apocytochrome *c* binding protein; PP, processing peptidase; PP II, second processing protease; $\Delta\psi$, membrane potential.

requires import receptors, translocation contact sites and perhaps retranslocation sites. The first import step depends on the membrane potential (Fig. 1D). A second class of intermembrane space proteins, including cytochrome *c*, is imported independently of the membrane potential as the mature size protein. Targeting sequences in these proteins have not been defined yet. Cytochrome *c* does not appear to have a specific receptor at the outer surface of the outer membrane. It seems that cytochrome *c* first inserts into the lipid phase of the outer membrane, thereby making contact with the binding protein in the intermembrane space which then acts as an import receptor and mediates translocation in a specific and irreversible fashion. At some step in this sequence heme is added to the apo-protein, which is probably the step that makes import irreversible (Fig. 1E).

PERSPECTIVES

During the last years a number of steps in the import of mitochondrial proteins have been elucidated. The existence of matrix targeting sequences in mitochondrial precursor proteins is well documented and general features of these sequences have been described. There are, however, proteins—such as the ADP/ATP carrier—which do not contain these N-terminal sequences. What are the targeting signals of these proteins? Some intermembrane space proteins are processed in two separate steps by different proteases. It is not clear yet whether the second prepeptide stops the transfer of the polypeptide and thereby has a function in sorting (8), or whether it serves as a distinct second sorting signal which mediates a second transmembrane movement. The latter seems to be the case for the FeS protein (20). Since it has been possible to experimentally dissect the pathway it appears possible to settle this question in the near future.

To a large degree the evidence for the existence of the various components of the import machinery (cytosolic factors, surface receptors, translocation contact sites, modifying enzymes, *etc.*) is only indirect. The components are well defined by their functions, but with a few exceptions (*e.g.*, the processing peptidase) none of the components have been isolated and characterized so far. Since import of precursors can be blocked at defined steps and thereby import intermediates can be accumulated, the isolation of defined components of the import machinery should now be possible. New techniques to incorporate photoreactive amino acid analogues into newly synthesized proteins (24) and the possibility to synthesize single species of precursors in a coupled transcription translation system (25) should greatly facilitate the isolation of components involved in recognition and import of mitochondrial proteins. With the isolated components, interaction of the precursors with the import machinery may then be studied at the molecular level in reconstituted systems.

SUMMARY

Most mitochondrial proteins are synthesized in the cytoplasm as precursor proteins and are then selectively imported into the various subcompartments of the mitochondrion. This requires the interaction of specific targeting signals in the newly synthesized proteins with the import machinery on the mitochondrion itself. Since mitochondria are organelles surrounded by two membranes, thus being composed of four compartments, the initial recognition reaction must be linked to a complex sorting system. Additionally, specific sorting signals on the precursor proteins for the various subcompartments must exist. We attempt here to summarize what is known about the targeting signals and the machinery involved in import.

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