PROTEIN TRANSFER AND ORGANELLE BIOGENESIS

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Synthesis and Assembly of Mitochondrial Proteins

DONALD W. NICHOLSON AND WALTER NEUPERT

I. INTRODUCTION

Eukaryotic cells can perform a variety of metabolic tasks with high efficiency owing to the compartmentalization of functions within cellular organelles. The organelles are delineated by membrane structures and contain specific subsets of proteins related to their role within the cell. The uniqueness of each type of organelle is maintained by processes in

the cell which sort and deliver the individual components in a highly specific manner. In this chapter, we discuss the biogenesis of mitochondria and specifically the processes which are involved in the import of proteins into these organelles.

Mitochondria are not synthesized *de novo*. Instead, control of the number of mitochondria within a cell occurs by division and fusion events. In the steady state there is a constant synthesis and turnover of mitochondrial proteins. Mitochondria grow and are maintained by incorporating newly synthesized material into preexisting organelles. The mitochondrion itself has a genetic apparatus which is discrete from that of the rest of the cell, but because of its relatively small size it can only account for a small percentage of the hundreds of mitochondrial proteins. The remainder are imported from the cytoplasm.

Because mitochondria contain two membranes, the organelle can be spatially divided into four distinct compartments: the outer membrane, the inner membrane, the matrix, and the intermembrane space. These too are specialized in function so that imported proteins must be correctly and specifically sorted within the mitochondrion as well. The pathways by which this occurs can be resolved into a number of discrete steps. In general, most imported mitochondrial proteins are synthesized on free ribosomes in the cytosol as precursors containing amino-terminal extensions. They are released into a cytostolic pool and are rapidly taken up by mitochondria. Receptors on the outer surface of the outer mitochondrial membrane seem to be involved in the initial recognition process. Proteins which must be transported to or through the inner membrane do so via translocation contact sites where the inner and outer membranes come close enough together to be spanned simultaneously. This step is energy dependent and specifically requires an electrochemical potential across the inner membrane. Precursor proteins are proteolytically processed to their mature size by a metal ion-dependent matrix peptidase, in some cases in combination with a second proteolytic event. The imported proteins are sometimes modified further by covalent or noncovalent attachment of cofactors. Many proteins are also assembled into larger complexes composed of several different subunits. In this chapter, we shall examine, in chronological order, each individual step which occurs during the import of mitochondrial proteins.

II. PRECURSOR PROTEINS

A. Properties of Precursor Proteins

Proteins which are imported into mitochondria are synthesized as precursors that differ from their mature counterparts in a number of ways: (1)

most, but not all, are synthesized as higher molecular weight proteins with amino-terminal peptide extensions which are proteolytically removed during or following import; (ii) a number of precursor proteins are covalently modified when imported while some acquire cofactors which are associated with the protein in a tight but noncovalent manner; (iii) precursor proteins differ in conformation from their mature form; (iv) precursor proteins often aggregate in the cytosol, whereas mature proteins in mitochondria are more likely to be found as monomers, dimers, or as subunits in heterologous complexes; (v) precursor proteins accumulated in the cytosol are far less stable than their correctly imported mature equivalent. Changes which accompany the maturation of imported proteins occur for one of two reasons. Some (i.e., removal of amino-terminal extensions) are connected to the import process, while others (i.e., acquisition of cofactors) are required for functional properties once inside the mitochondrion.

Of the more than 60 different proteins examined to date which must be imported into either the matrix or the inner membrane, almost all contain amino-terminal presequences (for an extensive list see Hay *et al.*, 1984, or Harmey and Neupert, 1985). Possible exceptions of the matrix proteins include 2-isopropylmalate synthase (Gasser *et al.*, 1982a; Hampsey *et al.*, 1983; Beltzer *et al.*, 1986), extramitochondrially expressed yeast bI4 maturase (Banroques *et al.*, 1987), and 3-oxoacyl-CoA thiolase from rat (Arakawa *et al.*, 1987), none of which appear to contain an amino-terminal extension. Some proteins of the inner membrane are known to be imported in their mature size, namely, the ADP/ATP carrier (Zimmermann *et al.*, 1979b; Hatalová and Kolarov, 1983), the uncoupling protein of brown adipose tissue (Freeman *et al.*, 1983; Ricquier *et al.*, 1983; Bouillaud *et al.*, 1986; Ridley *et al.*, 1986), two subunits (14 and 11 kDa) of the bc_1 complex (Teintze *et al.*, 1982; van Loon *et al.*, 1983c; Maarse and Grivell, 1987), the ubiquinone-binding protein of the bovine bc_1 complex (Nishikimi *et al.*, 1986), and sweet potato cytochrome oxidase subunit Vc (Nakagawa *et al.*, 1987). Proteins which are imported into the intermembrane space seem to be divided in this regard. Some, like precursors to cytochrome *c* peroxidase (Maccecchini *et al.*, 1979b; Reid *et al.*, 1982), cytochrome b_2 (Reid *et al.*, 1982; Daum *et al.*, 1982b; Gasser *et al.*, 1982b), and sulfite oxidase (Mihara *et al.*, 1982b) carry amino-terminal extensions, while others, like cytochrome *c* (Korb and Neupert, 1978; Zimmermann *et al.*, 1979a; Matsuura *et al.*, 1981) and adenylate kinase (Watanabe and Kubo, 1982), do not.

Although only a few of the proteins which are imported to the outer membrane have been examined, none appears to be synthesized as a higher molecular weight precursor. These include the channel-forming porin protein (Freitag *et al.*, 1982; Mihara *et al.*, 1982a; Gasser & Schatz,

1983) and three outer membrane proteins of unknown function (70, 45, and 14 kDa; Gasser and Schatz, 1983). One possible exception is a 35 kDa protein from rat liver mitochondria (Shore *et al.*, 1981) which appears to migrate on sodium dodecyl sulfate–polyacrylamide gels as a precursor of slightly greater molecular size. Whether this represents a precursor protein containing a peptide extension or is a gel artifact is not entirely clear and will require sequencing data to verify.

The trend that emerges is that the deeper into the mitochondrion a protein must be imported, the more likely it is to be synthesized as a precursor of higher molecular weight. As will be discussed in the following section, the amino-terminal prepiece carries targeting information which is necessary to direct imported proteins to their correct intramitochondrial location. In addition, the prepieces, which are generally hydrophilic, confer different solubility properties on the precursor proteins that may allow for their transfer through the cytosol and are important for subsequent import.

While proteolytic processing is the most prevalent covalent modification of imported mitochondrial proteins, there are a number of other changes which occur in many proteins during or following import. For example, protoheme is covalently attached to cytochromes *c* and c_1 via cysteine residues in the corresponding apoproteins. Iron–sulfur centers (nonheme iron) are constructed in subunits of complexes I, II, and III of the respiratory chain, again via cysteine residues. In addition, many mitochondrial proteins are modified by tight but noncovalent bonding of coenzymes and cofactors (i.e., NAD, FAD, heme *a* and *b* and metal ions such as Cu). These proteins are predominantly constituents of the respiratory chain, and the cofactors which are associated with them are primarily involved in their unique roles as electron carriers. In some cases, however, such modifications also appear to be important for events in the import pathway. Covalent attachment of heme to apocytochrome *c* (holocytochrome *c* lacking heme), for instance, initiates conformational changes along the polypeptide chain which pull the protein through the outer membrane to its functional location in the intermembrane space (Hennig and Neupert, 1981; Nicholson *et al.*,1987). Cytochrome c_1 is proteolytically processed in two distinct steps, the second of which has been suggested to be preceded by covalent attachment of the heme to the intermediate size apoprotein (Gasser *et al.*, 1982b; Ohashi *et al.*, 1982). Likewise, the second processing step of the Fe/S protein of the bc_1 complex may be dependent on formation of the Fe/S cluster (F.-U. Hartl and W. Neupert, unpublished).

Precursor proteins are also distinguishable from their mature counterparts in conformational arrangement. Apocytochrome *c* (prepared by

chemical removal of the heme group from holocytochrome *c*, then renatured to an import-competent form) shows a nearly featureless circular dichroism (CD) pattern. Following interaction with negatively charged lipids in model membranes, which is believed to represent the first event in its import into mitochondria, up to 35% α-helical structure is expressed (Rietveld *et al.*, 1985). The disordered structure of the precursor cytochrome *c* is folded into a highly ordered stable conformation when heme is attached to form holocytochrome *c* (Fisher *et al.*, 1973). The difference between precursor and mature forms of cytochrome *c* is thus dependent on events occurring during its import, namely, interactions with phospholipids and covalent attachment of heme. These conformational changes can be detected *in vitro* with differential antibodies raised against apo- and holocytochrome *c* which do not cross-react (Korb and Neupert, 1978) or by differential proteolytic sensitivity in solution (Basile *et al.*, 1980).

The precursor to the ADP/ATP carrier does not bind carboxyatractyloside whereas the mature form does. This reflects conformational differences in the proteins which can be distinguished by their binding properties to columns of hydroxylapatite in the presence of carboxyatractyloside. *In vitro* imported and correctly assembled ADP/ATP carrier, when solubilized from mitochondria with detergent, behaves like authentic mature ADP/ATP carrier and passes through these columns while the precursor protein does not (Zimmermann and Neupert, 1980; Schleyer and Neupert, 1984). Since ADP/ATP carrier is not proteolytically processed during import, the binding properties to hydroxylapatite serve as useful criteria for establishing whether correct import and assembly has occurred. Such criteria are important for determining whether a protein imported *in vitro* acquires the properties of the mature protein *in vivo*. In some cases, for example, for proteins which are not proteolytically processed, import can only be studied by following these changes.

Precursor conformation is important for import. For example, although CD spectra for apocytochrome *c* show no detectable secondary structure (Rietveld *et al.*, 1985), binding of the protein to mitochondria is sensitive to denaturation by a single freeze–thaw cycle (H. Köhler and W. Neupert, unpublished). Similarly, the apocytochrome *c* produced by a mutant of *Neurospora crassa*, in which the carboxy-terminus is 19 amino acids longer than wild type apocytochrome *c* (the final 27 amino acids being of an unrelated sequence), cannot bind or be imported into mitochondria, most likely because of conformational perturbation (Stuart *et al.*, 1987). On the other hand, the import of a fusion protein containing mouse dihydrofolate reductase linked to the presequence of cytochrome oxidase IV can be blocked by methotrexate, which stabilizes the folding of the dihydrofolate reductase moiety. In this case, the protein must be at

least partially unfolded to be imported into mitochondria (Eilers and Schatz, 1986). Recent evidence indicates that most precursor proteins are maintained in, or "defolded" into, an import-competent conformation by the hydrolysis of nucleoside triphosphates (Pfanner and Neupert, 1986; Pfanner *et al.*, 1987; see Section V).

Most newly synthesized precursor proteins tend to aggregate. Though this has not been well characterized *in vivo*, it is frequently observed *in vitro* in either homologous or heterologous (i.e., reticulocyte lysate) translation systems. The ADP/ATP carrier, for example, is present in soluble complexes with apparent molecular weights of 120K and 500K (Zimmermann and Neupert, 1980). Aggregation has also been demonstrated for ATPase IX (Schmidt *et al.*, 1983b) and for cytochrome oxidase V (Neupert and Schatz, 1981). In all of these cases it is not clear whether aggregation occurs as homo- or heterooligomers. The precursor to rat ornithine carbamoyltransferase is transported to mitochondria as a 5 S complex (approximately 90 kDa) containing an unidentified import factor (Argan and Shore, 1985). Similarly, the import of the F_1 ATPase β subunit into yeast mitochondria is dependent on a cytosolic factor believed to be a 40 kDa protein which binds to the precursor proteins and enables their correct association with mitochondria (Ohta and Schatz, 1984). These last two examples suggest that aggregation may occur in a heterooligomeric fashion for some imported proteins. On the other hand, the precursor to rat mitochondrial fumarase (fumarate hydratase) was reported to form homooligomeric aggregates containing six to eight molecules (Ono *et al.*, 1985).

Beside the nonspecific interaction of proteins in solution, aggregation of precursor proteins may occur for specific reasons as well. Since many imported mitochondrial proteins have a membrane localization and are therefore at least partially hydrophobic, there probably exists some means for disguising these parts of the molecule and allowing their solubility in the cytosol. The amino-terminal prepiece may confer some solubility in aqueous environments, while aggregation of precursor proteins, presumably via their hydrophobic domains, may also contribute in this respect. The best example of this is ATPase subunit IX [proteolipid or dicyclohexylcarbodiimide (DCCD)-binding protein], one of the most hydrophobic proteins known. It contains a long polar prepiece (66 amino acids in *Neurospora crassa*) that accounts for the solubility of the highly hydrophobic mature sequence (81 amino acids) in aqueous environments (Viebrock *et al.*, 1982). The protein also forms aggregates, perhaps to further aid its solubility in the cytosol. Cytochrome *c*, on the other hand, is a soluble protein of the intermembrane space which is not imported

with a prepiece and behaves as a monomer or dimer in solution (H. Köhler and W. Neupert, unpublished). Another possible role of aggregation is that it is required for import-competent transport and binding of precursors to mitochondria (Section VI).

Once precursor proteins are synthesized, they are rapidly cleared from the cytosol and imported into mitochondria. Precursors can be accumulated *in vivo* by growing cells in the presence of uncouplers of oxidative phosphorylation. Under these conditions they cannot be imported and are pooled in the cytosol. In contrast to the relative stability of mature mitochondrial proteins, the accumulated precursors (in general) are rapidly degraded. For example, yeast cells grown to early exponential phase and then treated with the uncoupler carbonyl cyanide *N*-chlorophenylhydrazone (CCCP) accumulated large amounts of some mitochondrial precursor proteins (Reid and Schatz, 1982a). In pulse-labeling experiments, the proteins were degraded at different rates. The precursor of cytochrome c_1 was unstable and was degraded with a half-life of about 10 min. On the other hand, the precursor to F_1 ATPase β subunit was more stable and was degraded with a half-life of 50 min. In similar experiments, the precursor of aspartate aminotransferase, accumulated in chick embryo fibroblast cultures treated with CCCP, was degraded with a half-life of about 5 min (Jaussi *et al.*, 1982), and the precursor of carbamoyl-phosphate synthase in rat liver explants, in which proteolytic processing was blocked, was degraded with a half-life of 2–3 min (Raymond and Shore, 1981). The apparent relative stability comparing precursor with mature proteins is probably also related to the normal subcellular location of the proteins (i.e., cytosol versus mitochondria) and the degradative processes that exist there.

Maturation of imported mitochondrial proteins occurs within the mitochondrion itself and not in the cytosol immediately following synthesis. This occurs in this sequence for two reasons: (1) many maturation events are involved in the import pathway itself and/or can only take place after certain import steps have occurred, and (2) development of functional characteristics within the mitochondrion is consistent with the theme of compartmentation of processes in eukaryotic cells.

B. Cotranslational versus Posttranslational Transport

Two mechanisms exist in eukaryotic cells by which proteins may be synthesized and transported across biological membranes. In cotranslational transport, synthesis begins on soluble cytoplasmic polysomes. As the nascent polypeptide chain appears from the ribosome it is directed,

with the accompanying polysome, to the target membrane and is simultaneously inserted into or through the membrane as chain elongation continues. Cotranslational import is the primary means by which proteins are transported across the membranes of the endoplasmic reticulum. It appears that in this case the cotranslational nature, probably with a few exceptions, is obligatory (i.e., a completed polypeptide chain can never enter the transport pathway). It is likely that the polypeptide chain otherwise folds in such a way that it is transport incompetent. The function of the signal recognition particle and docking protein is in arrest and dearrest of the elongation process to guarantee that the nascent polypeptide is kept in a translocation competent state for a sufficient period of time. In posttranslational transport, protein synthesis also begins on soluble cytoplasmic polysomes; however, complete polypeptide synthesis and release into the cytosol occurs before transport into or across membranes begins. Posttranslational import is the mechanism predominant in mitochondria, chloroplasts, microbodies, and probably nuclei. In mitochondria, import may occur with some cotranslational characteristics, though it is clearly not an obligatory process.

In cotranslational compared to posttranslational import, the way in which organelle targeting information is processed is different. For example, in the mechanism for microsomal targeting, the signal recognition particle and docking protein serve as common components for most proteins destined for the endoplasmic reticulum (ER). On the other hand, targeting information for proteins which are posttranslationally transported must be self-contained.

Import of mitochondrial proteins can occur posttranslationally, though early evidence pointed toward a cotranslational mechanism. Cytoplasmic 80 S ribosomes were coisolated with purified yeast mitochondria (Kellems and Butow, 1972). These ribosomes were tightly bound to mitochondria, and only one-third could be released by incubation at high ionic strength. The remaining two-thirds could only be released when their nascent polypeptide chains were dissociated from the ribosome with puromycin (Kellems *et al.*, 1974), suggesting that the ribosomes were anchored via the newly synthesized nascent polypeptide chain which was presumably undergoing cotranslational (vectorial) transport. Furthermore, the mitochondria-associated ribosomes were enriched in mRNAs coding for mitochondrial proteins. When yeast mitochondria were isolated with their accompanying cytoplasmic ribosomes and placed in a readout system to complete polypeptide chain synthesis, over 80% of the products remained associated with the mitochondria and were imported to a location not accessible to externally added proteases (Ades and Butow, 1980a,b). The

distribution of total message for mitochondrial proteins, however, was not exclusively associated with these mitochondria-bound ribosomes. Although the mRNA from mitochondria-bound ribosomes was enriched for mitochondrial proteins, indicating that the association was specific, not all mitochondrial proteins were preferentially synthesized on them (Suissa and Schatz, 1982). For example, while 60% of the translatable mRNA for F_1 ATPase β subunit or cytochrome *c* peroxidase was associated with mitochondria-bound polysomes, more than 95% of the message for cytochrome oxidase V or VI and porin was associated with free polysomes. In no case was the translatable message exclusively associated with mitochondria-bound polysomes. This suggested that cotranslational import was not necessarily the transport mechanism used by all imported mitochondrial proteins.

Evidence for posttranslational import of mitochondrial proteins came from studies both *in vivo* and *in vitro:*

1. Extramitochondrial pools of a number of mitochondrial proteins have been detected in pulse-labeling experiments *in vivo*. The appearance of label in mitochondrial proteins showed a lag compared to total cellular protein in *Neurospora crassa* (Hallermayer *et al.*, 1977). The labeled mitochondrial proteins first appeared in a cytosolic pool and then in mitochondria. Similarly, in yeast, mitochondrial precursor proteins first appeared in a cytosolic pool and were then subsequently imported into mitochondria and converted to their mature forms (Reid and Schatz, 1982b; Schatz, 1979).

2. Posttranslational import in the absence of protein synthesis has been demonstrated both *in vivo* and *in vitro*. When protein synthesis was blocked with cycloheximide immediately following pulse-labeling *in vivo*, the precursor pool of mitochondrial proteins in the cytosol decreased with concomitant posttranslational uptake by mitochondria and conversion to the mature forms during the subsequent chase (Hallermayer *et al.*, 1977; Schatz, 1979; Reid and Schatz, 1982b; Teintze *et al.*, 1982). Similarly, mitochondrial precursor proteins which were accumulated *in vivo* by growing cells in the presence of CCCP could be subsequently chased into mitochondria by removing the inhibitory effects of CCCP with 2-mercaptoethanol (Reid and Schatz, 1982a,b) or cysteamine (Jaussi *et al.*, 1982). This too was unaffected by the presence of cycloheximide and therefore occurred posttranslationally.

3. Perhaps the most convincing evidence that transport is possible in the absence of protein synthesis comes from *in vitro* studies where translation systems were programmed with poly(A)-containing RNA to synthe-

size precursor proteins. If further translation following synthesis was blocked by the addition of cycloheximide or if ribosomes were removed by centrifugation and the resulting supernatants incubated with isolated mitochondria, the precursor proteins were rapidly imported into mitochondria and processed to their mature form (Harmey *et al.*, 1977; Korb and Neupert, 1978; Maccecchini *et al.*, 1979a; Zimmermann and Neupert, 1980). The same effect is observed regardless of whether a heterologous (i.e., reticulocyte lysate) or homologous (from cell cytosol) system is used. Posttranslational transport has been demonstrated *in vitro* for all imported mitochondrial proteins studied so far and in many cases *in vivo* as well.

In mitochondria, protein transport normally occurs posttranslationally although some cotranslational import (which is clearly not an obligatory process) may occur as a consequence of the amino-terminal targeting prepiece being exposed before complete polypeptide synthesis is finished. The prepiece might then initiate the import process before chain elongation is complete. Whether this actually occurs *in vivo* is not clear since the early experiments with mitochondria-bound ribosomes did not distinguish whether the nascent polypeptide chains were concomitantly transported with translation or transported only after complete synthesis. In any case, the enrichment of mRNAs for mitochondrial proteins in mitochondria-bound ribosomes has provided a useful means for isolating and screening for genes for imported proteins. Clones for 16 different genes of imported yeast mitochondrial proteins were identified by selective hybridization to these enriched mRNAs (Suissa *et al.*, 1984).

III. TARGETING AND SORTING SEQUENCES

The intracellular sorting of proteins synthesized on cytoplasmic polysomes is a fairly specific process. The signals which direct these events are contained within the newly synthesized protein itself. In addition, components of the target organelle, such as receptors, recognize these signals and facilitate import and sorting.

A. Fusion Proteins

Delineation of the exact regions in precursor proteins which are responsible for mitochondrial targeting and intramitochondrial sorting has been demonstrated by gene fusion experiments in which parts of a mitochon-

drial precursor protein can be attached to a nonmitochondrial "passenger" protein. Generally, three observations have been made: (i) the cleavable amino-terminal prepiece of imported mitochondrial proteins fused to nonmitochondrial proteins [such as mouse dihydrofolate reductase (DHFR) or *Escherichia coli* β-galactosidase] can correctly mediate mitochondrial targeting and frequently intramitochondrial sorting of the passenger protein; (ii) mitochondrial proteins in which the prepiece has been removed cannot be imported into mitochondria; and (iii) distinct regions of the polypeptide chain of proteins which are not proteolytically processed contain the information necessary for targeting and sorting. It has been suggested that some mitochondrial precursor proteins also have carboxy-terminal extensions that are removed during import and maturation, though the significance of these findings is as yet unclear (Okamura *et al.*, 1985; Power *et al.*, 1986; Patterson and Poyton, 1986).

Cytochrome oxidase IV (COX IV) from yeast, an inner membrane protein, is synthesized as a precursor with a 25 amino acid amino-terminal prepiece (Maarse *et al.*, 1984). When progressively truncated parts of the COX IV presequence were fused to DHFR, the first 12 amino acids, but no less, directed DHFR to the mitochondrial matrix both *in vivo* and *in vitro* (Hurt *et al.*, 1984b, 1985a). When the entire 25 amino acid presequence or the first 22 amino acids were fused to DHFR, proteolytic processing by the matrix peptidase also took place, albeit at an alternative site in the latter case. Since the fusions were directed to the matrix instead of the inner membrane, even when the first 53 amino acids of the COX IV precursor were fused to DHFR (Hurt *et al.*, 1984a), the intramitochondrial sorting information must be contained within the mature part of COX IV. Mature COX IV, prepared from either pre-COX IV *in vitro* or with fusions *in vivo*, was neither imported nor bound to mitochondria (Hurt *et al.*, 1984a). In fact, removal of only the first 7 amino acids of the prepiece prevented COX IV import (Hurt *et al.*, 1985b).

Similarly, the targeting information for yeast pre-ATPase $F_1\beta$, which contains about a 20 amino acid prepiece and is imported to the matrix, exists within the first 27 amino acids of the precursor protein (Douglas *et al.*, 1984; Emr *et al.*, 1986). In fusion studies, as few as 39 amino acids from the amino terminus of pre-$F_1\beta$ (shorter fusions were not examined) could direct invertase to mitochondria *in vivo*, but 169 amino acids were required to direct β-galactosidase (presumably due to a folding artifact). Internal deletion studies of a fusion between pre-$F_1\beta$ amino acids 1–380 and β-galactosidase narrowed down the portion with the targeting information to the first 27 amino acids of the $F_1\beta$. In all cases, however, the importable fusions were misdirected to the inner membrane. Deletion

proteins made from authentic $F_1\beta$ in which amino acids were removed between residues 10 and 36 of the pre-$F_1\beta$ were fully import competent, while deletions within the first 10 amino acids abolished import (Vassarotti *et al.*, 1987a).

The amino-terminal prepiece of both rat and human ornithine carbamoyltransferase (OTC), which is 32 amino acids long and directs the protein to the matrix, can also target nonmitochondrial passenger proteins to the matrix *in vitro* (Horwich *et al.*, 1985b; Nguyen *et al.*, 1986). In contrast to the case of COX IV, the targeting information does not appear to reside in the very amino terminus of the prepiece since deletion studies in which either the amino or carboxy terminus of the prepiece were removed produced proteins which were still import competent. Instead, the targeting information appears to reside between amino acids 8 and 22 of the 32 amino acid prepiece, while the flanking regions contribute to import efficiency (Horwich *et al.*, 1986). The shortest targeting sequence identified to date is from the prepiece of 5-aminolevulinate synthase, in which the amino-terminal 9 amino acids fused to β-galactosidase correctly directs the fusion protein to the mitochondrial matrix *in vivo*, though with low efficiency (Keng *et al.*, 1986).

Proteins which do not contain removable prepieces also contain specific targeting information. In yeast ADP/ATP carrier this is contained within the first 115 amino-terminal amino acids but has not been further resolved (Adrian *et al.*, 1986). Since the ADP/ATP carrier is a tripartite protein having three segments (each of approximately 100 amino acids) which have a high degree of homology (Saraste and Walker, 1982), then similar targeting ability might also exist in the other two segments of the protein. [Interestingly, the bovine phosphate carrier protein, which has a high degree of structural homology with the ADP/ATP carrier and would presumably follow a similar import pathway, is synthesized as a precursor with a 49 amino acid amino-terminal extension (Runswick *et al.*, 1987)]. In the 70 kDa outer membrane protein from yeast, the targeting and sorting functions are contained in the first 41 amino-terminal amino acids. Deletion and fusion studies have identified two critical regions: amino acids 1 through 21 are required for mitochondrial targeting while the overlapping amino acid sequence 10 through 37 is necessary for sorting via membrane anchoring (Riezman *et al.*, 1983c; Hase *et al.*, 1984, 1986). As for proteins containing removable prepieces, the information exists in the amino-terminal end of the protein. In fact, the amino-terminal 12 amino acids of the 70 kDa protein fused to mature COX IV could target the fusion protein to mitochondria and restore cytochrome oxidase function in COX IV-deficient mutants *in vivo* (Hurt *et al.*, 1985b).

That the amino-terminal prepiece carries specific mitochondrial targeting information is confirmed by comparing differences in alcohol dehydrogenase (ADH) isozymes which have different subcellular locations. ADH I and II are located in the cytoplasm while ADH III is a mitochondrial protein. They are 80–90% identical in sequence except for a 27 amino acid prepiece in ADH III. If the presequence of ADH III was removed it could not be imported into mitochondria. If the ADH III prepiece was fused to cytosolic ADH II, then the fusion protein was imported into the mitochondrial matrix. Therefore, ADH II is a cytosolic protein since it lacks an amino-terminal targeting sequence while ADH III is a mitochondrial protein since it contains the targeting sequence (van Loon and Young, 1986).

The mitochondrial targeting function of amino-terminal extensions has also been suggested by studies of other proteins in which isozymes having different subcellular locations are encoded by the same gene. For example, the gene encoding yeast histidyl-tRNA synthase has two in-frame translation starts, both of which are expressed (Natsoulis *et al.*, 1986). Mutations destroying the first start codon resulted in a respiratory deficient (Pet^-) phenotype without affecting the cytoplasmic isozyme suggesting that the longer mRNA encodes the mitochondrial isozyme while the shorter message encodes the cytoplasmic form. Similarly, the yeast 2-isopropylmalate synthase gene contains multiple in-frame transcription start sites (Beltzer *et al.*, 1986) which produce two related proteins of different sizes in cell-free translation mixtures (Hampsey *et al.*, 1983). Only the larger of these two proteins was imported into mitochondria, suggesting that selection of the appropriate transcription and translation start sites controls the subcellular location of the protein and that mitochondrial targeting information is contained in the amino terminus of the larger precursor protein. This is also the case for a 20 kDa protein from *Neurospora crassa* (M. Tropschug, H. Köhler, R. A. Stuart, and W. Neupert, in preparation). A single gene encodes the mature-size cytosolic form and a larger precursor (24 kDa), which is imported into mitochondria. During import, the amino-terminal extension is removed in two proteolytic steps.

Mitochondrial prepieces appear to be able to direct almost any passenger protein into mitochondria. For example, in addition to fusions with DHFR and β-galactosidase, it has been demonstrated that a mitochondrial presequence (in this case from COX IV) can direct a chloroplast-encoded protein (ribulose-1,5-bisphosphate carboxylase, large subunit) into mitochondria (Hurt *et al.*, 1986b) and that ATPase subunit VIII, normally a mitochondrial gene product, can also be imported back into

mitochondria when fused to a mitochondrial-targeting sequence (Gearing and Nagley, 1986). In the latter case, the 66 amino acid prepiece of ATPase subunit IX could mediate import of ATPase VIII, while the shorter 40 amino acid COX VI leader could not. It is clear then that targeting information itself resides in the amino-terminal extension of most precursor proteins. The efficiency of import with a given prepiece, however, is significantly affected by the passenger protein (van Steeg *et al.*, 1986).

B. Properties of Prepiece Sequences

The prepiece sequences for a number of imported mitochondrial proteins have now been determined (Table I). They vary in length between 20 and 80 amino acids. Although the prepieces do not share extensive sequence homology (except perhaps between equivalent proteins in different organisms), they do have several similar characteristics including the following: (i) a high content of positively charged basic amino acids (particularly arginine) which are distributed somewhat randomly throughout the prepiece; (ii) an absence or near absence of negatively charged acidic amino acid residues; (iii) a high content of hydroxylated amino acids (particularly serine); and (iv) a propensity to form amphiphilic α-helical structures. Only a few exceptions exist, the most striking of which is the amino-terminal sequence of the 17 kDa subunit IV of yeast bc_1 complex which contains an extremely high content of acidic amino acids (van Loon *et al.*, 1984); however, import of this protein into mitochondria has not been examined. Another deviation from these general properties occurs in human OTC, which, unlike its counterpart in rat, contains no hydroxylated amino acids. The placement of positively charged amino acids is well conserved between human and rat OTC but the hydroxylated amino acids in rat OTC have been replaced in human OTC by asparagine (instead of threonine) and phenylalanine (instead of serine).

How these amino-terminal prepieces direct proteins to mitochondria and how they facilitate transport across membranes is not entirely clear. One possibility is that they are recognized by specific receptors on the mitochondrial surface. Another is that, because of their amphiphilic helical nature, they can penetrate lipid bilayers. Finally, because they are positively charged, they may be electrophoretically drawn toward the mitochondrial matrix by the potential across the inner membrane (inside negative). It is possible that all of these events are involved. For example, a membrane potential is necessary for the import of all proteins which must be directed to the inner membrane or matrix. Similarly, if positively charged amino acids in the prepiece are replaced by uncharged amino

acids, then import is abolished (Horwich *et al.*, 1985a). Specific amino acids, and not just net positive charge, also appear to be important. In human OTC, arginine 23 of the 32 amino acid prepiece is critical for both import and proteolytic processing; however, when it is replaced by an amino acid supporting α-helical structure, the import function is conserved (Horwich *et al.*, 1986).

The ability to form amphiphilic helical structures may be a critical feature of presequences. In a theoretical analysis of many mitochondrial presequences, von Heijne identified regions which can be folded into helices with high hydrophobic moment (von Heijne, 1986a). The segments of highest hydrophobic moment correlated very well with the critical regions identified by fusion studies for yeast COX IV and 70 kDa protein, and human OTC (see Table I). In another study, removal of the basic amphipathic α-helix of the ATPase $F_1\beta$ prepiece prevented import into mitochondria (Vassarotti *et al.*, 1987b). When mutations were selected that restored the correct *in vivo* localization of ATPase $F_1\beta$, it was found that these modifications specifically replaced acidic amino acids at the amino-terminus with basic or neutral amino acids that support amphipathic helix formation.

Some of the physical properties of prepieces have been examined with synthetic prepiece peptides. Synthetic peptides of the COX IV prepiece are soluble in aqueous solutions but were able to penetrate phospholipid monolayers or disrupt liposomes having a diffusion potential negative inside but not of the opposite polarity (Roise *et al.*, 1986). A synthetic peptide equivalent to the first 27 amino acids of pre-OTC assumed an amphiphilic helical conformation that was induced by the presence of anionic phospholipids and could perturb the bilayer of synthetic liposomes (Epand *et al.*, 1986). Synthetic prepiece peptides dissipated the membrane potential in isolated mitochondria (Ito *et al.*, 1985; Gillespie *et al.*, 1985; Roise *et al.*, 1986); however, in the presence of reticulocyte this did not appear to occur (Gillespie *et al.*, 1985). In this case, a synthetic peptide of amino acids 1–27 of the rat OTC prepiece completely blocked the import of pre-OTC, while amino acids 16–27 did not. The peptide (1–27) also blocked the import of pre-malate dehydrogenase (a matrix protein) and pre-thermogenin (uncoupling protein of the inner membrane), suggesting common components are shared in the import pathway of these proteins.

Artificial presequences, encoded by synthetic oligonucleotides, that were fused to the mature part of COX IV were able to mediate import into mitochondria both *in vitro* and *in vivo* if the balance of basic, hydrophobic and hydroxylated amino acids was similar to that of authentic presequences (Allison and Schatz, 1986). In all cases, the import-competent extensions were surface active, indicating that targeting may not depend

TABLE I

Comparison of Amino-Terminal Prepieces of Imported Mitochondrial Proteins

	Sequence*	Notes†
	OUTER MEMBRANE	
1	MKSFITRNKTAILATVAATGTAIGAYYYYNQLQQQQRGKKNTINKDEKK...	(a)
	INTERMEMBRANE SPACE	(b)
2	MTTAVRLLPSLGRTAHKRSLYLFSAAAAAAAAAATFAYSQSHKRSSSSPGGGSNHGWNNWGKAAALASTTPLVHVASV...	
3	MLKYKPLLKISKNCEAAILRASKTRLNTIRAYGSTVPKSKSFEQDSRKRTQSWTALRVGAILAATSSVAYLNWHNGQIDNEPKLDMNKQK	
	INNER MEMBRANE - C SIDE	
4	MAPVSIVSRAAMRAAAAPARAVRALTTSTALQGSSSSTFESP...	(b)
5	MFSNLSKRWAQRTLSKSFYSTATGAASKSGKLTQKLVTAGVAAAGITASTLLYADSLTAEAMTAAEHGLHA...	(b)
	- INTRINSIC OR UNKNOWN	
6	MLRNTFTRAGGLSRITSVRFAQTHALSNAA...	
7	MLRTPTVSALVRNVAVRAAKPTMAVRAASTMPISNPT...	
8	MLATRVFSLIGRRAISTSVCVRAHGSVVKSED...	(c)
9	MLARGLPLRSALVKACPPILSTVGEGWGHHRVGTGEGAGISTKTPRPYS...	
10	MLSRAIFRNPVINRTLLRARPGAYHATRLTKNTFIQSRKYSDAHDEETFE...	
11	MASTRVLASRLASQMAASAKVARPAVRVAQVSKRTIQTGSPLQTLKRTQMTSIVNATTRQAFQKRAYSSEIAQAMV...	(b)
12	MQTTGALLISPALIRSCTRGLIRPVSASFLSRPEIQSVQPSYSSGPLQVARREFQTSVVSRDIDTAAKFIG...	(b)
13	MYTCAKFVSTPSLIRRTSTVLSRSLSAVVVRRPETLTDESHSSLAVVPRPLTTSLTPSRSFQTSAISRDIDTAAKFIG...	(b)
	- M SIDE	
14	MLSLRQSIRFFKPATRTLCSSRYLLQQKPVVKTAQ...	
	MATRIX	
15	MSAILSTTSKSFLSRGSTRQCQNMQKALFA...	
16	MLSKLASLQTVAALRRGLRTSVASATSVATKKTEQ...	
17	MALLQSRLLLSAPRRAAATARASSWWSHVEMG...	
18	MALLHSGRVLSGVASAFHPGLAAAASARASWWAHVEMGP...	
19	MFAKTAAANLTKKGGLSLLSTTARRTKVTLPDLKWD...	
20	MNSLRIARAALRVRPTAVRAPLQRRGYAEAVADKIK...	(d)
21	MQRSIFARFGNSSAAVSTLNRLSTTAAPHAKNGYATA...	
22	MLRTSSLFTRRVQPSLFSRNILRLQSTAAIPKTQKGV...	(e)
23	MLFNLRILLNNAAFRNGHNFMVRNFRCGQPLQNKVQLKGRDL...	
24	MLSNLRILLNKAALRKAHTSMVRNFRYGKPVQSQVQLKGRDL...	
25	MSALLPRLLTRTAFKASGKLLRLSSVISRTFSQTTTSYAAAFDRSKP...	(f)
26	MTRILTACKVVKTLKSGFGLANVTSKRQWDFSRPGIRLLSVKAQTAHI...	(g)
27	MVLPRLYTATSRAAFKAAQSAPLLSTSWKRCMASA...	(h)
28	MASRRLLASLLRQSAQRGGGLISRSLGNSIPKSASRASSRASPKGFLLNRAVQYATSAAAPASQP...	(i)
29	MTSLWGKGTGCKLFKFRVAAAPASGALRRLTPSASLPPAQLLLRAVRRRSHPVRDYAAQTSPSPKAGAA...	(h, j)
30	MAARLLRVASAALGDTAGRYRLLVRPRAGAGGLRGSRGPGLGGGAVATRTLSVSGRAQSSSEDKITVH...	

TABLE I (*continued*)

Sequence*	Notes†
OTHER SEQUENCES OF INTEREST	(k)
31 MAAVIAKSSVSAAVARPARSSVRPMAALKPAVKAAPVAAPAQANQMMVWTPVNNK...	
32 MDMLELVGEYWEQLKITVPVVAAAEDDDNEQHEEKAAEGEEKEEENGDE...	
33 MPQSFTSIARIGDYILKSPVLSKLCVPVANQFINLAGYKKLGLKFDDLIA...	
34 MKIQLVRWHCSRNALWNRAFYSTRKATKNASSATPATMTSMVSQRQDLFM...	
35 MSNKQAVLKLISKRWISTVQRADFKLNSEALHSNATVFSMIQPTGCFHLG...	
36 MLSRSLNKVVTSIKSSSIIRMSSATAAATSAPTANAANALKASKAPKKGK...	
37 MVKESIIALAEHAASRASRVIPPVKLAYKNMLKDPSSKYKPFNAPKLSNR...	(l)
38 MTVLYAPSGATQLYFHLLRKSPHNRLVVSHQTRRHLMGFVRNALGLDPPP...	
39 MVNWQTLFMVSLRRQGSSSRYRYKFNMENITHQVFPRCKQAFKKTNLSYE...	
40 MSSSQVVRDSAKKLVNLLEKYPKDRIHHLVSFRDVQIARFRRVAGLPNVD...	

* The amino-terminal prepiece sequences of imported mitochondrial precursor proteins are listed (by the single letter amino acid code) in groups according to the intramitochondrial location of the mature protein. Above the primary sequence, basic (lysine and arginine), acidic (aspartate and glutamate), and hydroxylated amino acids (serine and threonine) are identified (+, −, and ·, respectively). Proteolytic cleavage sites which yield intermediate or mature proteins are indicated by an arrow or by a bracket above the sequence where only the general region of proteolytic processing is known (i.e., based on apparent molecular weight differences between precursor and intermediate or mature proteins). The segment of the prepiece identified by von Heijne (1986a) as having the highest hydrophobic moment (18 residue window, Eisenberg *et al.*, 1984) when plotted in a helical wheel projection (Schiffer and Edmundson, 1967) is indicated by a box. Proteins: (1) 70 kDa protein (yeast), Hase *et al.* (1983); (2) cytochrome c peroxidase (yeast), Kaput *et al.* (1982), Reid *et al.* (1982); (3) cytochrome b_2 (yeast), Guiard (1985), Gasser *et al.* (1982b); (4) Rieske Fe/S of bc_1 compiex (*N. crassa*), Harnisch *et al.* (1985), Hartl *et al.* (1986); (5) cytochrome c_1 (yeast), Sadler *et al.* (1984), Gasser *et al.* (1982b); (6) cytochrome oxidase V (yeast), Koerner *et al.* (1985); (7) cytochrome oxidase V (*N. crassa*), Sachs *et al.* (1986); (8) cytochrome oxidase IV (bovine), Lomax *et al.* (1984); (9) cytochrome *P*-450 (SCC) (bovine), Morohashi *et al.* (1984); (10) cytochrome oxidase VI (yeast), Wright *et al.* (1984); (11) ATPase IX (*N. crassa*), Viebrock *et al.* (1982), Schmidt *et al.* (1984); (12) ATPase IX-P1 (bovine), Gay and Walker (1985); (13) ATPase IX-P2 (bovine), Gay and Walker (1985); (14) cytochrome oxidase IV (yeast), Maarse *et al.* (1984); (15) citrate synthase (yeast), Suissa *et al.* (1984); (16) ornithine aminotransferase (rat), Mueckler and Pitot (1985), Simmaco *et al.* (1986); (17) aspartate aminotransferase (chicken), Jaussi *et al.* (1985); (18) aspartate aminotransferase (porcine), Joh *et al.* (1985); (19) Mn-superoxide dismutase (yeast), Marres *et al.* (1985); (20) ATPase F_1 δ subunit (*N. crassa*), Kruse and Sebald (1984); (21) 5-aminolevulinate synthase (yeast), Keng *et al.* (1986), Urban-Grimal *et al.* (1986); (22) alcohol dehydrogenase iso-III (yeast), Young and Pilgrim (1985); (23) ornithine carbamoyltransferase (human), Horwich *et al.* (1984); (24) ornithine carbamoyltransferase (rat), McIntyre *et al.* (1984), Takiguchi *et al.* (1984), Kraus *et al.* (1985); (25) mEF-Tu (yeast), Nagata *et al.* (1983); (26) carbamoyl-phosphate synthase I (rat), Nyunoya *et al.* (1985); (27) ATPase $F_1\beta$ subunit (yeast), Takeda *et al.* (1985); (28) ATPase F_1 β (*Nicotiana plumbaginifolia*), Boutry and Chua (1985), Kobayashi *et al.* (1986); (29)

on specific amino acid sequences but rather on the overall composition of the prepiece and its amphipathic nature. Sequences with these characteristics may reside at the amino-terminus of mitochondrial precursor proteins, where they are exposed and can be active, but they also appear to exist within nonmitochondrial proteins, although they are apparently masked such that they do not normally direct these proteins to mitochondria (Hurt and Schatz, 1987).

C. The Stop Transport Model

Based on the sequence characteristics of mitochondrial precursor proteins, Hurt and van Loon (1986) proposed a model for intracellular target-

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ATPase $F_1\beta$ (human), Ohta and Kagawa (1986); (30) adrenodoxin (bovine), Okamura *et al.* (1985); (31) ribulose-1,5-bisphosphate carboxylase small subunit (*Chlamydomonas reinhardtii*), Schmidt *et al.* (1979), Hurt *et al.* (1986a); (32) subunit VI of bc_1 complex (yeast), van Loon *et al.* (1984); (33) 14 kDa subunit of bc_1 complex (yeast), De Haan *et al.* (1984); (34) threonyl-tRNA synthase (yeast), Pape *et al.* (1985); (35) tryptophanyl-tRNA synthase (yeast), Myers and Tzagoloff (1985); (36) histidyl-tRNA synthase (yeast), Natsoulis *et al.* (1986); (37) 2-isopropylmalate synthase (yeast), Beltzer *et al.* (1986); (38) MSS51 (COX I pre-mRNA maturase, yeast), Faye and Simon (1983); (39) CBP2 (cytochrome *b* pre-mRNA maturase, yeast), McGraw and Tzagoloff (1983); (40) CBP6 (yeast), Dieckmann and Tzagoloff (1985).

† Notes: (a) Not an actual prepiece (i.e., not proteolytically removed) but amino terminus is able to specifically target β-galactosidae to mitochondria (Hase *et al.*, 1984, 1986). (b) Precursors are processed in two proteolytic steps. This has not yet been demonstrated for bovine ATPase IX (P1 or P2) but it may occur in two steps like *N. crassa* ATPase IX. (c) Bovine COX IV is equivalent to COX V in yeast or *N. crassa* (Gregor and Tsugita, 1982). (d) Possible cleavage site based on homology with *N. crassa* ATPase IX processing sites. Grouped with matrix proteins since it is probably imported as a matrix protein and then assembled into ATPase. (e) Possible cleavage site based on alignment with cytosolic ADH II. (f) Possible cleavage site based on alignment with bacterial mature sequence. (g) An alternate region with similar hydrophobic moment exists between residues 22 and 39. (h) ATPase $F_1\beta$ (a subunit of ATPase at the inner face of the inner membrane) is grouped with matrix proteins since it is probably imported as a matrix protein and then assembled into ATPase. (i) Possible cleavage site based on homology with amino terminus of mature $F_1\beta$ from *Ipomoea batatas* (Kobayashi *et al.*, 1986). (j) Possible cleavage site based on homology with bovine mature sequence. (k) The presequence of chloroplast RuBPCase (31) is presented since the first 35 amino-terminal amino acids can direct DHFR or mature COX IV to mitochondria (Hurt *et al.*, 1986). The first 50 amino acids of the 17K subunit VI of the bc_1 complex (32; determined from the nuclear gene sequence)—which contains an unusually high content of acidic amino acids—are presented, though import has not been examined. Sequences 33–40 are amino-terminal sequences (determined from the nuclear gene sequence) for putative mitochondrial proteins or proteins for which import into mitochondria has not been characterized. The first 50 amino acids from the presumed start site are presented. (l) Apparently imported into mitochondrial matrix without proteolytic processing (Gasser *et al.*, 1982a; Hampsey *et al.*, 1983).

ing and intramitochondrial sorting of imported mitochondrial proteins. In this model, mitochondrial presequences (or amino-terminal sequences in noncleavable proteins) can be divided into distinct domains: (1) matrix targeting domains; (2) stop transport domains; and (3) proteolytic cleavage sites. The presence or absence and arrangement of these domains determines the targeting and sorting of the accompanying mature piece of the protein. The matrix targeting domain (the amino-terminal positively charged region of the prepiece) directs the attached protein to mitochondria and mediates its transfer across both mitochondrial membranes to the matrix. If this domain is followed by a stop transport domain (a long uninterrupted sequence of uncharged amino acids capable of forming a transmembrane anchor), then the transfer of the protein is halted at either the outer or inner membrane, depending on subtle differences in the size and strength of the stop transport region. Further sorting or simply removal of the prepiece can then occur by proteolytic processing.

For example, in this model ADH III is a matrix protein because it contains the amino-terminal matrix targeting domain but no subsequent stop transport sequence. Inner membrane proteins also contain the matrix targeting domain but are halted at the inner membrane because of stop transfer domains within the mature part of the sequence. The matrix targeting sequence is then removed by proteolytic processing to prevent further import. For intermembrane space proteins, such as cytochrome b_2, the stop transport domain is in the prepiece so that when transport is halted at the inner membrane and proteolytic cleavage occurs at the outer face of the inner membrane the mature part of the protein is released into the intermembrane space. Cytochrome c_1 is proposed to follow a similar mechanism but remains associated with the inner membrane because of a carboxy-terminal anchor. Indeed, when the first 64 amino acids of cytochrome c_1 (containing the entire 61 amino acid presequence) was fused to DHFR, the fusion protein was directed to the inner membrane and DHFR was released into the intermembrane space following processing (van Loon *et al.*, 1986). Identical results were observed when the cytochrome c_1 prepiece was fused to the mature part of COX IV (van Loon *et al.*, 1987). Outer membrane proteins are simply halted at the outer membrane by a stop transport domain with presumably different characteristics from the inner membrane stop transport region.

The stop transport model accounts for the intramitochondrial sorting of many imported proteins. For the most part, it assumes a relatively simple linear arrangement of targeting and sorting domains, which may not be the case for all proteins. It does not, however, account for the import mechanism of intermembrane space proteins which do not contain presequences, such as cytochrome *c* or adenylate kinase. Other movements

may also be possible which the data supporting the stop-transport model cannot yet exclude. For example, the Fe/S protein of the bc_1 complex, which like cytochrome c_1 is also synthesized with a long prepiece (that is processed in two steps), is fully transported into the matrix, where it is processed by the matrix peptidase, and is then redirected back to the intermembrane space (Hartl *et al.*, 1986; see Section XIB). In *Neurospora crassa*, cytochrome c_1 follows a similar transport pathway through the matrix (F.-U. Hartl, J. Ostermann, and W. Neupert, in preparation). In yeast, however, the imported cytochrome c_1 was never found on the matrix side of the inner membrane (van Loon and Schatz, 1987).

D. Heterologous Import

The precursor proteins and import machinery of mitochondria from different organisms share many common features. This can be demonstrated in heterologous import experiments in which the mitochondrial precursor protein from one organism can be imported into the mitochondria of another. For example, the Fe/S protein from *Neurospora crassa* bc_1 complex could be imported into yeast mitochondria (Teintze *et al.*, 1982), and, similarly, either ADP/ATP carrier or ATPase subunit IX could be imported into rat liver mitochondria (Schleyer *et al.*, 1982). This is not at all surprising since conservation of complicated import machinery is likely to be high during evolution. What is interesting, however, is that the precursor to *N. crassa* ATPase subunit IX could be imported into yeast mitochondria and be correctly processed (Schmidt *et al.*, 1983a; Fig. 1). In *Neurospora*, ATPase IX is a nuclear gene product which is synthesized on cytoplasmic polysomes (Jackl and Sebald, 1975; van den Boogaart *et al.*, 1982b) while in yeast it is coded for by mitochondrial DNA and synthesized inside the mitochondrion (Tzagoloff and Meagher, 1972; Macino and Tzagoloff, 1979; Hensgens *et al.*, 1979). The mature proteins are similar, and their sequences are 53% homologous in overlapping regions; however, the *Neurospora* pre-ATPase IX, which must be imported from the cytosol, contains a long 66 amino acid amino-terminal prepiece which presumably directs it to the mitochondrial inner membrane. A similar prepiece is absent in the yeast mitochondrial gene product.

Heterologous import has also been demonstrated between different subcellular organelles. When the prepiece (transit peptide) of chloroplast ribulose-1,5-bisphosphate carboxylase (small subunit) was fused to either DHFR or the mature part of cytochrome oxidase IV, the resulting fusion proteins were imported into mitochondria *in vivo*, albeit with lower efficiency than with authentic mitochondrial prepieces. In the latter case, the fusion protein could restore cytochrome oxidase activity *in vivo* when

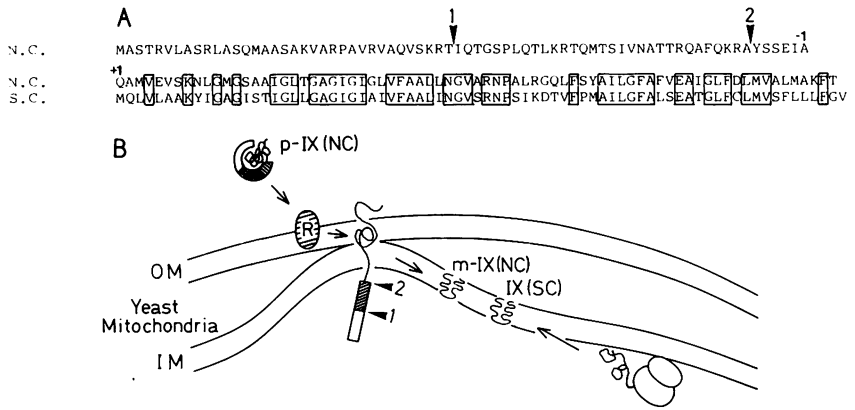

Fig. 1. *Neurospora crassa* pre-ATPase subunit IX is imported into yeast mitochondria. (A) Comparison of amino acid sequences of ATPase IX from *N. crassa* (N.C.) and *Saccharomyces cerevisiae* (S.C.) beginning with the amino terminus. The two sites of proteolytic processing of the *N. crassa* ATPase IX prepiece are indicated by arrows. Identical amino acid residues are indicated by boxes. (See text for references.) (B) *Neurospora crassa* ATPase IX is a nuclear gene product synthesized in the cytosol with a long prepiece. It can be imported into and processed by yeast mitochondria (*in vitro*) where ATPase IX is a mitochondrial gene product. Left: Sequence of events in the heterologous import of *N. crassa* ATPase IX. Right: Synthesis of yeast ATPase IX on membrane-bound mitochondrial ribosomes and insertion into the inner membrane. OM, Outer membrane; IM, inner membrane; R, receptor; p, precursor; m, mature.

used to transform a COX IV-deficient mutant of yeast (Hurt *et al.*, 1986a,b). When compared to mitochondrial prepiece sequences, the chloroplast transit peptide shares many common features (e.g., high content of basic and hydroxylated amino acids with no acidic amino acids; Table I). What subtle differences exist in the targeting prepieces that correctly direct proteins to either chloroplasts or mitochondria in photosynthetic cells are unknown.

IV. RECEPTORS

While much information is obviously contained within specific regions of mitochondrial precursor proteins, there must exist complementary structures within mitochondria themselves to decode and process it. One obvious possibility is that receptors are involved in the initial recognition of proteins imported from the cytosol.

Proteinaceous components on the outer surface of mitochondrial membranes were first demonstrated by shaving isolated mitochondria with low concentrations of protease which did not penetrate or destroy the outer

membrane (Gasser *et al.*, 1982a; Zwizinski *et al.*, 1984; Pfaller and Neupert, 1987). Following this treatment, the specific binding of precursor proteins to the outer membrane was blocked and import was abolished.

The binding of precursor proteins to mitochondria is independent of import and precedes all other events in the import pathway. For example, mature outer membrane porin is in a location which is protected from externally added proteases. If the insertion of precursor porin into the lipid bilayer of the outer membrane was inhibited by importing at 0°C, the binding of the protein to mitochondria was unaffected and it remained in a protease-susceptible location at the outer face of the outer membrane. When mitochondria containing the bound porin were reisolated and warmed to 25°C, the protein was subsequently imported from its receptor sites to the protease-protected location (Freitag *et al.*, 1982). Similarly, the import of cytochrome *c* could be blocked with the heme analog deuterohemin which prevents covalent attachment of heme to the precursor apocytochrome *c* and subsequent translocation across the outer membrane. Under these conditions, apocytochrome *c* could still bind to mitochondria independently of import. When the inhibition by deuterohemin was reversed by adding excess amounts of hemin, cytochrome *c* was subsequently imported from its receptor sites into the intermembrane space (Hennig and Neupert, 1981).

Proteins such as the ADP/ATP carrier, which require a membrane potential for import, could be stalled at their receptor sites by disrupting the potential with CCCP. On reestablishing the membrane potential, the bound precursor could be imported (Zwizinski *et al.*, 1983; Pfanner and Neupert, 1985). In these cases, however, the binding to deenergized mitochondria was slow, though clearly independent of a membrane potential. Binding of pre-ATPase $F_1\beta$ to deenergized mitochondria did not occur at all (Zwizinski *et al.*, 1984), though for unknown reasons. The binding of membrane potential-dependent proteins to deenergized mitochondria may be poor because of the low capacity of specific sites which may be occupied by precursors with higher affinity.

Receptors for imported mitochondrial proteins are specific in many regards as was demonstrated by the receptors for precursors to cytochrome b_2 and citrate synthase in yeast (Riezman *et al.*, 1983b). First, binding was specific to the outer mitochondrial membrane. These precursor proteins were able to bind to whole mitochondria or to isolated outer membrane vesicles (Riezman *et al.*, 1983a) but not to inner membrane preparations. Binding was also specific for precursor proteins. When the partially processed intermediate form of the cytochrome b_2 precursor was generated by treatment with an extract of the mitochondrial matrix (con-

taining the processing peptidase), the intermediate size cytochrome b_2 did not bind to mitochondria. Similarly, binding of the mature form did not occur either. The same observation has been made with cytochrome *c* where mature holocytochrome *c* did not recognize the binding sites in *Neurospora* mitochondria (Hennig *et al.*, 1983) and even large excesses could not displace prebound apocytochrome *c* (H. Köhler and W. Neupert, unpublished). Last, binding was specific for mitochondrial proteins. For example, the binding of cytosolic hexokinase and glyceraldehyde-3-phosphate dehydrogenase to whole yeast mitochondria or isolated outer membrane vesicles was negligible (Riezman *et al.*, 1983b).

How many different receptors exist to mediate the recognition and binding of imported mitochondrial proteins? It is clear from the large number of mitochondrial proteins and the limited number associated with the outer membrane that it would be impossible, if not just simply impractical, for a different receptor to exist for each protein. Nevertheless, experiments have shown that many mitochondrial proteins do not share common binding sites so that a single receptor for all imported proteins is not the case either. For example, the binding of porin to yeast mitochondria was not affected when the mitochondria were pretreated with low concentrations of trypsin that were sufficient to abolish the binding of F_1 ATPase β subunit and cytochrome b_2 (Gasser and Schatz, 1983). This suggests that they are bound by different receptors having different trypsin sensitivities. Similarly, shaving *Neurospora* mitochondria with elastase inhibited the binding and import of ADP/ATP carrier and porin but had no effect on ATPase $F_1\beta$ (Zwizinski *et al.*, 1984).

By this criterion it is difficult to assign possible common binding sites for the few mitochondrial proteins that have been studied to date. In addition, proper competition studies are limited by the difficulties of preparing sufficient amounts of mitochondrial precursor proteins. In three cases this has been overcome: (1) apocytochrome *c*, the precursor to holocytochrome *c*, can be prepared by chemical cleavage of the heme group and renaturation of the protein by dialysis from urea (Ambler and Wynn, 1973); (2) a water-soluble form of porin can be made by subjecting isolated porin to acid precipitation and then resolubilizing the protein at alkaline pH in the absence of detergent (Pfaller *et al.*, 1985); and (3) the precursor to F_1 ATPase β subunit can be accumulated then purified from yeast *rho*$^-$ mutants grown in the presence of CCCP (Ohta and Schatz, 1984). In all three cases, the resulting precursor proteins were import competent and retained characteristics of their precursors synthesized in cell-free systems. Apocytochrome *c* does not compete for the binding of any mitochondrial proteins tested so far, including cytochrome c_1, the Fe/S protein of bc_1 complex, ATPase $F_1\beta$, ATPase IX, or ADP/ATP carrier

(Zimmermann *et al.*, 1981; Teintze *et al.*, 1982). It appears to have a unique receptor. Porin, however, is able to compete for the binding and import of the ADP/ATP carrier, suggesting a common receptor (Pfaller and Neupert, 1987). Binding competition has not yet been examined using the ATPase $F_1\beta$ precursor purified from yeast.

The binding of cytochrome *c* to *Neurospora* mitochondria is the best characterized of imported mitochondrial proteins so far. When radiolabeled apocytochrome *c* was bound to mitochondria in the presence of deuterohemin (to prevent subsequent import), it could be completely displaced by adding unlabeled apocytochrome *c* but not holocytochrome *c* (Hennig *et al.*, 1983). Apocytochrome *c* from different species could also displace the precursor protein to varying degrees. Titration of the binding sites on mitochondria by Scatchard analysis indicated that there were 90 pmol of high affinity binding sites for apocytochrome *c* per milligram of mitochondrial protein. These sites had an association constant (K_a) of $2.2 \times 10^7\ M^{-1}$ (Table II).

When mitochondria were solubilized with octyl glucoside and the resulting extract was reconstituted into liposomes, the high affinity binding sites were also reconstituted (Köhler *et al.*, 1987). Using this procedure to identify high affinity binding for apocytochrome *c*, a putative receptor protein was identified and purified to homogeneity. The protein, when reconstituted into liposomes, bound apocytochrome *c* with similar affinity compared to whole mitochondria. In addition, the purified protein could compete with mitochondria for the binding of apocytochrome *c*. The unusual feature of the apocytochrome *c* binding protein, however, is that it is a soluble protein of the intermembrane space. This explains why more than a 10-fold higher concentration of proteases are required to abolish apocytochrome *c* binding to mitochondria compared to other imported proteins. Although the binding of apocytochrome *c* to the protein meets all criteria for specific interaction of a protein with a receptor—namely, (i)

TABLE II

Mitochondrial Receptors for Imported Precursor Proteins

Precursor protein	Number of binding sites (pmol/mg mitochondrial protein)	Association constant (K_a)	Reference
Apocytochrome *c*	90	$2.2 \times 10^7\ M^{-1}$	Hennig *et al.* (1983)
Porin	5–10	$2 \times 10^8\ M^{-1}$	Pfaller *et al.* (1985, 1987)
ADP/ATP carrier	1.7	$1.1 \times 10^9\ M^{-1}$	Schmidt *et al.* (1985)

rapid and reversible binding, (ii) saturable and limited in number, and (iii) specificity—the location in the intermembrane space is unexpected. Cytochrome *c* appears to have an unusual import pathway which is not representative of the mechanism for other imported proteins. Apocytochrome *c* is able to spontaneously insert into lipid bilayers in a nonspecific manner with low affinity (Rietveld *et al.*, 1983, 1985, 1986a,b; Rietveld and Kruijff, 1984; Dumont and Richards, 1984). A possible explanation of the binding protein's location is that it recognizes the partially inserted apocytochrome *c* and then binds it from the inner face of the outer membrane, perhaps to mediate exposure of the cysteine sulfhydryl groups to the heme attaching enzyme. The apocytochrome *c* binding protein and the heme attaching enzyme are distinct proteins (Nicholson *et al.*, 1987).

The high affinity binding of porin to mitochondria was sensitive to treatment of whole mitochondria with very low concentrations of trypsin, indicating that the receptor is exposed to the outer surface of the outer membrane (Pfaller and Neupert, 1987). As for cytochrome *c*, the high affinity binding sites for porin could be solubilized with detergent and reconstituted into liposomes. Using this approach, it should be possible to purify the porin receptor.

The identification of putative receptor proteins mediating import into mitochondria has also been successful using other methods. For example, antibodies raised against the total outer mitochondrial membrane of yeast blocked the import of the preCOX IV-DHFR fusion protein (Ohba and Schatz, 1987a). Antibodies raised against a 45 kDa outer membrane protein also blocked import while sera against other outer membrane proteins did not. The Fab fragments of the anti-45 kDa antibodies alone also blocked import. In another study, a 30 kDa protein from the outer membrane of rat mitochondria was specifically cross-linked to the synthetic prepiece (amino acids 1–27) of pre-OTC (Gillespie, 1987). Mild pretreatment of mitochondria with trypsin reduced both cross-linking of the prepiece peptide to the 30 kDa protein and the import of pre-OTC into mitochondria.

V. ENERGY REQUIREMENTS

Precursor proteins bind to mitochondria independently of other events in the import pathway; however, subsequent translocation into the mitochondrion is dependent on energy in most cases. Generally, energy is required for (i) all proteins having an amino-terminal extension, (ii) all proteins which are inserted into or translocated across the inner membrane, and (iii) only for import to the first processing stage for those

proteins which are proteolytically processed in two steps. Where import requires energy, it is needed in the form of an energized inner membrane, specifically, the membrane potential component ($\Delta\psi$) of the total protonmotive force (Δp).

The primary energy source required for import was first thought to be ATP (Nelson and Schatz, 1979). It was later shown, however, that an energized inner membrane was the necessary energy form. Even a low potential of 20–40 mV, about one-tenth the normal value of 230 mV (Mitchell and Moyle, 1969), can drive the import of mitochondrial proteins (Pfanner and Neupert, 1985).

The requirement for an electrochemical potential and not ATP as the immediate energy source was clearly demonstrated *in vitro* in a series of experiments by Schleyer *et al.*, (1982) in which high intramitochondrial ATP plus a dissipated membrane potential versus low ATP plus a normal membrane potential were compared in *Neurospora crassa* mitochondria. In the first case (high ATP/no membrane potential), the membrane potential was dissipated by the protonophore CCCP, and intramitochondrial ATP was elevated via the ADP/ATP carrier by adding ATP externally. Oligomycin was added to inhibit the F_0F_1 ATPase and prevent the hydrolsis of ATP which would generate a small potential. Under these conditions, high internal concentrations of ATP in the absence of an energized inner membrane could not drive import. In the opposite case (low ATP/normal membrane potential), intramitochondrial ATP was depleted by preincubating mitochondria in the presence of oligomycin (to inhibit the membrane potential-driven synthesis of ATP) and carboxyatractyloside (to block the ADP/ATP carrier and prevent the translocation of external ATP into mitochondria). Under these circumstances, the electrochemical potential in the absence of ATP was sufficient to power import. In yeast mitochondria, import was blocked when the membrane potential was dissipated with CCCP but not when ATP synthesis was inhibited by oligomycin (Gasser *et al.*, 1982a). Furthermore, ATP-supported import in cyanide-inhibited mitochondria was blocked by either carboxyatractyloside (so ATP could not enter the matrix) or oligomycin (which would prevent ATP hydrolysis), demonstrating that ATP-stimulated import was a consequence of the small electrochemical gradient generated by the hydrolysis of ATP by the F_0F_1 ATPase.

The electrochemical potential (or total protonmotive force) Δp is the sum of the membrane potential ($\Delta\psi$) from the separation of charged species across the inner membrane and the force exerted by the pH gradient (ΔpH) ($\Delta p = \Delta\psi - Z\,\Delta$pH). It is the membrane potential component of the total protonmotive force which is responsible for powering import. This was first suggested by experiments where the ionophore nigericin, which

exchanges K^+ for H^+ in a stoichiometric way across the inner membrane, did not affect the import of ADP/ATP carrier or ATPase IX into *Neurospora* mitochondria (Schleyer *et al.*, 1982). Since nigericin leads to the breakdown of the proton gradient without affecting the membrane potential, it is the latter which drives import of precursor proteins. This was substantiated in experiments where the physiological membrane potential was inhibited by antimycin A/oligomycin and then a valinomycin-induced potassium diffusion potential could drive the import of the ADP/ATP carrier into *Neurospora* mitochondria (Pfanner and Neupert, 1985). This import was not abolished by protonophores like CCCP which dissipate the proton gradient and, in the absence of a K^+ diffusion potential, would normally dissipate the accompanying membrane potential. Furthermore, the establishment of a ΔpH did not power import. The importance of the membrane potential component of the total protonmotive force is not surprising since ΔpH makes only a relatively small contribution to Δp under physiological conditions.

The energy necessary for import is not needed for proteolytic processing itself. For example, proteins that are transported to the inner membrane or matrix, but which are not proteolytically processed, require an energized inner membrane. This has been demonstrated for the ADP/ATP carrier (Schleyer *et al.*, 1982; Pfanner and Neupert, 1985) and for the matrix-localized 2-isopropylmalate synthase (Hampsey *et al.*, 1983). In addition, the purified matrix processing peptidase does not require any form of energy to process protein outside of whole mitochondria (G. Hawlitschek and W. Neupert, unpublished). Instead, energy is required for interaction with the mitochondrial inner membrane. Evidence for this is 3-fold. First, all precursor proteins which must be translocated into or across the inner membrane require a membrane potential for import. This is also true for intermembrane space proteins which require proteolytic processing (e.g., cytochrome b_2, cytochrome c_1, and cytochrome *c* peroxidase). In these cases, the precursor protein must at least partially penetrate the inner membrane to reach the matrix peptidase for the first processing event (Daum *et al.*, 1982b; Gasser *et al.*, 1982b; Reid *et al.*, 1982; Teintze *et al.*, 1982). Second, proteins that are imported into the intermembrane space but do not come in contact with the inner membrane, such as cytochrome *c* (Zimmermann *et al.*, 1981), and those imported into the outer membrane, such as porin (Freitag *et al.*, 1982; Mihara *et al.*, 1982a) and the major outer membrane polypeptides (Gasser and Schatz, 1983), do not require an energized inner membrane for import. Finally, when import of cytochrome c_1 and F_1 ATPase β subunit was performed at 7°C, an intermediate could be trapped in which the amino-terminal prepiece could be processed by the matrix peptidase, but the

major portion of the precursor was still outside the mitochondrion and could be digested by externally added proteases (Schleyer and Neupert, 1985). Import to this stage was dependent on energy; however, subsequent translocation of the proteins completely into mitochondria was independent of a membrane potential when chased at 25°C. Therefore, only import of the amino terminus through the inner membrane required a membrane potential while transport of the rest of the protein did not.

Proteolytic processing, however, is not obligatory for import past the energy-dependent step. Precursors to F_1 ATPase β and IX subunits could be partially imported into mitochondria without processing in the presence of *o*-phenanthroline, which blocks the matrix processing peptidase. The precursors could then be chased to the mature size by adding Mn^{2+} in a step that did not require a membrane potential (Zwizinski and Neupert, 1983). The energy-dependent step precedes and is independent of proteolytic processing.

Exactly why the membrane potential is required for import is not clear. One possibility is that it produces an electrophoretic driving force (negative inside) on the positively charged prepieces which mediates pentration into or through the inner membrane (Fig. 2). Another is that it induces transient conformational changes in lipid and protein organization which allow the initial entry of the precursor protein into or through the membrane barrier. Both effects probably contribute to energy-dependent import.

Not all energy requirements are strictly for an energized inner mem-

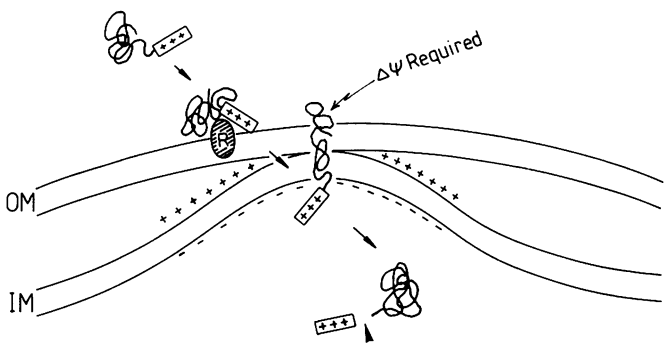

Fig. 2. A membrane potential ($\Delta\psi$) is required for the import of proteins which must go to or through the inner membrane. Energy is not required for binding to receptors but is necessary for penetration of the positively charged amino-terminal prepiece through the inner membrane. Subsequent translocation of the remainder of the protein through the inner membrane, or movement away from contact sites (i.e., inner membrane proteins), does not require the continued presence of $\Delta\psi$. Proteolytic processing is also independent of energy. OM, Outer membrane; IM, inner membrane; R, receptor.

brane. For example, degredation of endogenous ATP, by the enzyme apyrase, reduced the import of ATPase $F_1\beta$ into *Neurospora crassa* mitochondria even in the presence of a membrane potential, whereas subsequent addition of ATP or GTP restored import (Pfanner and Neupert, 1986). Nonhydrolyzable ATP analogues also blocked the import of mitochondrial precursor proteins (Pfanner and Neupert, 1986; Eilers *et al.*, 1987; Chen and Douglas, 1987), indicating that cleavage of the nucleoside triphosphate (NTP) phosphodiester bond is necessary. It has been suggested that NTPs maintain or confer an import-competent conformation in mitochondrial precursor proteins. This is supported by experiments in which the proteolytic sensitivity of precursor proteins is greater in the presence of NTPs (Pfanner *et al.*, 1987; Verner and Schatz, 1987), indicating that a less folded conformation is sustained by NTP hydrolysis and that such a conformation is necessary for import. The requirement for NTPs depends primarily on the mature part of the precursor protein. For example, precursors having identical presequences but different mature polypeptides required different concentrations of NTPs for optimal import (Pfanner *et al.*, 1987). It appears that NTPs are necessary for confering import-competence during all steps that precede and include the interaction of the precursor with the outer membrane (Pfanner *et al.*, 1987; Eilers *et al.*, 1987). In addition, maturation steps for some imported proteins require NADH. For example, the covalent attachment of heme to apocytochrome *c* and concomitant translocation across the outer membrane is dependent on NADPH in yeast (Basile *et al.*, 1980) and NADH in *N. crassa* (Nicholson *et al.*, 1987). Similarly, the second processing step of cytochrome c_1, which is accompanied by covalent heme attachment, is also dependent on NADH (Teintze *et al.*, 1982; Schleyer and Neupert, 1985). The second processing step of the Fe/S protein of the bc_1 complex requires NADH (F.-U. Hartl and W. Neupert, unpublished), presumably for the Fe/S cluster formation. Maturation of cytochrome oxidase II, a mitochondrial translation product, also requires NADH (W. Driever, R. Cook, and W. Neupert, unpublished). These proteins all have the common feature of having iron, either in heme or as nonheme Fe/S clusters.

VI. CYTOSOLIC COFACTORS

By virtue of the elaborate mixtures which are required for the cell-free synthesis of precursor proteins, import reactions *in vitro* contain many components. In the few cases where mitochondrial precursor proteins could be purified or where minimal amounts of cell-free translation mixtures have been used, a number of potential "cytosolic" cofactors which are required for import have been identified. These cofactors are present

both in reticulocyte lysates and in homologous postribosomal cytosol preparations. They fall into three broad classes: (i) soluble low molecular weight components; (ii) proteins; and (iii) RNA.

When a reticulocyte lysate translation mixture containing the newly synthesized precursor to rat ornithine carbamoyltransferase (OTC) was passed over a Sephadex G-25 column, the precursor, recovered in the excluded fraction, could no longer by imported into mitochondria. Import of the precursor could be restored by fresh unlabeled reticulocyte lysate mixture but not by its individual components, including Mg^{2+}, K^+, or ATP (Argan *et al.*, 1983). The reticulocyte lysate itself, without additives for translation, completely restored import. In an independent report, the postribosomal supernatant of the reticulocyte lysate, which had been dialyzed, stimulated the import of pre-OTC severalfold (Miura *et al.*, 1983). The stimulating activity of the added dialyzed lysate was inactivated by either pretreatment with trypsin or heat denaturation, suggesting that the cofactor is a protein. The cofactor was further characterized by examining requirements for the import of pre-OTC, which had been purified by immunoaffinity chromatography, into mitochondria (Argan and Shore, 1985). When pre-OTC was mixed with the untreated lysate, the cofactor bound the precursor to form a 5 S complex. The pre-OTC in the isolated 5S complex was imported into mitochondria without a requirement for extra lysate. When mitochondria were pretreated with reticulocyte lysate and then reisolated, import of purified pre-OTC did not occur. However, when pre-OTC plus reticulocyte lysate was mixed with mitochondria at 4°C, 50% of the pre-OTC bound to the mitochondrial surface and could be imported when the mitochondria were isolated and incubated at 30°C. The import factor appears to be necessary for the import-competent delivery and binding of pre-OTC to mitochondria.

The purified precursor to the F_1 ATPase β subunit is poorly imported into yeast mitochondria. In the presence of the cytosolic fraction from yeast or reticulocytes, however, import and processing was stimulated 4- to 8-fold (Ohta and Schatz, 1984). The cofactor was nondialyzable, protease sensitive, and had an apparent molecular mass of 40 kDa. Similar stimulation of import of the *in vitro* synthesized cytochrome b_2 precursor by unlabeled reticulocyte lysate indicated that cofactors may be necessary for other proteins as well. Again, the cofactor appeared to aid the correct association of the precursor with the mitochondrial surface.

A cytoplasmic RNA component has also been implicated in the import of several proteins into rat liver mitochondria. Posttranslational treatment with RNase of reticulocyte lysates containing newly synthesized precursor proteins inhibited subsequent import into mitochondria (Firgaira *et al.*, 1984). The cofactor and OTC precursor fractionated as a 400 kDa

complex with characteristics of a ribonucleoprotein. On the other hand, the cofactor which stimulated the import of purified OTC precursors was not sensitive to RNase (Argan and Shore, 1985). The import of F_1 ATPase β subunit and citrate synthase precursors into yeast mitochondria was also inhibited by pretreatment of the reticulocyte lysate (in which they were synthesized) with RNase; however, removal of the ribosomes by centrifugation abolished the sensitivity of import to RNase, suggesting that the RNA cofactor is not a specific component necessary for import but that degraded ribosomes inhibit import (Burns and Lewin, 1986). In contrast, RNase treatment of reticulocyte lysate postribosomal supernatants containing newly synthesized ATPase $F_1\beta$ inhibited binding and import into *Neurospora crassa* mitochondria (N. Pfanner and W. Neupert, unpublished). The role of putative RNA cofactors requires further investigation.

Protein and RNA cofactors present in cytosolic fractions may serve a number of possible functions during import. For example, they may stabilize precursor proteins against premature proteolytic digestion in the cytosol or mediate conformational arrangements which are necessary for import competence. These cofactors appear to be necessary for the specific binding of precursors to mitochondria but not for subsequent stages of import. Specific cofactors have not yet been isolated so that it cannot be ruled out that they may act in a nonspecific manner by, for instance, preventing the small amounts of precursor proteins from aggregating. Whether such cofactors are important *in vivo* is unknown.

The import of apocytochome *c* into mitochondria is also dependent on a cytosolic or reticulocyte lysate cofactor. In this case, however, the stimulatory component is a low molecular weight, heat-stable factor which is not sensitive to proteases. In yeast, the cofactor can be substituted by an NADPH-regenerating system (Taniuchi *et al.*, 1982), but in *N. crassa* the cofactor appears to serve some other function (Nicholson *et al.*, 1987). It is not involved in the binding of apocytochrome c to mitochondria, but is necessary for enzymatic attachment of heme and subsequent translocation across the outer membrane.

VII. TRANSLOCATION CONTACT SITES

Proteins which are imported into the mitochondrial matrix or inner membrane must cross two membrane barriers to reach their final location. The question arises as to whether transport across the outer and inner membrane occurs in two distinct steps, with a soluble intermediate in the intermembrane space, or whether the inner and outer membranes come

close enough together to be spanned and crossed in a single event. For proteins imported into the inner membrane or matrix of *Neurospora crassa* mitochondria, the latter mechanism appears to be the case. These translocation contact sites have been demonstrated for the import of the F_1 ATPase β subunit and cytochrome c_1 (Schleyer and Neupert, 1985), the Fe/S protein of bc_1 complex (Hartl *et al.*, 1986), and the ADP/ATP carrier (Pfanner and Neupert, 1987).

Membrane-spanning intermediates could be detected when translocation was impeded by importing the precursors of ATPase $F_1\beta$ or cytochrome c_1 at low temperatures (4–12°C). Under these conditions, translocation intermediates were accumulated which had penetrated far enough through the inner membrane to be processed by the matrix-localized processing peptidase but which still had the major portion of the polypeptide exposed to the outer surface of the outer membrane since they could be digested with externally added proteases (Schleyer and Neupert, 1985). Import to this stage was dependent on a membrane potential. When translocation intermediates spanning contact sites were accumulated this way, they could be subsequently chased into a protease-insensitive location by raising the temperature to 25°C in a step that was independent of the membrane potential. Similarly, complete transport across the mitochondrial membranes could be blocked by prebinding the precursor proteins to specific antibodies generated against the mature size proteins. When incubated with mitochondria, the amino terminus of the antibody-bound precursors penetrated far enough into the matrix to be proteolytically processed while the major part of the precursor was retained by the antibodies outside the mitochondria in a location susceptible to externally added proteases. These experiments indicate that (i) the ATPase $F_1\beta$ and cytochrome c_1 precursors first entered the matrix by their amino termini in a step that is dependent on a membrane potential; (ii) the membrane potential is required only for import of the amino-terminal prepiece which is then sufficient to trigger the transmembrane movement of the remainder of the polypeptide chain; and (iii) transport across the mitochondrial membranes occurs via translocation contact sites in which the outer and inner membranes come close enough together to be simultaneously spanned by the imported polypeptide.

Sites of contact between the outer and inner mitochondrial membranes have been visualized by transmission electron microscopy (Hackenbrock, 1968). They appear to be stable structures since they are visible in mitochondria in both the condensed and orthodox conformations. It was estimated that there were 115 of these sites in a 1 μm diameter mitochondrion from rat liver. Similar regions of contact have been observed in mitochondria that were prepared for electron microscopy by freeze-etching (van

Venetie and Verkleij, 1982). Interpretation of the fracture plane, which jumped back and forth between the outer and inner limiting membranes, suggested that semifusion of the membranes had occurred in which nonbilayer lipids (specifically hexagonal II phase lipids) were involved.

Recently, the sites where mitochondrial precursor proteins form translocation intermediates spanning both membranes were correlated to the morphological contact regions (Schwaiger *et al.*, 1987). The precursor of the F_1 ATPase β subunit, synthesized in reticulocyte lysate, was prebound to a specific antibody directed against the mature protein and then incubated with mitochondria. The antibody prevented the protein from being completely imported into mitochondria, but the amino-terminal prepiece was removed by the matrix-localized processing peptidase. When the antibody-bound pre-$F_1\beta$ was tagged by protein A–gold and visualized by electron microscopy, the gold particles were exclusively localized in regions of contact between inner and outer membranes. These contact sites had three distinct characteristics. First, they appeared to be stable structures. Mitochondria which had been treated with low concentrations of digitonin to disrupt the outer membrane still contained regions of contact between the inner membrane and outer membrane fragments. This agrees with the persistent occurrence of contact sites in condensed versus orthodox states. Second, contact site formation did not require precursor proteins. Mitochondria which had been pretreated with digitonin were able to mediate protein import. Therefore, contact sites seem to be preformed to facilitate import as opposed to being formed as a consequence of the presence of precursor proteins. And third, the outer membrane was necessary for contact site-mediated import. When mitochondria were pretreated with digitonin (to create mitoplasts), then incubated with antibody-bound $F_1\beta$, none of the protein A–gold particles was associated with the inner membrane, but they were associated with the remaining contact regions. Similarly, intact mitochondria which were shaved with trypsin then treated with digitonin did not import pre-$F_1\beta$ while the nonprotease-treated mitochondria (also treated with digitonin) did. While this indicates that second sites on the inner membrane that recognize precursor proteins do not exist, it has been demonstrated in yeast that import into trypsin-inactivated mitochondria can be restored if the outer membrane is disrupted by osmotic shock (Ohba and Schatz, 1987b).

How contact sites mediate transmembrane transport of proteins is unclear, particularly for proteins directed to the inner membrane. One possibility is that the formation and dissipation of contact sites is in constant flux so that proteins are translocated into the sites and then pulled into the mitochondrion with the inner membrane as they are dissipated. Another

possibility is that the imported proteins migrate laterally from the contact regions. Finally, inner membrane proteins may be completely transported through the contact sites to the matrix and then redirected back to the inner membrane. Whether physical contact between the two membranes is necessary for import is unknown. The shortest precursor protein shown to be imported via contact sites so far is the Fe/S protein of the bc_1 complex (231 amino acids) (Hartl *et al.*, 1986). Calculation of the minimal distance which the membranes must come together to be spanned by pre-Fe/S indicates that they do not necessarily have to make contact, but they must come very close together (i.e., 10–20 nm, Fig. 3). The constituents and events occurring at contact sites remain unclear.

VIII. PROTEOLYTIC PROCESSING

During or shortly following the translocation step, the amino-terminal prepiece of many proteins directed to the inner membrane or matrix is removed by a specific protease which is located in the matrix (Böhni *et al.*, 1980; Mori *et al.*, 1980; Schmidt *et al.*, 1984). This occurs very rapidly *in vivo*. For example, in pulse–chase experiments in rat liver explants, the precursor for carbamoyl-phosphate synthase was imported into mitochondria and processed with a half-life of 2 min (Raymond and Shore, 1981). Similarly, in yeast, the F_1 ATPase β subunit precursor was imported and processed with a half-life of 0.5 min (Reid and Schatz, 1982b). Proteolytic processing must normally occur immediately on exposure of the precursor proteins to the matrix protease since precursor proteins cannot normally be detected in mitochondria. Processing, however, is not obligatory for import since the precursors to F_1 ATPase β and IX subunits could be imported into mitochondria when proteolytic processing was blocked with *o*-phenanthroline (Zwizinski and Neupert, 1983). Similarly, the precursor of the bc_1 complex Fe/S protein could be imported and did accumulate in the mitochondrial matrix when processing was blocked (Hartl *et al.*, 1986).

The matrix proteases identified and characterized in yeast, rat, and *Neurospora* mitochondria share similar properties: (1) the protein is a soluble component of the mitochondrial matrix; (2) it has a neutral pH optimum; (3) it is not affected by inhibitors of serine proteases [i.e., 4-nitrophenyl phosphate (PMSF)]; (4) it is sensitive to divalent cation chelators (i.e., *o*-phenanthroline); and (5) activity is stimulated by divalent cations such as Co^{2+}, Mn^{2+}, and Zn^{2+} (which can also reverse chelator inhibition; Böhni *et al.*, 1980, 1983; McAda and Douglas, 1982; Mori *et al.*, 1980; Miura *et al.*, 1982; Conboy *et al.*, 1982; Schmidt *et al.*, 1984). In

whole mitochondria, only *o*-phenanthroline inhibits processing activity, whereas chelators such as ethylenediaminetetraacetic acid (EDTA) or bathophenanthroline, which cannot penetrate the inner membrane, do not. Partially purified preparations of the protease are inhibited by all these chelators and also by nucleoside triphosphates (Böhni *et al.*, 1983). In addition, activity was reported to be specifically inhibited in whole cells (isolated hepatocytes) by rhodamine 123 and 6G, and it was described to occur without affecting the membrane potential (Morita *et al.*, 1982; Kolarov and Nelson, 1984; Kuzela *et al.*, 1986). The underlying mechanism is not understood, but inhibition could not be overcome with excess divalent cations.

The matrix-located peptidase has been partially purified from yeast mitochondria. In one report, activity was purified 200-fold over whole mitochondria (McAda and Douglas, 1982). When analyzed by gel filtration, the protease had an apparent molecular weight of 150,000, and activity correlated best with a 59 kDa protein subunit identified by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). In another study where the protease activity was enriched 100-fold over whole mitochondria, the partially purified enzyme migrated with an apparent molecular weight of 115,000 but displayed 10 protein bands on SDS–PAGE, none of which corresponded to the 59 kDa band identified in the former study (Böhni *et al.*, 1983). The matrix protease was found to be a nuclear gene product which itself must be imported into mitochondria.

The matrix-localized peptidase has been purified to homogeneity from *N. crassa* mitochondria. Activity was enriched 5000-fold over whole mitochondria by a series of purification steps and displayed two bands on SDS–PAGE (52 kDa and 57 kDa; Hawlitschek *et al.*, 1988). When the two subunits were separated, neither was able to process precursor proteins. Activity was fully restored, however, when they were recombined. Activity of the matrix-localized processing peptidase depends on the presence of both proteins, though the contribution of each is as yet unclear.

Some imported mitochondrial proteins are proteolytically processed in two distinct steps. This has been demonstrated in yeast cytochrome b_2 (Gasser *et al.*, 1982b; Reid *et al.*, 1982; Daum *et al.*, 1982b), yeast and *Neurospora* cytochrome c_1 (Gasser *et al.*, 1982b; Ohashi *et al.*, 1982; Teintze *et al.*, 1982; Schleyer and Neupert, 1985), yeast cytochrome *c* peroxidase (Reid *et al.*, 1982), *Neurospora* ATPase subunit IX (Schmidt *et al.*, 1984), and the Fe/S protein of bc_1 complex in yeast (Sidhu and Beattie, 1983) and *Neurospora* (Hartl *et al.*, 1986). The precursor for rat liver OTC was originally thought to be processed in two steps as well, with the transient formation of an intermediate size protein (Mori *et al.*, 1980; Kraus *et al.*, 1981; Miura *et al.*, 1982; Conboy *et al.*, 1982); how-

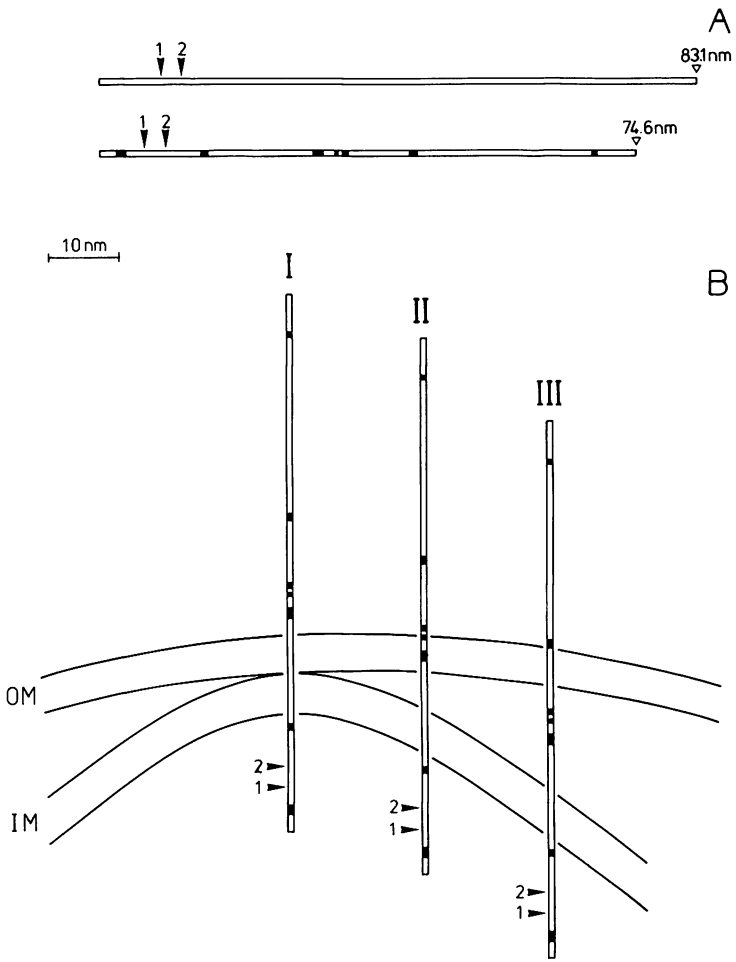

Fig. 3. Proximity of inner and outer membranes is required for simultaneous crossing by imported precursor proteins. Components are drawn approximately to scale. (A) The shortest protein demonstrated to be imported via translocation contact sites to date is the Fe/S protein of the bc_1 complex (231 amino acids). The approximate linear length (at 0.36 nm/residue) is 83.1 nm. When compression owing to a helical structure is included (yielding 0.15 nm/residue), as predicted from the primary sequence (Chou and Fasman, 1974; Argos *et al.*, 1978), the contour length is 74.6 nm. Helical regions are indicated by shaded boxes. Sites of proteolytic processing are indicated by arrowheads. (B) Translocation intermediates are shown where the amino terminus protrudes far enough into the matrix to be processed by the chelator-sensitive matrix peptidase but the protein still has a major part outside the mitochondrion where it is accessible to externally added proteases. Three contact proximities are illustrated: (I) membranes in contact, (II) a 5 nm space between membranes as observed by electron microscopy (Schwaiger *et al.*, 1987), and (III) estimated maximum

ever, evidence now suggests otherwise since: (i) "intermediate" OTC can be found outside of mitochondria while the processing peptidase is located in the mitochondrial matrix (Kolansky *et al.*, 1982); (ii) intermediate size OTC cannot be detected *in vivo* (Mori *et al.*, 1981; Morita *et al.*, 1982); and (iii) apparent "intermediate" OTC is found in the absence of mitochondria and does not associate with mitochondria *in vitro* (Argan *et al.*, 1983).

The first and second processing events appear to be performed by distinct proteases in different submitochondrial locations, with the possible exception of *N. crassa* ATPase IX processing. The ATPase IX precursor is imported to the inner membrane where its amino-terminal extension protrudes into the matrix and is processed in two steps by the chelator-sensitive matrix peptidase (Schmidt *et al.*, 1984). Evidence that the same enzyme is responsible for both cleavage steps includes the following: (1) both processing steps take place in the matrix, (2) both steps have the same sensitivity to chelating agents, (3) the two cleavage sites share amino acid sequence homology (hydrophobic-polar-Lys-Arg-small/bulky hydrophobic), and (5) the purified processing peptidase performs both cleavage steps (G. Hawlitschek and W. Neupert, unpublished).

Other than ATPase IX, precursor proteins which are processed in two steps share common features (Table III): (i) their respective mature forms are exposed to the intermembrane space (cytochrome b_2 and cytochrome *c* peroxidase are soluble components of the intermembrane space, whereas cytochrome c_1 and Fe/S of bc_1 complex are attached to the inner membrane but with their bulk protruding into the intermembrane space); (ii) they all make membrane-potential dependent contact with the inner membrane; (iii) the first proteolytic processing event occurs by the chelator-sensitive matrix peptidase; and (iv) the second processing event is catalyzed by a different protease.

Evidence indicates that different proteases are involved in two-step processing. First, whereas the first processing step is clearly performed by the matrix protease, the second step of cytochrome b_2 maturation is not sensitive to chelators (Daum *et al.*, 1982b). The sensitivity of the second step of bc_1 complex Fe/S protein processing to *o*-phenanthroline is believed to be due to inhibition of the Fe/S cluster formation (Hartl *et al.*, 1986), which, like attachment of heme to intermediate cytochrome c_1

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separation which could still account for experimental observations (Schleyer and Neupert, 1985). Distances greater than 10–20 nm would probably produce detectable fragments from the imported part of the protein following external protease digestion. Mitochondrial membranes are assumed to be 5 nm thick. Insertion of the first processing site 10 nm past the inner face of the inner membrane is allowed for accessibility by the matrix peptidase.

TABLE III

Topology and Requirements for Mitochondrial Precursor Proteins Processed in Two Steps[a]

Precursor protein	Final location[b]	Two steps in vivo?	First step		Intermediate location[b]	Second step		Reference
			Energy[c]	o-Phe-sensitive		Energy[c]	o-Phe-sensitive[d]	
Cytochrome b_2 (yeast)	IMS	+	+	+	O-IM	−	−	Gasser *et al.* (1982b), Reid *et al.* (1982), Daum *et al.* (1982a,b).
Cytochrome *c* Peroxidase (yeast)	IMS	?	+	+				Reid *et al.* (1982), Maccecchini *et al.* (1979b)
Cytochrome c_1 (yeast)	O-IM	+	+	+	O-IM			Gasser *et al.* (1982b), Ohashi *et al.* (1982)
Cytochrome c_1 (*Neurospora*)	O-IM	+	+	+	O-IM	+(NADH)		Teintze *et al.* (1982), Schleyer and Neupert (1985), B. Schmidt and W. Neupert (unpublished) Hartl *et al.* (1987)
Fe/S of bc_1 complex (*Neurospora*)	O-IM	+	+	+	M	+(NADH)	+	Hartl *et al.* (1986)
ATPase IX (*Neurospora*)	IM	+	+	+			+	Schmidt *et al.* (1984)

[a] Features of import for proteins which are processed in two steps are summarized. The energy for the first processing step is required for transport of the precursor to or through the inner membrane and not for proteolytic activity itself. Features of the second processing step are described only where conversion of the intermediate to the mature form has been examined independently of the first processing step. Areas left blank were not determined.

[b] IMS, intermembrane space; O-IM, outer surface of inner membrane; M, matrix.

[c] Energy required is a membrane potential unless otherwise indicated.

[d] o-Phe, o-phenanthroline.

(Ohashi *et al.*, 1982), might precede the second proteolytic processing step. Second, the topological arrangement of the intermembrane cytochromes b_2 and c_1 (and probably cytochrome *c* peroxidase) presumably exposes the second processing site to the intermembrane space (Daum *et al.*, 1982b; Ohashi *et al.*, 1982; Reid *et al.*, 1982). Third, the partially purified matrix peptidase cleaves cytochome b_2, c_1, and cytochrome *c* peroxidase precursors only to their intermediate size (Gasser *et al.*, 1982b; Reid *et al.*, 1982). Finally, a mutation in yeast blocks the second processing step of cytochrome b_2 without affecting the first step (Pratje and Guiard, 1986).

Two-step processing of these proteins has been demonstrated *in vivo*, with the exception of cytochrome *c* peroxidase (Reid *et al.*, 1982; Maccecchini *et al.*, 1979b) in which an intermediate size protein cannot be detected (presumably for kinetic reasons). For some proteins, NADH is required for the second processing step. As discussed in Section V, this may be necessary for heme attachment or Fe/S cluster formation and not for proteolytic processing itself. In these cases, such modifications appear to be obligatory and precede the second processing step.

Mutants have been isolated in yeast which are defective in proteolytic processing activity. In an attempt to identify essential components in the pathway of protein import, two complementation groups of temperature-sensitive mutants, defective in the import of mitochondrial proteins, were identified (Yaffe and Schatz, 1984). Termed *mas1* and *mas2* (mitochondrial assembly), the mutants were normal at the permissive temperature (23°C) but accumulated F_1 ATPase β subunit precursor at 37°C and stopped growing after 2–3 generations. The *mas1* mutants were deficient in the matrix-localized protease activity and could not process a number of mitochondrial precursor proteins (Yaffe *et al.*, 1985). The phenotype suggested that processing of imported proteins is essential for mitochondrial function (though processing is not necessary for import). Another temperature-sensitive mutation in yeast has also been identified which was defective in the second processing step of cytochrome b_2 but not cytochrome *c* peroxidase (Pratje *et al.*, 1983; Pratje and Guiard, 1986). In addition, the mutant was unable to process the precursor of cytochrome oxidase subunit II, a mitochondrial gene product. Assuming that the mutation is in a structural gene coding for a processing enzyme, this suggests that (i) the second processing protease differs from the first step protease; (ii) the processing of the mitochondrial gene product COX II is catalyzed by the same protease, and probably in the same location, as a second step protease; and (iii) the second processing step for cytochrome b_2 and cytochrome *c* peroxidase occurs by different proteases. All evidence taken together, the minimum number of distinct processing peptidases in mitochondria now appears to be three.

TABLE IV

Comparison of Sites of Proteolytic Processing of Imported Mitochondrial Precursor Proteins[a]

One-step Processing	−10 +10
1. Cytochrome oxidase IV (*N. crassa*)	...RSIATTVVRCNAGTKPVPPH...
2. Cytochrome oxidase V (*N. crassa*)	AAKPTMAVRAASTMPISNPT
3. Cytochrome oxidase IV (yeast)	RTLCSSRYLLQQKPVVKTAQ
4. Cytochrome oxidase V (yeast)	GLSRITSVRFAQTHALSNAA
5. Cytochrome oxidase VI (yeast)	KNTFIQSRKYSDAHDEETFE
6. Mn-superoxide dismutase (yeast)	SLLSTTARRTKVTLPDLKWD
7. Aspartate aminotransferase (chicken)	PRRAAATARASSWWSHVEMG
8. Ornithine aminotransferase (rat)	RGLRTSVASATSVATKKTEQ
9. Ornithine carbamoyltransferase (rat)	RNFRYGKPVQSQVQLKGRDL
10. Carbamoyl-phosphate synthase I (rat)	WDFSRPGIRLLSVKAQTAHI
11. Aspartate aminotransferase (porcine)	GLAAAASARASWWAHVEMGP
12. Adrenodoxin (bovine)	RTLSVSGRAQSSSEDKITVH
13. Cytochrome oxidase IV (bovine)	RAISTSVCVRAHGSVVKSED
14. Cytochrome *P*-450 (SCC) (bovine)	HRVGTGEGAGISTKTPRPYS
15. Ornithine carbamoyltransferase (Human)	RNFRCGQPLQNKVQLKGRDL

Two-step processing	First Site	Second Site	
+10	−10	+10	−10
16. ATPase IX (*N. crassa*)	VRVAQVSKRTIQTGSPLQTL	TTRQAFQKRAYSSEIAQAMV	[b]
17. Rieske Fe/S of bc_1 complex (*N. crassa*)	AAAPARAVRALTTSTALQGS	LTTSTALQGSSSSTFESP	[c]
18. Cytochrome *c* peroxidase (yeast)	?	NWGKAAALASTTPLVHVASV	—
19. Cytochrome b_2 (yeast)	?	LNWHNGQIDNEPKLDMNKQK	—
20. Cytochrome c_1 (yeast)	?	LYADSLTAEAMTAAEHGLHA	—
21. ATPase IX P1 (bovine)	?	REFQTSVVSRDIDTAAKFIG	[d]
22. ATPase IX P2 (bovine)	?	RSFQTSAISRDIDTAAKFIG	[d]

[a] The amino acid sequences flanking the sites of proteolytic processing of imported mitochondrial proteins are listed by the single letter amino acid code (left to right, amino to carboxy terminus). The vertical lines indicate the sites of cleavage. Basic, acidic, and hydroxylated amino acids are indicated by the symbols +, −, and ·, respectively, above the primary sequence. The proteins are grouped by the organism in which they occur and then according to rough evolutionary order. Only sequences in which the cleavage sites have been positively identified are presented. References are the same as those indicated in Table I. In addition, the processing site for cytochrome oxidase IV from *Neurospora crassa* was determined by Sachs *et al.* (1986).

[b] The second processing step of *N. crassa* ATPase IX is catalyzed by the same protease as the first step (Schmidt *et al.*, 1984). Comparison of the second processing site with first site sequences might be more appropriate.

[c] Only eight amino acids preceding the second processing site are presented since the first processing step occurs at this position.

[d] Homology of bovine ATPase IX presequences (P1 and P2) with ATPase IX from *N. crassa* suggests that the bovine prepieces might also be processed in two steps. This has not been demonstrated yet.

What are the recognition sites for proteolytic processing? A comparison of the amino acid sequences flanking the cleavage sites (Table IV) does not indicate a clear consensus sequence. There may be a number of reasons why very few trends are apparent. (1) the site specificity may vary between different organisms; however, a certain degree of conservation must exist since heterologous import and processing can occur. (2) Processing could be catalyzed by more than one chelator-sensitive matrix protease with different specificities. First site processing and processing of proteins in which the prepiece is removed in a single step, however, all appear to be catalyzed by the same chelator-sensitive matrix peptidase. The partially purified matrix peptidase from yeast processed precursors to F_1 ATPase β and α subunits, cytochrome oxidase IV, citrate synthase (Böhni *et al.*, 1983), and cytochrome oxidase V (Cerletti *et al.*, 1983). Similarly, the purified *Neurospora* matrix peptidase processed all precursor proteins tested to date (G. Hawlitschek and W. Neupert, unpublished). (3) The specificity of the matrix peptidase may depend on regions within the prepiece or mature part of the protein distal from the actual cleavage site. For example, cytochrome oxidase IV is normally processed between amino acids 25 and 26 to remove the 25 amino acid prepiece. When only the first 22 amino acids of the COX IV prepiece were fused to DHFR, thus removing the normal cleavage site, the fusion protein was still processed, but between amino acids 17 and 18 instead (Hurt *et al.*, 1985a). The sequence of amino acids flanking the new cleavage site show no homology to the authentic site, suggesting that other elements in the prepiece may contribute to the signal for processing. Processing of a fusion protein consisting of the presequence of pre-COX IV and DHFR by the solubilized matrix peptidase was blocked when even small deletions were made at the very amino terminus of the prepiece (Hurt *et al.*, 1987).

In some cases, the specificity of processing has requirements in the mature sequence. Correct processing of the rat OTC prepiece occurred when the first 60 amino-terminal amino acids of the pre-OTC, containing the 32 amino acid prepiece, were fused to asparagine synthase; however, incorrect processing at a site 14 amino acids closer to the amino terminus occurred when only the first 37 amino acids (still containing the full 32 amino acid prepiece) were fused (Nguyen *et al.*, 1986). The processing of human pre-OTC, also containing a 32 amino acid prepiece, was inhibited when deletions or substitutions were made between amino acids 8 and 22 of the prepiece. In addition, the glutamine at position -1 was also critical for proteolytic cleavage (Horwich *et al.*, 1986). Similarly, small deletions up to 17 amino acids away from the ATPase $F_1\beta$ cleavage site also prevented processing (Vassarotti *et al.*, 1987a).

How processing peptidases recognize the correct sites of cleavage is unclear. Undoubtedly, conformation plays an important role in the recognition of cleavage sites. The alkali-denatured OTC precursor, for example, was not processed by a partially purified form of the matrix peptidase (Miura *et al.*, 1986). Sequences around the cleavage sites show only minor similarities (Table IV, sequences 1–17). (1) Position −1 rarely has a charged amino acid. In lower eukaryotes it is usually a hydrophobic amino acid while in higher eukaryotes it is more often an uncharged polar amino acid. (2) In position −2, 10 of 17 amino acids are positively charged, mostly in lower eukaryotes. When not positively charged, the −2 amino acid is usually hydrophobic with an aliphatic side chain. (3) Charged amino acids are also rare in the +1 and +2 positions, but hydroxylated amino acids (particularly serine) frequently occur. Despite no obvious consensus sequence, however, proteolytic processing is highly specific. Neither mature mitochondrial proteins nor nonmitochondrial proteins are cleaved by the matrix-located peptidase (Böhni *et al.*, 1983).

IX. MITOCHONDRIAL GENE PRODUCTS

Not all mitochondrial proteins are nuclear gene products which are imported from their site of synthesis in the cytosol. A small number (<10%) are coded for by the mitochondrial genome and are synthesized on 70 S mitochondrial ribosomes which are associated with the inner face of the inner membrane. The mitochondrial genomes in a number of species have been completely or partially sequenced. They vary in size from 17 kilobases in humans (Anderson *et al.*, 1981) to over 200 kilobases in plants (Palmer and Shields, 1984). Despite this variation, however, they code for a similar complement of proteins. These usually include apocytochrome *b*, cytochrome oxidase subunits I, II, and III, ATPase subunits VI and VIII, and several subunits from the NADH dehydrogenase complex (for review, see Breitenberger and RajBhandary, 1985). Yeast, fungi, and plant mitochondrial genomes also code for other proteins. For example, yeast and fungi mitochondria contain genes for the S5 protein of the small ribosomal subunit, ATPase subunit IX (which is dormant in *Neurospora crassa*), and intron-coded proteins involved in RNA maturation. Plant mitochondrial genomes also encode ATPase $F_1\alpha$. Finally, information coding for mitochondrial transfer RNAs and for ribosomal RNAs are contained within the genome.

Why mitochondria need a distinct genome at all and why the specific proteins that they encode are so highly conserved is unknown. One theory is that the hydrophobic nature of the mitochondrial gene products

necessitates their synthesis within the organelle. This, however, seems unlikely to be the only reason since ATPase IX, a very hydrophobic protein, is synthesized in the cytosol of *N. crassa* containing a hydrophilic amino-terminal prepiece to mask the hydrophobic mature part (Jackl and Sebald, 1975; van den Boogaart *et al.*, 1982b). In fact, the ATPase IX prepiece has been shown to be able to mediate the import of ATPase VIII, an authentic mitochondrial gene product, back into mitochondria (Gearing and Nagley, 1986), although the shorter COX VI prepiece did not. On the other hand, the S5 ribosomal protein is water soluble but is still a mitochondrial gene product in some species. A second possibility is that the mitochondrial genome is an evolutionary remnant and that most of the genes from the bacterial endosymbiont were transferred to the nucleus of the host cell, but the process was incomplete. A third possibility is that the mitochondrial gene products form nucleating points around which the remainder of respiratory complexes are built. This cannot be absolutely critical though, since ATPase, for example, is assembled into a functional, albeit somewhat less efficient, complex when the subunits encoded by the mitochondrial genome are absent (Schatz, 1968; De Jong *et al.*, 1979; Marzuki and Linnane, 1985).

A final hypothesis is that the cotranslational protein export mechanism existed prior to the evolutionary endosymbiotic event. Accordingly, the genes for proteins which had amino acid sequences within them that resembled the export signal sequence were retained in the mitochondrial genome so that they would not be mistakenly exported. Indeed, it has been determined that most mitochondrial gene products from *Xenopus* and yeast seem to contain signal sequence-like segments near the amino terminus (von Heijne, 1986b). Eukaryotic cells maintain a distinct genome in the mitochondrion at great expense, since a large number of proteins must be imported simply to accommodate a separate protein synthetic system.

Mitochondrial protein synthesis occurs on ribosomes which are associated with the inner mitochondrial membrane, suggesting that translation is coupled to the insertion of the newly synthesized proteins into the membrane. Even the soluble S5 ribosomal protein, however, is synthesized on membrane-bound ribosomes (Marzuki and Hibbs, 1986). Cytochrome oxidase II is synthesized as a larger precursor in *N. crassa* (Machleidt and Werner, 1979; van den Boogaart *et al.*, 1982a) and in yeast (Sevarino and Poyton, 1980). In both cases, the higher molecular weight precursor can be chased to the mature size protein in the absence of protein synthesis (Sevarino and Poyton, 1980; Driever *et al.*, 1987). Furthermore, the newly synthesized COX II precursor from *Neurospora* was not integrated into the inner membrane, demonstrating that assembly can

occur posttranslationally. Processing of the COX II precursor was catalyzed by a protease which is located in the intermembrane space, and formation of the mature size protein is dependent on NADH but not on a membrane potential. Cytochrome oxidase II is probably not representative of the other mitochondrial gene products since it is synthesized with an amino-terminal prepiece while others from the mitochondrial genome are not (with the possible exception of COX I; Burger *et al.*, 1982).

X. ASSEMBLY AND COORDINATION

Many of the proteins which are imported into mitochondria are subunits of respiratory complexes. Therefore, assembly into functional complexes represents the final step in the import pathway. What sequence of events occurs during assembly, and how is the supply of subunits coordinated and regulated?

Since two genetic systems are responsible for the synthesis of proteins for most respiratory complexes, it would seem logical that they would be synchronized in some way. No doubt there is some long-term regulation of the mitchondrial genome by the nucleus since most of the proteins comprising the mitochondrial transcription/translation system are nuclear gene products. In the short term, however, the two systems do not appear to be tightly coupled. For example, when synthesis of mitochondrial gene products was blocked *in vivo* by growth of *Neurospora crassa* on chloramphenicol, thereby inhibiting the synthesis of cytochrome *b* of the bc_1 complex, normal amounts of cytochrome c_1 were synthesized in the cytosol (Weiss and Kolb, 1979). Furthermore, cytochrome c_1 was imported normally and assembled into a cytochrome *b*-deficient complex. Similarly, when overexpression of the 11 kDa subunit (a nuclear gene product) of the bc_1 complex was induced in yeast cells by transformation, it did not affect the rate of synthesis or degradation of the other subunits of the complex (van Loon *et al.*, 1983a). The same was observed when individual subunits of the bc_1 complex (also nuclear gene products) were overexpressed, suggesting that stringent coupling does not exist (van Loon *et al.*, 1983b).

Stringent coordination may not occur at the level of translation for the nuclear gene products either. Cytoplasmically made subunits for the ATPase, the bc_1 complex, and cytochrome oxidase are synthesized as individual subunits, not as polyproteins (Lewin *et al.*, 1980; Mihara and Blobel, 1980; van Loon *et al.*, 1983c). Notwithstanding this apparent lack of coordination, excess unincorporated subunits do not accumulate in mitochondria. One suggestion has been that surplus amounts of unas-

sembled subunits are simply eliminated by proteolytic digestion (Luzikov, 1986) regardless of the waste of cellular resources by such a process.

Despite the apparent lack of coordination of synthesis of subunits in these experiments, nuclear genes are involved in regulating the expression of the mitochondrial genome. This appears to occur at three levels: (i) control of mRNA processing, (ii) control of translation, and (iii) post-translational modification (e.g., proteolytic processing of pre-COX II by a nuclear-coded protease; Pratje *et al.*, 1983; Pratje and Guiard, 1986). These systems have been studied best in *Saccharomyces cerevisiae*. Yeast mitochondrial genes contain introns, separating the exons of a gene, which must be spliced out prior to translation. These intervening sequences are removed from pre-mRNAs by self-splicing mechanisms and by protein-assisted splicing. In the latter case, these proteins (termed mRNA maturases) are encoded by either mitochondrial intron open reading frames (Weiss-Brummer *et al.*, 1982; Carignani *et al.*, 1983; Guiso *et al.*, 1984; Jacq *et al.*, 1984) or are nuclear gene products (Faye and Simon, 1983; Pillar *et al.*, 1983; McGraw and Tzagoloff, 1983; Dieckmann *et al.*, 1984) and have been shown to be necessary for processing of COX I and apocytochrome *b* pre-mRNAs. The interesting feature of these studies is that the maturation proteins appear to be specific for individual mitochondrial gene transcripts so that the expression of mitochondrial gene products can be selectively controlled by the activity of the different nuclear-encoded mRNA maturases.

Nuclear gene products are also required for the specific translation of mitochondrial mRNAs (Fox, 1986). For example, the yeast *pet494* mutant has normal levels of fully processed COX III mRNA. A nuclear gene product was found to be required to promote the translation of the COX III mRNA that appeared to interact with the 5′-untranslated leader (Müller *et al.*, 1984). Similar nuclear gene products were also required for the specific translation of apocytochrome *b* (CPB6, Dieckmann and Tzagoloff, 1985; and MK2, Rödel *et al.*, 1985) and for COX II (PET111; Fox, 1986). Though they serve similar functions, there seems to be little homology between the PET494, CBP6, and PET111 gene products. They all appear, however, to act on the 5′-untranslated mRNA leader sequence in a specific way so that translation of mitochondrial-encoded proteins can be independently controlled.

Compared to the loose coordination between nuclear and mitochondrial genomes, the assembly of individual subunits into functional complexes is a more ordered process. The best studied examples of complex assembly to date are the F_0F_1 ATPase and cytochrome oxidase. When the synthesis of yeast mitochondrial gene products was blocked by growth in the presence of chloramphenicol (De Jong *et al.*, 1979) or as in *rho*$^-$ mutants

(Schatz, 1968), a correctly assembled functional ATPase was still produced which was loosely associated with the inner membrane but no longer sensitive to oligomycin. This suggests that the mitochondrial gene products (subunits VI, VIII, and IX) are not essential for either assembly or function but are required for stability of the complex and confer oligomycin sensitivity. Sequential assembly of the ATPase subunits was demonstrated in yeast mutants lacking each of the mitochondrially encoded proteins (Marzuki and Linnane, 1985). The mutant lacking subunit IX was deficient in the assembly of both VI and VIII, while the mutant lacking subunit VIII could assemble IX but not VI. This indicated that the relative order of assembly of the mitochondrial gene products was IX then VIII then VI and that sequential assembly was necessary. Each of these mutants had a functional but unstable ATPase.

A large number of components are required for the functional assembly of cytochrome oxidase. In a *N. crassa* mutant lacking COX I (COX I, II, and III are mitochondrial gene products) only the assembly of subunits V and VI occurred (Nargang *et al.*, 1978). In pulse–chase experiments in rats, COX II and III were immediately assembled while COX I arrived only after a long chase (Wielburski *et al.*, 1982). Taken together, this suggests that COX II and III are assembled first, followed by COX I, which in turn is necessary for the subsequent assembly of COX V and VI. In a yeast mutant lacking COX IV, no cytochrome oxidase activity was observed, although the mutant still contained the other mitochondrial COX subunits. This suggested that COX IV is necessary for the proper assembly of cytochrome oxidase (Dowhan *et al.*, 1985).

Cofactors such as heme, oxygen, and copper are also necessary for cytochrome oxidase assembly. In yeast, a mutant lacking heme contained no cytochrome oxidase activity. Although the mitochondria still contained subunits II, III, and IV, it had only low amounts of COX I and IV and no V or VI. The residual subunits were not assembled (Saltzgaber-Müller and Schatz, 1978). In rats, heme was shown to be necessary for the assembly of subunit I with the preassembled COX II and III (Wielburski and Nelson, 1984). COX I was predominantly associated with COX III. Yeast cells grown anaerobically did not contain assembled cytochrome oxidase. When shifted to an oxygen environment, however, subunits I and II were immediately assembled with VI and VII (Woodrow and Schatz, 1979). Cytochrome oxidase is not assembled in copper-depleted *N. crassa,* although both nuclear and mitochondrial gene products are synthesized normally (Werner *et al.*, 1974).

The coordination and assembly of functional mitochondrial complexes occurs by a series of events which are dependent on components other than the mitochondrial subunits alone. Whether assembly is controlled by

factors other than the simple stoichiometric availability of components is unknown.

XI. OVERVIEW

A. Import Pathway Models

Owing to the diversity of mitochondrial proteins and their topological locations, a variety of different pathways exist to faciliate their import. We summarize what is known about the import pathways of representative proteins and what can be hypothesized.

Porin: Outer Membrane (Fig. 4). The precursor to porin differs from the mature form only in conformational arrangement, thereby allowing its solubility in the cytosol. It does not contain an amino-terminal prepiece and does not require an energized inner membrane for import. Preporin binds to its receptor at the outer face of the outer membrane and is inserted into the outer membrane where it is protected from externally added proteases. It then forms dimers and trimers. The porin pathway is probably representative of most outer membrane proteins (Pfaller *et al.*, 1985; Pfaller and Neupert, 1987, and references therein).

Cytochrome *c*: Intermembrane Space (Fig. 5). Cytochrome *c* is synthesized as apocytochrome *c* which does not contain covalently attached heme and has a loosely ordered conformation. Apocytochrome *c* spontaneously inserts partway through the lipid bilayer of the outer membrane. It is then sequestered at the inner face of the outer membrane by an apocytochrome *c* binding protein which in turn exposes the cysteine

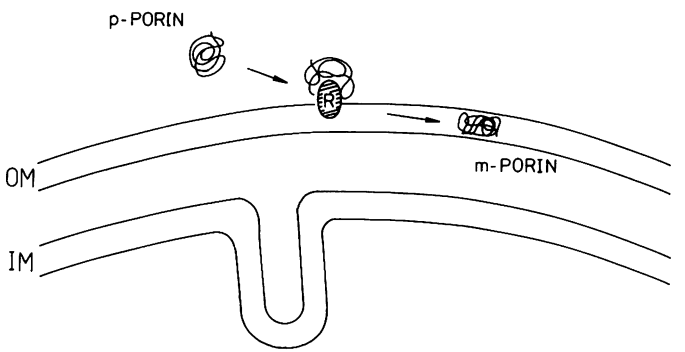


Fig. 4. Import pathway of porin into mitochondria. OM, Outer membrane; IM, inner membrane; R, receptor; p, precursor; m, mature.

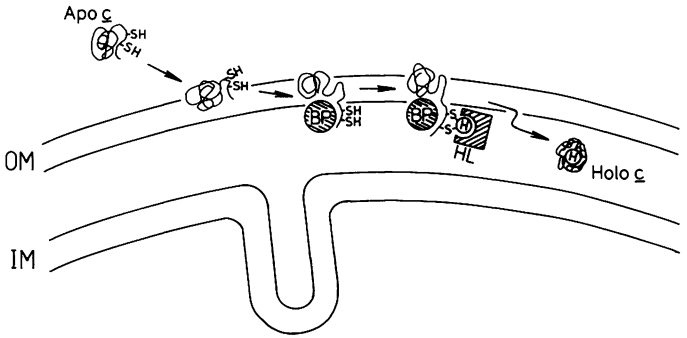


Fig. 5. Import pathway of cytochrome *c* into mitochondria. OM, Outer membrane; IM, inner membrane; Apo *c*, apocytochrome *c*; Holo *c*, holocytochrome *c*; BP, apocytochrome *c* binding protein; H, heme; HL, cytochrome *c* heme lyase.

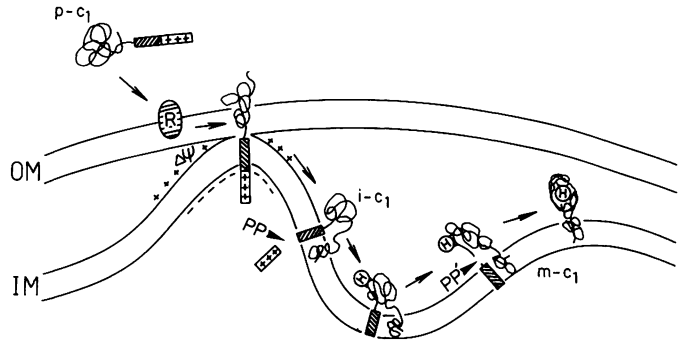


Fig. 6. Import pathway of cytochrome c_1 into mitochondria (according to Hurt and van Loon, 1986). OM, Outer membrane; IM, inner membrane; p, precursor; i, intermediate; m, mature; R, receptor; $\Delta\psi$, membrane potential; PP, processing peptidase; H, heme. Boxes represent prepiece segments.

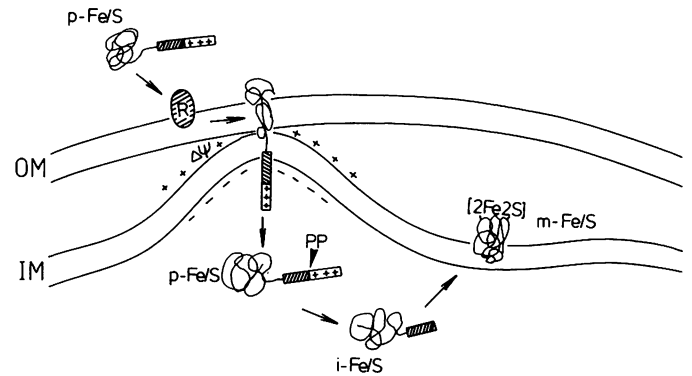


Fig. 7. Import pathway of the Rieske Fe/S protein of bc_1 complex into mitochondria. Abbreviations are as in Fig. 6.

thiols to the intermembrane space. Heme is enzymatically attached by cytochrome *c* heme lyase, and the resulting conformational change pulls the protein through the outer membrane. Holocytochrome *c* then migrates to its functional location in association with cytochrome *c* reductase and cytochrome *c* oxidase at the outer face of the inner membrane (Hennig and Neupert, 1981; Nicholson *et al.*, 1987, and references therein).

Cytochrome c_1: Intermembrane Space, Inner Membrane (Fig. 6). The cytochrome c_1 precursor is synthesized with a two-domain prepiece. Pre-c_1 binds to its receptor at the outer face of the outer membrane. The amino terminus is then translo‹ ted through the inner membrane via contact sites in a step which is dependent on a membrane potential. The first part of the prepiece is removed by the chelator-sensitive matrix peptidase to generate intermediate c_1. The carboxy terminus, which eventually anchors the mature protein to the inner membrane, is embedded into the inner membrane at some point following translocation across the outer membrane. Heme is covalently attached to intermediate c_1, and the second part of the prepiece is then removed yielding the mature c_1 which is then assembled into the bc_1 complex (Ohashi *et al.*, 1982; Schleyer and Neupert, 1985; Hurt and van Loon, 1986, and references therein.) The same basic pathway may be followed by cytochrome *c* peroxidase, which is released as a soluble protein into the intermembrane space following the second proteolytic step because it does not contain a carboxy-terminal anchoring segment. This pathway demonstrates the principles of the stop transfer model suggested by Hurt and van Loon (1986). Another possibility is that pre-c_1 is completely translocated into the matrix, then redirected back to the inner membrane like the Fe/S protein of the bc_1 complex (Fig. 7). This has been demonstrated to be the case in *Neurospora crassa* and also for the import of cytochrome b_2 into yeast mitochondria (Hartl *et al.*, 1987).

Fe/S Protein of bc_1 Complex: Inner Membrane (Fig. 7). The precursor to the Fe/S protein is synthesized with a two-part prepiece. Pre-Fe/S binds to its receptor on the outer membrane and is then completely translocated into the mitochondrial matrix via translocation contact sites in a step which is dependent on the membrane potential. The matrix-located pre-Fe/S is then processed to the intermediate size protein by the chelator-sensitive processing peptidase. The Fe/S protein is then directed back across the inner membrane to the outer face of the inner membrane. The protein is processed to its mature form by removal of the second half of the prepiece and formation of the Fe/S cluster, but when and where this occurs are unknown (Hartl *et al.*, 1986).

ADP/ATP Carrier: Inner Membrane (Fig. 8). The ADP/ATP carrier is synthesized without a prepiece but contains stretches of positively

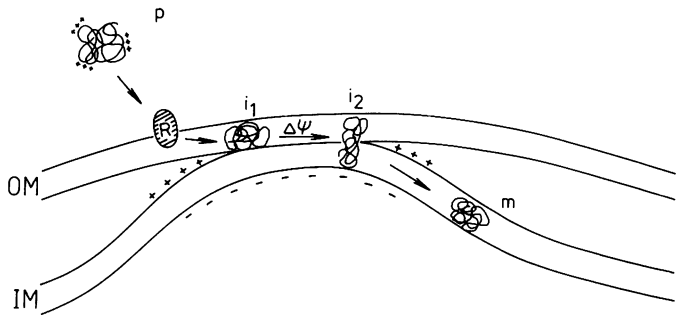


Fig. 8. Import pathway of ADP/ATP carrier into mitochondria. Abbreviations are as in Fig. 6.

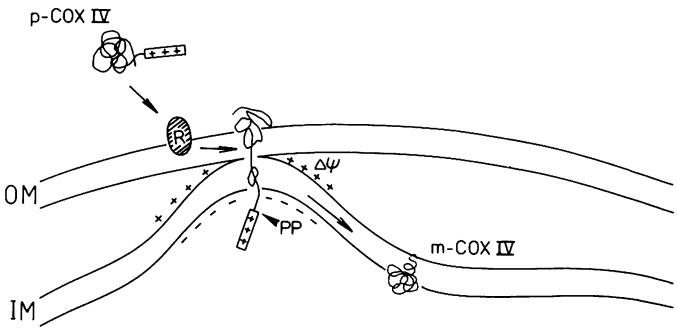


Fig. 9. Import pathway of cytochrome oxidase subunit IV into mitochondria. Abbreviations are as is Fig. 6.

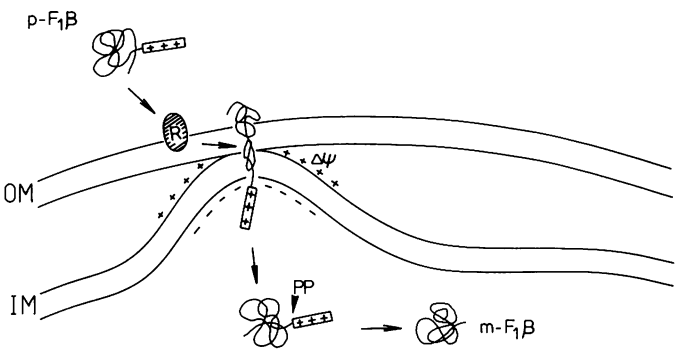


Fig. 10. Import pathway of F_1 ATPase β subunit into mitochondria. Abbrevations are as in Fig. 6.

charged amino acids which resemble mitochondrial targeting sequences. The precursor binds to its receptor and is inserted into the mitochondrial membrane where it is protected from externally added proteases. The first intermediate (i_1) is past the receptor stage but before the membrane potential-dependent stage of import. The second intermediate (i_2) is found in the presence of a membrane potential and is transported to the inner membrane via translocation contact sites. The imported ADP/ATP carrier then undergoes a conformational change, in which it acquires properties of the mature protein, followed by the formation of dimers (Schleyer and Neupert, 1984; Pfanner and Neupert, 1987). The uncoupling protein from brown fat mitochondria has similar sequence and folding characteristics (Aquila *et al.*, 1985) and is probably imported in a similar manner.

Cytochrome Oxidase IV: Inner Membrane (Fig. 9). Pre-COX IV is synthesized with an amino-terminal prepiece in the cytosol. It binds to its receptor on the outer membrane and is subsequently transported to the inner membrane by translocation contact sites in a membrane potential-dependent step. The amino-terminal prepiece, which protrudes into the matrix, is removed by the chelator-sensitive peptidase, and the mature part of the protein remains integrated in the inner membrane where it is assembled into cytochrome oxidase (Hurt and van Loon, 1986, and references therein). This mechanism is probably representative of most imported inner membrane proteins which are processed in a single step.

F_0F_1 *ATPase* $F_1\beta$: Matrix (Fig. 10). Pre-$F_1\beta$ binds to its receptor and is transported completely into the matrix. This occurs via translocation contact sites and is dependent on a membrane potential. During or shortly following translocation, the mature $F_1\beta$ is generated by removal of the prepiece by the matrix peptidase. Mature $F_1\beta$ is then assembled with the other F_1 ATPase subunits at the inner face of the inner membrane. Most imported matrix proteins probably follow a similar pathway (Schleyer and Neupert, 1985, and references therein).

Cytochrome Oxidase II: A Mitochondrial Gene Product (Fig. 11). Most mitochondrial gene products are probably inserted directly into the inner membrane as they are synthesized on membrane-bound ribosomes (except the S5 ribosomal protein which is released into the matrix). COX II, on the other hand, is synthesized with a prepiece (in lower eukaryotes) and can be posttranslationally inserted into the inner membrane (B). Cotranslational insertion may occur *in vivo* (A) but is not obligatory. Pre-COX II is converted to mature COX II by removal of the prepiece by a protease located in the intermembrane space and noncovalent binding of heme *a* and copper. The sequence in which these changes occur is unknown, but is dependent on NADH. Mature COX II is then assembled into cytochrome oxidase (Driever *et al.*, 1987).

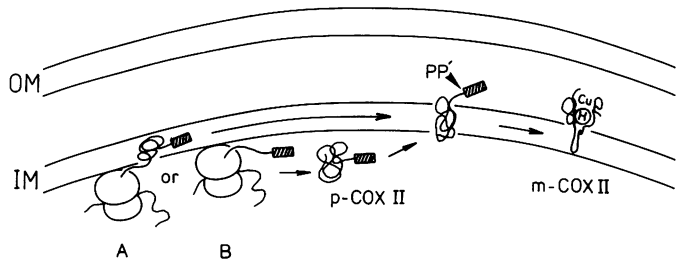


Fig. 11. Insertion pathway of cytochrome oxidase subunit II. Abbreviations are as in Fig. 6.

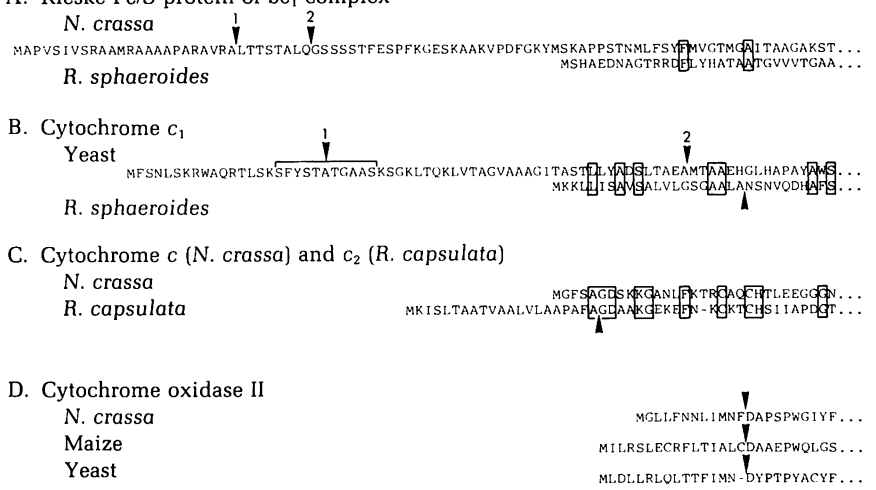
B. Evolutionary Considerations

A particularly interesting finding which emerged while examining the import of the Fe/S protein of bc_1 complex into *Neurospora crassa* mitochondria is that the precursor protein was completely translocated into the matrix where it was partially processed and then redirected back across the inner membrane to its functional location on the other side facing the intermembrane space. (Hartl *et al.*, 1986; see Fig. 7). This seemingly complex assembly pathway was explained in terms of a rerouting mechanism by which the Fe/S protein is returned to remnants of its "ancestral" assembly pathway. Following evolutionary gene transfer from the bacterial endosymbiont to the host cell nucleus, mechanisms had to evolve to return the gene product to its functional location. Rather than completely rebuild the means by which the protein was folded, assembled, and acquired the correct topology, the import pathway of the Fe/S protein has evolved to make use of preexisting mechanisms. To do this, the Fe/S protein had to be completely transported across both mitochondrial membranes back into the matrix. This was accommodated by adding a matrix targeting prepiece to the Fe/S protein (i.e., the first part of the pre-Fe/S prepiece) which allows it to enter the matrix by components which had evolved to mediate the import of other mitochondrial proteins. At this point, "ancestral" transport pathways take over to correctly insert the Fe/S protein into the inner membrane.

Indeed, the equivalent Fe/S protein from the photosynthetic bacteria *Rhodopseudomonas sphaeroides* is synthesized in the bacterial cytoplasm (comparable to the mitochondrial matrix) and transferred across the photosynthetic membrane to the side opposite the cytoplasm where, as in mitochondria, it is topologically opposed to the F_1 part of ATPase. This transfer is accompanied by a reduction in molecular weight (Gabellini *et al.*, 1985) which may be equivalent to processing of the mitochon-

Table V

Comparison of Amino-Terminal Sequences of Mitochondrial and Bacterial Proteins[a]



[a] (A – C) Amino acid sequences of the amino terminus of mitochondrial precursor proteins are compared to their equivalent in bacteria. Arrows indicate the sites of proteolytic processing. The estimated region containing the first processing site of yeast cytochrome c_1 is indicated by a bracket. The processing site for *R. sphaeroides* Fe/S protein is not known. Segments having identical amino acid sequences are marked by a box. Alignment inserts are indicated by (–). (D) The prepiece sequences of mitochondrial cytochrome oxidase subunit II. Sequences were taken from Harnisch *et al.* (1985) (*N. crassa* Fe/S protein of bc_1 complex), Gabellini and Sebald (1986) (*R. sphaeroides* Fe/S protein of bc_1 complex and cytochrome c_1), Sadler *et al.* (1984) (yeast cytochrome c_1), Lederer and Simon (1974) (*N. crassa* cytochrome c), Daldal *et al.* (1986) (*R. capsulata* cytochrome c_2), van den Boogaart *et al.* (1982a) (*N. crassa* COX II), Fox and Leaver (1981) (maize COX II), and Coruzzi and Tzagoloff (1979) (yeast COX II).

drial intermediate Fe/S to mature Fe/S. The bacterial Fe/S protein is homologous to the mature *Neurospora* Fe/S protein except in the targeting prepieces (Gabellini and Sebald, 1986). The amino terminus of the bacterial Fe/S protein has characteristics of a leader sequence for export through the cytoplasmic membrane, but the second part of the mitochondrial Fe/S protein prepiece is not comparable as might be predicted from this model (Table VA). Information to redirect the mitochondrial Fe/S protein from the matrix back across the inner membrane might not be contained in sequences analogous to bacterial leader sequences. In this regard, the second part of the cytochrome c_1 prepiece bears a close re-

semblance to the export leader sequence of the equivalent bacterial protein (Table VB).

The Fe/S protein of the bc_1 complex may have retained this elaborate import pathway because of stringent assembly requirements which necessitate its insertion into the inner membrane from the matrix side. On the other hand, cytochrome *c* is a soluble protein of the intermembrane space and is less likely to be so demanding. This is reflected in the way in which import pathway for cytochrome *c* has evolved. In the bacterium *Rhodopseudomonas capsulata,* the equivalent protein (cytochrome c_2) is synthesized as a precursor protein in the bacterial cytosol and is processed as it is secreted into the periplasmic space (comparable to the mitochondrial intermembrane space). Rather than via a redirected import pathway, mitochondrial cytochrome *c* is transported across just the outer membrane (Fig. 5). On evolutionary gene transfer to the host cell nucleus, the bacterial leader prepiece was removed (Table VC).

Some bacterial-like leader sequences remain in mitochondria. In lower eukaryotes such as *Neurospora,* yeast, and maize, for example, cytochrome oxidase II (a mitochondrial gene product) is synthesized with a prepiece having some characteristics of bacterial leader sequences (e.g., a stretch of hydrophobic amino acids following a charged amino terminus; Table VD). On the other hand, proteins which have no equivalent in bacteria, and therefore presumably did not evolve from the bacterial endosymbiont, may be imported by novel mechanisms which differ from most mitochondrial proteins. For example, the ADP/ATP carrier does not have an amino-terminal prepiece, but specific targeting information is contained within internal sequences that have accompanied the structural part of the protein during evolutionary formation.

Evolutionary remnants of the bacterial endosymbiont may still be present in the import pathways of mitochondrial proteins. The differing pathways could reflect specific assembly requirements of the various proteins and, in some cases, the relative time at which they were introduced as mitochondrial constituents.

C. Summary

Nearly the entire complement of mitochondrial proteins must be imported from the cytosol. To do this correctly, mitochondrial precursor proteins must be specifically targeted to mitochondria and then properly sorted to their functional submitochondrial location. In addition, the proper topological arrangement and assembly with other subunits must occur. Information to enable these processes is embodied within the precursor proteins themselves, and components required to facilitate these

events are molecules and structures within the mitochondrion. The import pathways for a variety of mitochondrial proteins have been resolved into a sequence of discrete but contiguous steps. In doing so, the components necessary for each step have frequently been identified at a molecular level. These can be divided into three broad classes, namely, components necessary for (i) specific targeting to mitochondria, (ii) sorting and translocation processes, and (iii) processing events. In the first group, targeting signals, usually contained within the amino-terminal prepiece of the precursor protein, mediate recognition of the remainder of the protein by mitochondria. Receptors on the surface of the mitochondrion bind the precursor protein and initiate its entry into mitochondria. In the second group, translocation contact sites facilitate transmembrane movement of most imported proteins. The constituents of these contact regions are unknown but probably contain specific proteins which are necessary for import. The membrane potential supplies the energy or the circumstances to initiate translocation. Features within the precursor proteins and perhaps processes which exist at the site of translocation guide the imported protein to its correct submitochondrial location. Finally, processing occurs in many forms. Prepieces are proteolytically removed by specific proteases, and some proteins are covalently or noncovalently modified. Refolding into an active conformation often occurs, and then assembly into a functional location marks the end of the import process.

The general sequence of events by which import occurs has been well-characterized for a number of proteins having different submitochondrial locations and different roles in mitochondrial function. The molecular mechanisms are now of greater interest and are the focus of current research.

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