Current Topics in Bioenergetics

Structure, Biogenesis, and Assembly of Energy Transducing Enzyme Systems

Edited by

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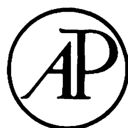

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CURRENT TOPICS IN BIOENERGETICS, VOLUME 15

Biogenesis of Mitochondrial Energy Transducing Complexes

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I. Introduction

Mitochondria are not formed de novo, but by growth and division of preexisting organelles. Newly synthesized components are assembled into preformed structures. Studying these biogenesis processes leads to the central question of how mitochondria maintain their continuity in space, function, and time, and, how the specific differentiation of mitochondria in various organisms and tissues is controlled. Proteins play the prominent role in these processes, in particular since they essentially determine the identity and function of the organelles. The lipid components are also of considerable importance, although their functions (besides their obvious structural role) are much less well understood.

The biogenesis and assembly of mitochondrial proteins prompts questions on how the information is genetically coded, transcribed, and translated, how the proteins are transported to their functional locations, and how they are assembled into preexisting structures. These processes are already enormously complex and are further complicated by the fact that the biogenesis of mitochondria requires two distinct genetic systems (Hay *et al.*, 1984; Harmey and Neupert, 1985).

For the purposes of this review, we have divided the complex biogenesis pathways of mitochondrial protein components into individual steps. In order to facilitate following the various steps, we first briefly summarize the most important principles that we know about mitochondrial biogenesis.

Mitochondria contain their own DNA and a complete system for replication, transcription, and translation. The mitochondrial DNA, however, codes for $<10\%$ of total mitochondrial protein.

The bulk of mitochondrial proteins are synthesized outside mitochondria on free cytoplasmic polysomes, and released as water-soluble precursor proteins into the cytosol. Nucleoside triphosphates are required to keep the precursor proteins in an import-competent conformation. The precursors bind to specific receptors on the mitochondrial surface and are subsequently translocated across the membrane(s) to their final destinations in one of the four mitochondrial compartments (outer membrane, intermembrane space, inner membrane, or matrix). Transport into or across the inner membrane is mediated by contact sites between outer and inner membranes and is dependent on a membrane potential across the inner membrane. Many precursor proteins, but not all, carry amino-terminal presequences which are removed during or after translocation by a matrix-located processing peptidase (Table I). Other processing steps during protein import include covalent modifications (e.g., attachment of heme) and conformational changes. The amino-terminal presequences

appear to carry sufficient information for the targeting of proteins to mitochondria.

Many proteins are assembled into large protein complexes in mitochondria. Several complexes are composed of both mitochondrially synthesized subunits and cytoplasmically synthesized subunits. These include the three proton-translocating complexes of the respiratory chain, the proton-translocating ATPase, and the small mitochondrial ribosomal subunit.

This review focuses on the biogenesis of mitochondrial energy-transducing complexes; however, the principles of biogenesis are common for all mitochondrial proteins. Therefore, we have referred to other proteins when the aspects discussed have not been sufficiently studied with proteins of energy-transducing complexes.

II. Biogenesis of Nuclear-Coded Mitochondrial Proteins

A. Posttranslational versus Cotranslational Transport

Studies since the mid-1970s have demonstrated two principal mechanisms for the translocation of proteins across biological membranes:

1. *Cotranslational transport.* During its synthesis on polysomes, the growing polypeptide chain is translocated across the membrane. For example, transport of proteins across the membrane of the endoplasmic reticulum (ER) usually involves the following steps (Blobel and Dobberstein, 1975; Meyer and Dobberstein, 1980; Walter *et al.*, 1981; Walter and Blobel, 1981a,b; Gilmore *et al.*, 1982; Meyer *et al.*, 1982; Gilmore and Blobel, 1985; Meyer, 1985; Wickner and Lodish, 1985): Translation starts on free cytoplasmic polysomes. When a certain part of the polypeptide is synthesized, it is recognized by the signal recognition particle (SRP), which binds to the peptide chain. In several cases the SRP induces elongation arrest. The complex of polysomes, polypeptide, and SRP binds to the ER via the docking protein (SRP receptor). The elongation arrest is released, and the growing peptide chain is inserted into and translocated across the membrane. Many precursor proteins contain amino-terminal signal sequences which are removed by a signal peptidase on the luminal face of the ER.

2. Posttranslational transport. In this case, the fully synthesized peptide chain is released from the polysome prior to membrane association,

TABLE I

CYTOPLASMIC PRECURSORS TO PROTEINS OF MITOCHONDRIAL ENERGY-TRANSDUCING COMPLEXES

Protein	Tissue	Presequence		Apparent molecular weight			Import requires membrane potential	References
		Amino acid residues	Net charge	Precursor	Intermediate	Mature		
Ubiquinone cytochrome-*c* reductase (bc_1 complex, complex III)								
Subunit I	*Neurospora*	N.D.[a]	N.D.	51,500		50,000	Yes	Teintze *et al.* (1982)
	Yeast	N.D.	N.D.	44,500		44,000	Yes	van Loon *et al.* (1983c)
Subunit II	*Neurospora*	N.D.	N.D.	47,500		45,000	Yes	Teintze *et al.* (1982)
	Yeast	N.D.	N.D.	40,500		40,000	Yes	van Loon *et al.* (1983c)
Subunit IV (cytochrome c_1)	*Neurospora*	N.D.	N.D.	38,000	35,000	31,000	Yes	Teintze *et al.* (1982)
	Rat hepatoma	N.D.	N.D.	33,000		30,000	N.D.	Kolarov and Nelson (1984)
	Yeast	61	+5	37,000	35,000	31,000	Yes	Ohashi *et al.* (1982); Sadler *et al.* (1984)
Subunit V (Fe-S protein)	*Neurospora*	32	+4	28,000	26,000	25,000	Yes	Teintze *et al.* (1982); Harnisch *et al.* (1985); Hartl *et al.* (1986)
	Rat hepatoma	N.D.	N.D.	30,000		24,000	N.D.	Kolarov and Nelson (1984)
	Yeast	N.D.	N.D.	27,000		25,000	Yes	Côté *et al.* (1979)

Subunit IV	*Neurospora*	—	—	14,000	14,000	N.D.	Teintze *et al.* (1982)
	Yeast	N.D.	N.D.	25,000	17,000	N.D.	van Loon *et al.* (1983c, 1984)
Subunit VII	*Neurospora*	N.D.	N.D.	12,000	11,500	Yes	Teintze *et al.* (1982)
	Yeast	—	—	14,000	14,000	N.D.	van Loon *et al.* (1983c); De Haan *et al.* (1984)
Subunit VIII	*Neurospora*	N.D.	N.D.	11,6000	11,200	N.D.	Teintze *et al.* (1982)
	Yeast	—	—	11,000	11,000	N.D.	van Loon *et al.* (1983c)
Cytochrome *c*	*Neurospora*	—	—	12,000	12,000	No	Korb and Neupert (1978); Zimmermann *et al.* (1979a, 1981)
	Rat liver	—	—	12,000	12,000	No	Matsuura *et al.* (1981)
Cytochrome oxidase (complex IV)							
Subunit IV	Rat hepatoma	N.D.	N.D.	18,000	16,500	N.D.	Hatalová and Kolarov (1983)
	Rat liver	N.D.	N.D.	19,500	16,500	N.D.	Schmelzer and Heinrich (1980); Heinrich (1982)
	Yeast	25	+5	17,000	14,000	Yes	Mihara and Blobel (1980); Maarse *et al.* (1984); Hurt *et al.* (1984b)
Subunit V	Rat liver	N.D.	N.D.	15,500	12,500	N.D.	Heinrich (1982)
	Yeast	20	+4	15,000	12,500	N.D.	Mihara and Blobel (1980); Lewin *et al.* (1980); Koerner *et al.* (1985)

TABLE—*Continued*

		Presequence		Apparent molecular weight				
Protein	Tissue	Amino acid residues	Net charge	Precursor	Intermediate	Mature	Import requires membrane potential	References
Subunit VI	Yeast	40	+9	17,000–20,000		12,500	N.D.	Mihara and Blobel (1980); Lewin *et al*. (1980); Wright *et al*. (1984)
Subunit VII	Yeast	—	—	5,000–7,500		5,000–7,500	N.D.	Mihara and Blobel (1980)
ATPase-F_1 complex								
Subunit 1 (α)	Yeast	N.D.	N.D.	64,000		58,000	Yes	Maccechini *et al*. (1979); Lewin *et al*. (1980)
Subunit 2 (β)	Human (HeLa cells)	N.D.	N.D.	54,500		51,500	N.D.	Ohta and Kagawa (1986)
	Neurospora	N.D.	N.D.	56,000		54,000	Yes	Zwizinski and Neupert (1983b)
	Yeast	N.D.	N.D.	56,000		54,000	Yes	Maccechini *et al*. (1979); Nelson and Schatz (1979); Lewin *et al*. (1980)
Subunit 3 (γ)	Yeast	N.D.	N.D.	70,000		34,000	Yes	Maccechini *et al*. (1979); Lewin *et al*. (1980); Suissa and Schatz (1982)
Subunit 4 (δ)	*Neurospora*	26	+7	165 aa[b]		139 aa	N.D.	Sebald and Kruse (1984)

F_1 Inhibitor	Yeast	21	N.D.	12,000		10,000	Yes	Yoshida *et al.* (1983a,b)
ATPase F_0 complex Subunit 9 (proteolipid)	Bovine	61	+5	136 aa		75 aa	N.D.	Gay and Walker (1985)
		68	+6	143 aa		75aa	N.D.	Gay and Walker (1985)
	Neurospora	66	+12	16,400	13,000	10,500	Yes	Michel *et al.* (1979); Zimmermann *et al.* (1981); Viebrock *et al.* (1982); Schmidt *et al.* (1983b, 1984)
ADP/ATP Carrier	*Neurospora*	—	—	32,000		32,000	Yes	Zimmermann *et al.* (1979b); Zimmermann and Neupert (1980); Schleyer *et al.* (1982); Arends and Sebald, (1984)
	Rat hepatoma	—	—	30,000		30,000	N.D.	Hatalová and Kolarov (1983)
Uncoupling protein	Rat brown adipose tissue	—	—	32,000		32,000	N.D.	Freeman *et al.* (1983); Ricquier *et al.* (1983)

[a] N.D., Not determined.
[b] aa, number of amino acid residues.

and subsequently translocated across the membrane(s). Proteins are transported posttranslationally into mitochondria, chloroplasts, peroxisomes, and glyoxysomes.

It should be noted that these two mechanisms are not necessarily mutually exclusive (as previously thought). Some proteins are translocated across the ER membrane in a posttranslational manner (Sabatini *et al.*, 1982; Hansen *et al.*, 1986; Mueckler and Lodish, 1986; Rothblatt and Meyer, 1986; Waters and Blobel, 1986; Zimmermann and Mollay, 1986). Likewise, mitochondrial protein import may be cotranslational in several cases (see below). In prokaryotes, protein translocation is not strictly coupled to protein synthesis and it can take place either during or after translation (Randall, 1983; Müller and Blobel, 1984; Wickner and Lodish, 1985). Apparently, some folding of precursors can take place prior to membrane contact which does not interfere with their translocation competence. In some cases co-versus posttranslational translocation may be a kinetic problem, rather than indicative of principal mechanistic differences.

Two types of experiments showed that most mitochondrial precursor proteins are imported posttranslationally.

The first evidence came from *in vivo* experiments. Varying times after pulse-labeling of precursor proteins, cells were lysed and fractionated. Mitochondrial proteins were then precipitated by specific antibodies. Since many precursor proteins differ in molecular weight or immunological properties from their mature counterparts, the processing of the precursor proteins could be followed. Mitochondrial precursor proteins appeared in a cytosolic fraction before they could be detected in mitochondria. Furthermore, translocation into mitochondria still occurred when protein synthesis was inhibited by cycloheximide (Hallermayer *et al.*, 1977; Schatz, 1979; Reid and Schatz, 1982; Teintze *et al.*, 1982).

In the second type of experiment, precursor proteins were synthesized *in vitro* in a cell-free translation system (e.g., rabbit reticulocyte lysate). After translation, the ribosomes were sedimented and mitochondrial precursor proteins were found in the postribosomal supernatant. When isolated mitochondria were added to this supernatant, the precursor proteins were transported into mitochondria (Harmey *et al.*, 1977; Korb and Neupert, 1978; Maccecchini *et al.*, 1979; Zimmermann and Neupert, 1980).

These experiments demonstrated that mitochondrial protein import is independent of translation. This does not exclude, however, that import can occur while the polypeptide chain is being elongated, i.e., by a cotranslational mechanism. Butow *et al.* showed that in yeast cells treated

with cycloheximide, cytoplasmic ribosomes were attached to mitochondria (Kellems and Butow, 1972; Kellems *et al.*, 1974; Ades and Butow, 1980). The messenger RNA associated with these ribosomes was specifically enriched for mRNA of mitochondrial proteins (Suissa and Schatz, 1982). Later studies showed that the amino-terminal parts of precursor proteins can be transported into the mitochondrial matrix while the carboxy-terminal ends are still exposed to the cytosol (Schleyer and Neupert, 1985). Thus, several features of a cotranslational transport mechanism are fulfilled. Although, as discussed above, the co- or posttranslational mode by which precursors are transported into mitochondria does not appear to contribute essential features to the mechanism of translocation across membranes.

B. Properties of Precursor Proteins

Mitochondrial precursor proteins differ from their mature counterparts in several regards. Most precursor proteins are synthesized with an amino-terminal peptide extension, termed "presequence." During or after import, the presequence is specifically cleaved ("proteolytic processing"). Precursor proteins without presequences are often covalently modified during import (e.g., attachment of heme to cytochrome *c*), or at least their conformation is different compared to the mature protein. For matrix proteins, however, it has not been shown that folding of mature parts in precursors is different from folding of mature matrix proteins.

All presequences known so far carry abundant positively charged amino acid residues and few, if any, negatively charged residues. Furthermore, in many cases the content of serines and threonines is relatively high (Kaput *et al.*, 1982; Viebrock *et al.*, 1982; Maarse *et al.*, 1984; Morohashi *et al.*, 1984; Sadler *et al.*, 1984; Sebald and Kruse, 1984; Suissa *et al.*, 1984; Takiguchi *et al.*, 1984; Wright *et al.*, 1984; Gay and Walker, 1985; Guiard, 1985; Harnisch *et al.*, 1985; Jaussi *et al.*, 1985; Joh *et al.*, 1985; Koerner *et al.*, 1985; Marres *et al.*, 1985; Mueckler and Pitot, 1985; Nyunoya *et al.*, 1985; Takeda *et al.*, 1985; Keng *et al.*, 1986; Ohta and Kagawa, 1986). Subunit VI of the bc_1 complex in yeast could be an exception, since it possesses a negatively charged amino-terminal sequence (van Loon *et al.*, 1984); however, it is unknown where or whether at all it is proteolytically processed.

Studies by Hurt *et al.* (1984a,b) have demonstrated that the presequence alone can be sufficient to direct a cytoplasmic protein, mouse dihydrofolate reductase (DHFR), into mitochondria. The experiments were performed by fusing the coding region for the presequence of cyto-

chrome oxidase subunit IV (pre cox IV) to the cDNA of DHFR. The fusion protein was even imported into mitochondria when only the first half (12 amino acid residues) of pre cox IV was fused to DHFR (Hurt *et al.*, 1985a). Horwich *et al.* (1985a) showed that the presequence of the matrix enzyme ornithine carbamoyl transferase can direct DHFR into mitochondria *in vivo* and *in vitro*. Similar experiments with fusion proteins containing *Escherichia coli* β-galactosidase or yeast invertase suggested that the 9 amino-terminal residues of δ-aminolevulinate synthase (Keng *et al.*, 1986) and the 27 amino-terminal residues of F_0F_1-ATPase subunit 2 (Douglas *et al.*, 1984; Emr *et al.*, 1986) can function as mitochondrial targeting signals. By sequence comparison of the three yeast alcohol dehydrogenase isoenzymes (ADH I–ADH III) and by gene fusion experiments, van Loon and Young (1986) concluded that a 27-residue amino-terminal extension of ADH III, not present in ADH I and ADH II, contains the information for directing ADH III to mitochondria. These experiments suggest that the presequence alone carries sufficient information for the targeting of proteins to mitochondria.

When the distal three out of four arginine residues in the presequence of ornithine carbamoyl transferase were replaced with glycine residues, the altered precursor could not be imported into mitochondria. In addition, it could no longer be proteolytically cleaved by a fraction containing the matrix-processing peptidase (Horwich *et al.*, 1985b). It remains to be determined whether the altered functions are caused by changes in the specific amino acid sequence, by a different secondary or tertiary structure, or simply by a loss of positive charges (see below).

Amino-terminal sequences could also be responsible for the transport of proteins to the correct intramitochondrial location. The 70-kDa protein of the yeast mitochondrial outer membrane, which is synthesized without a cleavable presequence, was manipulated by deleting different regions and by fusing amino-terminal parts to various proteins. Translocation into mitochondria was tested with the altered proteins (Riezman *et al.*, 1983c; Hase *et al.*, 1983, 1984; Hurt *et al.*, 1985b), and the following conclusions were drawn: All the information for targeting and anchoring of the 70-kDa protein are contained in the amino-terminal 41 amino acid residues; the extreme amino terminus (~12 amino acid residues) functions as a signal for directing proteins to mitochondria and into the mitochondrial matrix, whereas a subsequent stretch of uncharged amino acids could serve as a "stop-transfer" or "anchoring" sequence fixing the 70-kDa protein permanently into the outer membrane.

The ADP/ATP carrier (an inner membrane protein) is cytoplasmically synthesized without a presequence (Zimmermann *et al.*, 1979b; Zimmerman and Neupert, 1980). It possesses many positively charged amino acid

residues which frequently occur in clusters (Aquila *et al.*, 1982; Arends and Sebald, 1984). At least three sequences of ~20 amino acid residues are present in the carrier which could serve as internal signal sequences (see also Section II,E); they carry several positive charges, but no negative charges. Furthermore, these sequences are predicted to show α-helical structure (Aquila *et al.*, 1985), which could also be a feature of mitochondrial presequences (see below). Since the ADP/ATP carrier consists of three domains of 100 amino acid residues which show striking homology to each other, it has been postulated that it has originated by triplication of an ancestral gene (Saraste and Walker, 1982). Each domain contains a putative signal sequence. Studies by Adrian *et al.* (1986) have suggested that the first 115 amino acid residues of the protein carry sufficient information for targeting to mitochondria. Thus, multiple signal sequences could be present in a mitochondrial precursor protein. The mature carrier in the inner membrane is able to bind the specific inhibitor carboxyatractyloside and to pass over hydroxyapatite, in contrast to the precursor protein (Schleyer and Neupert, 1984). Therefore, folding of the mature protein and folding of the precursor appear to be different. This differential affinity for hydroxyapatite, which presumably reflects conformational differences, serves as a convenient and nondestructive test for the import and maturation of carrier.

The uncoupling protein (GDP-binding protein, 32,000 M_r protein, thermogenin) of brown adipose tissue shows many similarities to the ADP/ATP carrier. It is cytoplasmically synthesized without a presequence (Freeman *et al.*, 1983; Ricquier *et al.*, 1983). Its primary sequence is clearly homologous to that of the ADP/ATP carrier including the tripartite structure (Aquila *et al.*, 1985) and the putative internal signal sequences.

Another possible function of presequences besides this role in targeting is to confer solubility in aqueous compartments to precursor proteins. For example, subunit 9 of F_0F_1-ATPase (proteolipid or dicyclohexylcarbodiimide (DCCD)-binding protein) from the fungus *Neurospora crassa* is a very hydrophobic protein of 81 amino acid residues. Its presequence is long (66 amino acid residues) and hydrophilic (Viebrock *et al.*, 1982). Like most precursor proteins, subunit 9 is transported through the cytosol in the form of high-molecular-weight aggregates (Schmidt *et al.*, 1983b). The hydrophilic presequences probably face the outer side of the aggregates and thereby may be responsible for the water solubility of the precursor form.

The role of the positively charged presequences in membrane insertion with regard to the membrane potential will be discussed later (Section II,F).

A synthetic peptide containing the first 27 amino acids of the prese-

quence of rat liver ornithine carbamoyl transferase was used to examine whether import of a precursor can be inhibited by a presequence. Indeed, this peptide was observed to inhibit the import of pre-ornithine carbamoyl transferase into mitochondria. A synthetic peptide containing the amino acids 16–27 of the presequence did not affect import. The inhibitory effect could be overcome by increasing the concentration of pre-ornithine carbamoyl transferase in a competitive manner. In addition, in the *in vitro* import system (rabbit reticulocyte lysate) the mitochondria were not uncoupled. The synthetic peptide also inhibited import of two other matrix proteins and of the uncoupling protein. Gillespie *et al.* (1985) conclude that the synthetic presequence competes for an essential component of the import pathway (e.g., for a receptor), which is common for several proteins of the matrix and the inner membrane. In the absence of reticulocyte lysate, the synthetic peptide uncoupled isolated mitochondria. One explanation for its uncoupling properties may be that the peptide has an amphiphilic structure. This could have important consequences for the interaction of presequences with lipid bilayers. Similarly, Ito *et al.* (1985) reported that synthetic model peptides, which in some aspects resembled presequences of mitochondrial proteins (i.e., repeated short stretches of uncharged amino acids linked by basic amino acids), uncoupled oxidative phosphorylation of mitochondria. Recent studies with physiological and artificial mitochondrial targeting sequences suggested that the presence of positive charges and the formation of an amphiphilic α-helix on the surface of precursor proteins are important criteria for their function (Horwich *et al.*, 1985c, 1986; Allison and Schatz, 1986; Roise *et al.*, 1986; von Heijne, 1986; Hurt and Schatz, 1987).

C. Cytosolic Cofactors for Import

In the last few years, several groups have reported a requirement of cytosolic cofactor(s) for import of mitochondrial proteins (Argan *et al.*, 1983; Miura *et al.*, 1983; Ohta and Schatz, 1984; Argan and Shore, 1985; Pfanner and Neupert, 1987). Among these proteins are subunit 2 of F_0F_1-ATPase ($F_1\beta$) in yeast, the ADP/ATP carrier, and subunits 2 and 9 of F_0F_1-ATPase in *N. crassa*. The import-stimulating activity can be found in cytosolic fractions of yeast, *Neurospora,* and rabbit reticulocytes or erythrocytes. Sensitivity to proteases, denaturation by heating, nondialyzability, and gel filtration suggest that the cofactor in yeast is a protein with a molecular weight of ~40,000 (Ohta and Schatz, 1984). Possible functions of the cofactor include stabilization of precursor proteins, a role for specific (i.e., import-competent) binding to receptors, or a role in

translocation across the membrane(s). Our studies on the import of ADP/ATP carrier suggest that the cofactor may be required for import-competent binding (Pfanner and Neupert, 1987).

When a reticulocyte lystate containing *in vitro*-synthesized precursor proteins is treated with relatively high amounts of RNase at elevated temperatures (37°C), or in the presence of chelating agents, subsequent import of precursors into mitochondria is diminished. This procedure does not reduce the amount of precursor proteins and does not generate a mitochondrial poison. This suggests that the RNase-sensitive component may be in a ribonucleoprotein complex. This complex might also contain the precursor proteins themselves (Firgaira *et al.*, 1984). Import of *N. crassa* $F_1\beta$, for example, requires this "RNA cofactor" (Pfanner and Neupert, unpublished); however, its function remains unknown.

Recently, it was shown that nucleoside triphosphates are necessary to keep mitochondrial precursor proteins in import-competent conformation (see Section II,F). It was proposed that nucleoside triphosphate-dependent cytosolic cofactors are involved in this process (Pfanner *et al.*, 1987). Further studies have to clarify whether these proposed cytosolic cofactors are related to the cofactors described above.

D. Import Receptors

1. *The Apocytochrome c* Receptor

Apocytochrome *c*, the precursor of cytochrome *c*, is synthesized in the cytoplasm without an amino-terminal presequence. Transport to its final destination on the outer side of the inner membrane does not require a membrane potential. During or after translocation across the outer mitochondrial membrane, heme is covalently attached to form holocytochrome *c*. Apocytochrome *c* synthesized *in vitro* can be imported into isolated mitochondria. During import, it is rapidly converted to holocytochrome *c* and only a small fraction of apocytochrome *c* is recovered with subsequently reisolated mitochondria. When deuterohemin, a reversible inhibitor of the heme-attaching step, is added, a considerable amount of apocytochrome *c* is found associated with mitochondria and conversion to holocytochrome *c* is nearly abolished. This apocytochrome *c* can be displaced from mitochondria by addition of excess apocytochrome *c* (but not holocytochrome *c*), and is sensitive to externally added proteases. When the deuterohemin block is relieved by addition of protohemin, the bound apocytochrome *c* is converted to protease-resistant holocytochrome *c* (Korb and Neupert, 1978; Hennig and Neupert, 1981).

Scatchard plot analysis in the presence of deuterohemin using chemically prepared apocytochrome *c* shows two types of binding sites. The high-affinity binding sites are present at about 90 pmol/mg mitochondrial protein and have an association constant K_a of $2.2 \times 10^7 M^{-1}$ (Hennig *et al.*, 1983). Pretreatment of mitochondria with proteases reduces the number of high-affinity sites, but not the low-affinity sites. The high-affinity binding sites have been reconstituted into liposomes by using octylglucoside extracts of mitochondria. With this assay a mitochondrial protein which shows characteristics of an apocytochrome *c*-binding protein was purified. Notably, this protein seems to be localized on the inner side of the outer membrane (Köhler, Stuart, and Neupert, unpublished 1987). This result is consistent with the findings that chemically prepared apocytochrome *c* can spontaneously insert into lipid bilayers of a certain lipid composition (Rietveld *et al.*, 1983, 1985; Dumont and Richards, 1984; Rietveld and De Kruijff, 1984). Sodium carbonate treatment (pH 11.5), which releases soluble proteins and peripheral membrane proteins and leaves integral membrane proteins in the membrane fraction (Fujiki *et al.*, 1982a,b), suggests that, in contrast to holocytochrome *c*, the receptor-bound apocytochrome *c* is inserted into the lipid phase of the outer membrane.

The present model (Fig. 1) proposes that apocytochrome *c* can spontaneously and reversibly insert into the outer mitochondrial membrane. It then could bind specifically to a protein on the inner side of the outer

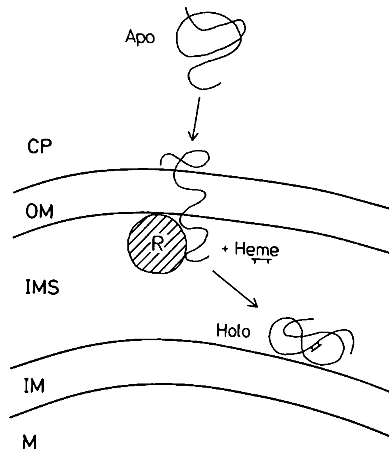

FIG. 1. Hypothetical import pathway for cytochrome *c*. CP, Cytoplasm; OM, outer mitochondrial membrane; IMS, intermembrane space; IM, inner mitochondrial membrane; M, matrix; Apo, apocytochrome *c*; Holo, holocytochrome *c*; R, receptor.

membrane. It is converted to holocytochrome *c* by covalent attachment of the heme group by an enzyme called cytochrome *c* heme lyase (or cytochrome *c* synthetase), and released to the outer face of the inner membrane (Chua and Schmidt, 1979; Hennig and Neupert, 1981; Veloso *et al.*, 1981; Taniuchi *et al.*, 1983; Visco *et al.*, 1985; Nicholson *et al.*, 1987).

2. *Import Receptors for Other Mitochondrial Proteins*

Binding and import of all other proteins tested so far (including cytochrome c_1 and subunit V of the bc_1 complex, subunits 2 and 9 of the F_0F_1-ATPase, and the ADP/ATP carrier) cannot be competed by apocytochrome *c*, even in excess amounts (Zimmerman *et al.*, 1981; Teintze *et al.*, 1982). Furthermore, import of these proteins is strongly reduced by pretreatment of mitochondria with very small amounts of trypsin ($<2 \mu g$/mg mitochondrial protein) (Zwizinski *et al.*, 1984), whereas import of apocytochrome *c* is only reduced by 10- to 20-fold higher amounts of trypsin. Mild trypsin treatment does not lead to destruction of the outer membrane; therefore, one can conclude that there exists a second type of receptors, which are proteins that are located on the outer face of the outer membrane. Pretreatment of mitochondria with elastase, for example, inhibits the import of ADP/ATP carrier but does not affect the import of F_0F_1-ATPase subunit 2, suggesting that a third class of receptors may exist (Zwizinski *et al.*, 1984).

Experiments using a water-soluble form of the outer membrane protein porin (prepared from the isolated membrane form) also showed high-affinity (5–10 pmol/mg mitochondrial protein; $K_a = 10^9 M^{-1}$) and low-affinity binding sites (Pfaller *et al.*, 1985). Water-soluble porin could compete for the specific binding of ADP/ATP carrier, suggesting that the same binding sites may be involved in the import of both precursors (Pfaller and Neupert, 1987).

Scatchard plot analysis of binding of the precursor of the ADP/ATP carrier also showed high-affinity (1.7 pmol/mg mitochondrial protein; $K_a = 1.1. \times 10^9 M^{-1}$) and low-affinity binding sites (Schmidt *et al.*, 1985). The precursor could be bound to the surface of deenergized mitochondria (deenergization blocks the import of many proteins; see Section II,F). When the mitochondria were reisolated and subsequently reenergized, the precursor was almost quantitatively imported to its functional location in the inner membrane (Zwizinski *et al.*, 1983; Pfanner and Neupert, 1987).

The precursor of cytochrome b_2, a protein of the intermembrane space,

binds to isolated mitochondria and to isolated outer membrane vesicles. Binding is energy-independent and is reduced by trypsin pretreatment of the membranes. Upon reenergization of mitochondria the precursor is imported from the binding sites. Experiments with the precursor of citrate synthase, a matrix protein, yielded similar results. The binding sites could be solubilized from outer membranes with nonionic detergent and reconstituted into liposomes (Riezman *et al.*, 1983a,b).

Other precursor proteins which need an energized inner membrane for import (including subunit V of the bc_1 complex, and subunit 2 ($F_1\beta$) of the F_0F_1-ATPase) are not efficiently imported when first bound to deenergized mitochondria (Pfanner and Neupert, 1985; Harth *et al.*, 1986). The capacity of specific binding sites might be relatively low for these proteins, or receptors are occupied by precursors having higher affinities. Under physiological conditions (energized mitochondria), precursor proteins are rapidly imported from their binding sites. When mitochondria are deenergized, the specific sites may become saturated and the remaining precursor proteins may be directed to unspecific binding sites. Interestingly, the precursor of $F_1\beta$ can not be found associated with deenergized mitochondria (Zwizinski et al., 1984). Presumably, the specific binding capacity is also low under these conditions, but alternatively unspecific binding does not take place.

As discussed above (Section II,B), a synthetic peptide containing the first 27 amino acids of ornithine carbamoyl transferase blocks import of different precursor proteins destined for the mitochondrial matrix or inner membrane. It is not known whether this occurs at the level of specific binding or at some other step of the pathway.

In summary, the present data suggest that at least two different types of import receptors exist. On the other hand, evidence is accumulating that even precursors destined for different mitochondrial compartments share components of the import apparatus, including receptors.

3. *Heterologous Import*

Several precursor proteins can be transported into mitochondria from other species, despite differences in primary structure compared to the corresponding native proteins of these species (e.g., precursor proteins of *N. crassa* are imported into mitochondria from rat and yeast) (Schleyer *et al.*, 1982; Teintze *et al.*, 1982).

In *N. crassa,* the precursor of ATPase subunit 9 ("proteolipid") is synthesized in the cytoplasm with an amino-terminal presequence (Viebrock *et al.*, 1982); in contrast, the proteolipid of yeast is synthesized within mitochondria and lacks a presequence (Tzagoloff and Meagher,

1972; Sierra and Tzagoloff, 1973; Hensgens *et al.*, 1979; Macino and Tzagoloff, 1979) (see also Section III). The precursor of *Neurospora* proteolipid can be imported into yeast mitochondria, where it is correctly processed by the processing peptidase in the matrix (Schmidt *et al.*, 1983a).

The fact that heterologous import is possible predicts general principles for specific recognition of mitochondrial precursor proteins and common import pathways which can be used by "foreign" precursor proteins.

E. Translocation Contact Sites

All cytoplasmically synthesized proteins which are destined for the mitochondrial matrix or inner membrane, and several proteins destined for the intermembrane space must be translocated across the outer membrane and completely or at least partially across the inner membrane. The question arises as to how proteins are translocated across two membranes. Are they transported across the membranes in separate steps, or is there a mechanism to cross both membranes at the same time? Electron-microscopic studies have demonstrated the existence of contact sites between mitochondrial outer and inner membranes (Hackenbrock, 1968; van Venetie and Verkleij, 1982); however, no biochemical function could be assigned to these morphological structures.

It has been shown that cytochrome c_1 and subunit 2 of F_0F_1-ATPase ($F_1\beta$) are imported through "translocation contact sites" (Schleyer and Neupert, 1985). Two different procedures yielded translocational intermediates of the precursor proteins spanning both membranes. In this topological arrangement, the amino-terminal part of the intermediates was cleaved by the matrix-located processing peptidase (see Section II,G), whereas a major part of the protein was still outside the outer membrane and was accessible to externally added proteases. In the first approach, import was performed at low temperature (4°–12°C). The accumulated intermediates could be completely imported by raising the temperature to 25°C, indicating that the intermediates were on the correct import pathway. Only the first import step into the intermediate position required a membrane potential. In a second approach, specific antibodies were prebound to precursor molecules. These antibody-bound precursor proteins were trapped at a similar intermediate position (Fig. 2).

Three conclusions can be drawn from these experiments: (1) Outer and inner mitochondrial membranes come close enough together that they can be spanned by polypeptide chains ("translocation contact sites"); (2) the amino-terminal ends of precursor proteins enter the matrix space first;

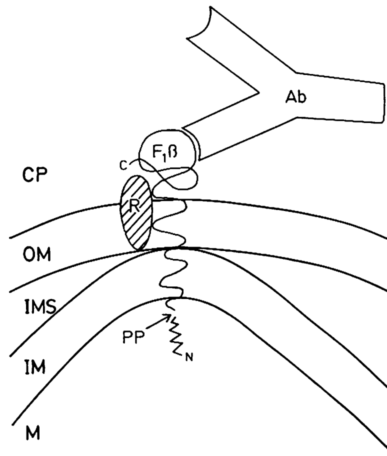

FIG. 2. Translocational intermediate of $F_1\beta$ spans contact site between outer and inner mitochondrial membranes. Ab, Antibody; N, amino terminus of $F_1\beta$; C, carboxy terminus of $F_1\beta$; R, receptor; PP, metal-dependent processing peptidase in the matrix. Other abbreviations are as in Fig. 1.

and (3) only import of the amino terminus requires a membrane potential $\Delta\psi$, while the majority of the polypeptide chain is translocated in the absence of $\Delta\psi$ (see Fig. 4).

Experiments were performed to determine whether the translocation contact sites were identical to the contact sites observed by electron microscopy. $F_1\beta$-Intermediates, trapped by specific antibodies, were labeled with protein A–gold particles which recognized the F_c domain of the antibodies and allowed visualization by electron microscopy. These studies confirmed that protein translocation did indeed occur via morphological contact sites (Schwaiger *et al.*, 1987).

Translocation contact sites could be transient structures which are formed to allow the import of precursor proteins and which are subsequently dissociated. For instance, cytochrome c_1, which ends up on the outside of the inner membrane, could be first transported into the described intermediate state via contact sites. Pre-cytochrome c_1 is proteolytically processed in two steps: the first part of the amino-terminal presequence is cleaved off by the matrix-localized processing peptidase, whereas the second portion which has been proposed to funtion as a "stop transfer signal" anchoring the precursor in the inner membrane (Kaput *et al.*, 1982; Hurt and van Loon, 1986; van Loon *et al.*, 1986) is removed by a different proteolytic activity that is probably present on the outer face of the inner membrane (see Section II,G). Thus the first processing event could take place while a part of cytochrome c_1 is inserted

into the contact site. The contact sites could then be disassembled, resulting in the concomitant translocation of the major part of the cytochrome c_1 molecule across the outer membrane. The second portion of the presequence would then be cleaved off by a protease on the outside of the inner membrane. Thereby release of the amino terminus of mature cytochrome c_1 from the inner membrane could occur while a carboxy-terminal portion became anchored in the inner membrane (Fig. 3).

On the other hand, translocation contact sites could be stable structures, and hydrophilic domains would have to completely cross the two membranes of the contact sites. According to this view, inhibition of the matrix-processing peptidase (see Section II,G) would lead to accumulation of precursors in the matrix. Indeed it was found that the precursor of subunit V of the bc_1 complex is completely transported into the mitochondrial matrix when the matrix-processing enzyme in intact mitochondria is inhibited by the addition of the metal chelator *o*-phenathroline. After relieving the processing block, the protein becomes translocated to its final destination on the outside of the inner membrane (see Fig. 5). The latter step does not require a membrane potential (Hartl *et al.*, 1986). Notably, the translocation of mitochondrially synthesized proteins into the inner membrane can occur in the absence of a membrane potential as well (see Section III). We suggest that at least several cytoplasmically synthesized proteins which end up in the inner membrane or intermembrane space are completely translocated into the matrix via translocation contact sites. After (proteolytic) processing, which can occur during or after the translocation (see Fig. 4), they may be sorted from the matrix to their functional locations. This view agrees with the "endosymbiont theory" of mitochondrial evolution, which postulates that mitochondria have originated in endocytosed prokaryotes (for review, see Gray and Doolit-

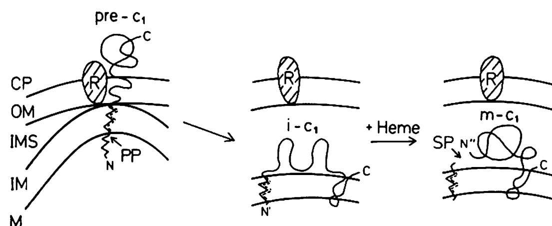

FIG. 3. Hypothetical import pathway for cytochrome c_1. Pre-c_1, i-c_1, and m-c_1 are precursor-, intermediate-, and mature-sized cytochrome c_1, respectively; N, N′, and N″ are the amino terminus of precursor-, intermediate-, and mature-sized cytochrome c_1, respectively; C, carboxy terminus; R, receptor, PP, processing peptidase; SP, second processing activity on the outer face of the inner membrane. Other abbreviations are as in Fig. 1.

tle, 1982). Thereby such proteins could use "ancestral" pathways developed early in evolution when they were still synthesized within mitochondria ("conservative model"). For example, early in evolution the precursor proteins may have been synthesized in the equivalent of the mitochondrial matrix (the cytoplasmic compartment of the endosymbiont) without the presequence, which is now cleaved off by the matrix-processing peptidase. This is illustrated by the different mechanisms by which F_0F_1-ATPase subunit 9 is synthesized in *N. crassa* and higher eukaryotes as compared to yeast and plants (see Sections II,D,3 and III). It should be noted that proteins exist, such as the ADP/ATP carrier and the uncoupling protein, for which no equivalent has been found in prokaryotes (Klingenberg, 1985). One might speculate that these proteins have been introduced into the organelles during or after the evolutionary endocytosis event. Interestingly, their precursors do not have cleavable presequences. With these proteins the signal sequences are apparently embedded in the mature proteins (also see Section II,B). The question arises as to how proteins like the ADP/ATP carrier are transported across two membranes. Recent studies in our laboratory show that it is also imported via translocation contact sites, emphasizing the important role of contact sites in mitochondrial protein import (Pfanner and Neupert, 1987). It should be noted that apocytochrome *c* contains no cleavable presequence and is not imported via translocation contact sites, although a prokaryotic equivalent exists for cytochrome *c*. Cytochrome *c* appears to use a unique biogenesis pathway (see Section VI,C).

Finally, contact sites could be organized in such a way that precursor proteins (for instance in the case of cytochrome c_1) can move away from the contact sites by lateral diffusion to the intermembrane space and, at the same time, the remaining untranslocated carboxy-terminal portion may cross the outer membrane. In such a model, translocation contact sites are stable structures, but the precursor proteins do not have to be completely transported into the matrix.

F. Energy Requirement of Import

Import of many proteins into mitochondria is inhibited *in vivo* and *in vitro* by uncouplers of oxidative phosphorylation (Hallermayer and Neupert, 1976; Nelson and Schatz, 1979; Zimmerman *et al.*, 1981). Protonophores dissipate the electrochemical potential across the inner membrane and deplete the matrix of ATP because of their stimulating effect on ATPase activity. Simply using these inhibitors was not adequate to determine whether the electrochemical potential or ATP or both were the required energy source.

Nelson and Schatz (1979) studied mitochondrial protein import in *rho*$^{-}$ mutants of yeast, which were deficient in mitochondrial protein synthesis and therefore lacked a functional respiratory chain and ATPase. Despite these deficiencies, precursor proteins could still be imported into mitochondria. On the assumption that *rho*$^{-}$ mutants have no electrochemical potential across the inner membrane, Nelson and Schatz concluded that only ATP and not the electrochemical potential is the direct energy source. Later experiments showed, however, that an energized inner membrane is required. It is possible that mitochondria from *rho*$^{-}$ mutants can still create a low electrochemical potential, e.g., by importing ATP via the electrogenic ADP/ATP carrier. It is now known that low potentials (20–40 mV) are sufficient to drive import (Pfanner and Neupert, 1985).

Schleyer *et al.* (1982) used protonophores, K^{+} ionophores (valinomycin), inhibitors of the respiratory chain (rotenone, antimycin A, and potassium cyanide), inhibitors of the F_0F_1-ATPase (oligomycin) and of the ADP/ATP carrier (carboxyatractyloside) to examine the nature of the energy requirement. These studies demonstrated that transport of precursors into or across the inner membrane required an energized inner membrane. Other groups (Gasser *et al.*, 1982a; Kolanski *et al.*, 1982) have obtained similar results.

A practical consequence of these experiments is that a combination of antimycin A (which blocks complex III of the respiratory chain) and oligomycin (which inhibits both formation and hydrolysis of ATP by the ATPase) inhibits protein import. Addition of ascorbate and *N*,*N*,*N*′,N′-tetramethylphenylenediamine (TMPD), which leads to a supply of electrons at the level of complex IV, restores import. Import can again be inhibited by potassium cyanide, which blocks complex IV (Schleyer *et al.*, 1982). Thus, precursor proteins can be bound to deenergized mitochondria and imported from the binding sites after reenergization (Zwizinski *et al.*, 1983; see Section II,D,2).

These experiments did not answer the question as to whether the total proton-motive force Δp (with the components membrane potential $\Delta\psi$ and ΔpH) or the membrane potential $\Delta\psi$ alone is the required energy source. For the import of ADP/ATP carrier we have shown that $\Delta\psi$ per se is the driving force (Pfanner and Neupert, 1985). Translocation of carrier from binding sites on the mitochondrial surface into the inner membrane can be driven by a valinomycin-induced potassium diffusion potential; this import cannot be abolished by addition of protonophores. Furthermore, imposing a ΔpH does not stimulate import. Thus, precursor import does not appear to be mediated by a Δp-driven proton movement, in contrast to many other processes which depend on an energized inner membrane.

All amino-terminal peptide extensions which have been sequenced until

now carry a net positive charge. The ADP/ATP carrier, which is synthesized without a peptide extension, carries abundant positive charges, and also clusters of positive charges (see Section II,B). Therefore, the role of the membrane potential could include an electrophoretic effect on the positively charged sequences mediating the penetration of these sequences into the energized mitochondrial inner membrane (Fig. 4). Another possible role of $\Delta\psi$ includes conformational changes in membrane lipids and proteins which may participate in facilitating the entry of precursor proteins into the inner membrane. The completion of translocation is independent of $\Delta\psi$ as discussed above (Fig. 4).

The majority of precursor proteins are partially or completely translocated across the inner membrane and thus require $\Delta\psi$. This is also the case with several proteins of the intermembrane space (e.g., cytochrome b_2 and cytochrome-*c* peroxidase), which must at least partially cross the inner membrane to reach the matrix-located processing peptidase (Gasser *et al.*, 1982b; Kaput *et al.*, 1982; Reid *et al.*, 1982). Precursor proteins which are not transported into or across the inner membrane, such as those of the outer membrane and cytochrome *c*, do not require a membrane potential for import (Zimmerman *et al.*, 1981; Freitag *et al.*, 1982; Mihara *et al.*, 1982; Gasser and Schatz, 1983).

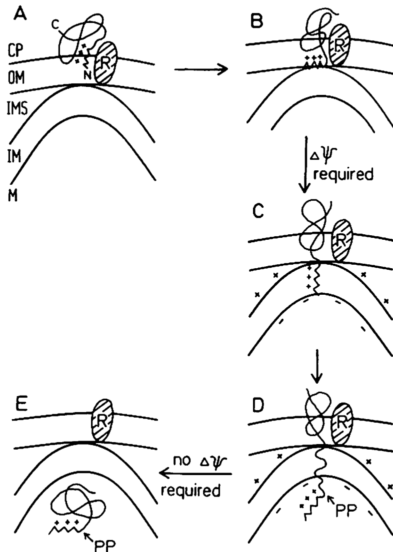

FIG. 4. Energy requirement for translocation of proteins into mitochondria. N, Amino terminus; C, carboxy terminus; R, receptor; $\Delta\psi$, membrane potential; PP, processing peptidase, which cleaves the precursor proteins at step (D) or (E). Other abbreviations are as in Fig. 1.

Recently, it was found that mitochondrial protein import required nucleoside triphosphates, e.g., ATP or GTP, independently of the requirement for a $\Delta\psi$. This was demonstrated for the import of $F_1\beta$, ADP/ATP carrier, fusion proteins between F_0-ATPase subunit 9 and dihydrofolate reductase, a fusion protein between the presequence of cytochrome oxidase subunit IV and dihydrofolate reductase, porin, cytochrome c_1, and cytochrome b_2 (Pfanner and Neupert, 1986; Pfanner, *et al.*, 1987; Eilers *et al.*, 1987; Chen and Douglas, 1987; Kleene *et al.*, 1987; Hartl *et al.*, 1987. Nucleoside triphosphates seem to be necessary to keep the cytosolic precursor proteins in an import-competent conformation (Pfanner, *et al.*, 1987).

G. Proteolytic Processing

During or after translocation, the amino-terminal presequences of imported proteins are proteolytically removed. For all mitochondrial precursor proteins which undergo a one-step cleavage, the proteolytic activity has been found in the mitochondrial matrix. Böhni *et al.* (1980) first detected this activity in hypotonic extracts of yeast mitochondria. It has also been described in rat liver and *Neurospora* mitochondria. The processing peptidases (or matrix proteases) from different organisms show very similar characteristics (Conboy *et al.*, 1982; McAda and Douglas, 1982; Miura *et al.*, 1982; Böhni *et al.*, 1983; Schmidt *et al.*, 1984) including solubility in detergent-free buffers; neutral pH optimum, inhibition by chelating agents such as EDTA, *o*-phenanthroline, and bathophenanthroline, and insensitivity to many other protease inhibitors including serine protease inhibitors (e.g., phenylmethylsulfonyl fluoride, PMSF). The processing activity is stimulated by addition of divalent cations such as zinc, cobalt, and manganese ions. Processing peptidase was localized in the matrix by two different approaches. First, it cofractionates with matrix marker proteins. Second, in intact mitochondria it cannot be inhibited by EDTA or bathophenanthroline, which are unable to penetrate the inner membrane, but it is inhibited by *o*-phenathroline, which can cross the inner membrane.

McAda and Douglas (1982) enriched the processing peptidase from yeast mitochondria about 200-fold. Following gel filtration, processing activity was found in a protein fraction with M_r 115,000. On sodium dodecyl sulfate (SDS)–polyacrylamide gels a major protein band with an apparent molecular weight of 59,000 and several minor bands of M_r 39,000 and 35,000 were visible. Böhni *et al.* (1983) enriched the processing peptidase from yeast mitochondria about 100-fold. The active enzyme fraction also had an apparent molecular weight of 115,000 as judged by gel filtra-

tion; of the ~10 protein bands resolved on SDS–polyacrylamide gels, however, none had an apparent molecular weight in the range of 59,000. Processing of subunit V of cytochrome oxidase with the partially purified protease yielded the correct amino terminus of the mature protein (Cerletti *et al.*, 1983). Starting with cell homogenates of *N. crassa*, the processing peptidase has been enriched 10,000-fold, corresponding to a 2,000-fold enrichment over mitochondria (Hawlitschek, Schmidt, and Neupert, in preparation). On silver-stained SDS–polyacrylamide gels, two protein bands were visible with apparent molecular weights of 57,000 and 52,000. Antibodies were prepared against the two proteins, and only the antibody directed against the larger one was able to precipitate the enzyme activity. This indicates that in *Neurospora* the processing peptidase is a protein component which constitutes <0.05% of total mitochondrial protein.

Yaffe and Schatz (1984) isolated temperature-sensitive yeast mutants which exhibit strongly reduced protein import into mitochondria (e.g., import of $F_1\beta$). They found two complementation groups (termed *mas1* and *mas2*) which represent single nuclear mutations. *In vitro* studies showed that the *mas1* mutant was deficient in the matrix-located processing activity (Yaffe *et al.*, 1985). The corresponding nuclear gene has now been cloned. It remains to be determined whether the *mas1* gene product is the processing enzyme per se or is somehow required for the activity of the enzyme.

Several precursor proteins are proteolytically cleaved in two steps. In all cases studied so far, the first processing event is carried out by the processing peptidase in the matrix. With F_0F_1-ATPase subunit 9 of *N. crassa*, this enzyme is also responsible for the second proteolytic processing. Radiosequence analysis proved that a true intermediate and a mature protein with the correct amino terminus were generated (Schmidt *et al.*, 1984). The second processing of cytochrome c_1 precursor and two precursors of proteins of the intermembrane space (cytochrome b_2 and cytochrome-*c* peroxidase) is carried out by different proteolytic activities (also see Section III). The processing activities appear to be located on the outside of the inner membrane and are not affected by chelating agents (Daum *et al.*, 1982a,b; Gasser *et al.*, 1982b; Ohashi *et al.*, 1982; Reid *et al.*, 1982; Teintze *et al.*, 1982).

It must be emphasized that proteolytic processing is not a prerequisite for translocation of proteins across mitochondrial membranes. When the processing peptidase is inhibited, precursor proteins are still transported into mitochondria (e.g., subunits 2 and 9 of F_0F_1-ATPase) (Zwizinski and Neupert, 1983) and subunit V of bc_1 complex (Hartl *et al.*, 1986). When the block in processing is relieved, the presequence is cleaved off without

any requirement for a membrane potential. Also, protein constructions which have a presequence but do not have a cleavage site are translocated into mitochondria (Hurt *et al.*, 1985a,b). Finally, as described above, several precursor proteins are synthesized without any peptide extension (also see Table I).

III. Biogenesis of Proteins Coded by Mitochondrial Genes

The mitochondrial genome of different species has now been completely (mammalians, insects, amphibians) or partially sequenced (fungi, plants). Interestingly, deviations from the general genetic code were found. Open reading frames (ORF) within introns code for proteins; probably for proteins involved in RNA splicing ("maturases") and for a protein of the small ribosomal subunit in *N. crassa*. This is discussed in other articles of this volume (for review, also see Breitenberger and RajBhandary, 1985). In mammalians, most proteins coded for by the mitochondrial genome belong to proton-translocating complexes of the inner membrane (i.e., to complexes I, III, and IV of the respiratory chain, and the F_0 part of the ATPase) (Chomyn *et al.*, 1983, 1985; Michael *et al.*, 1984). This also seems to be the case in other species. In *Neurospora*, probably most, if not all, so-called unassigned reading frames (URF) code for subunits of complex I (Ise *et al.*, 1985; Zauner *et al.*, 1985). In summary, proteins coded for by mitochondrial genome include several subunits of NADH dehydrogenase, cytochrome *b* of the bc_1 complex, subunits I, II, and III of cytochrome oxidase, and subunits 6 and 8 of the F_0F_1-ATPase. ATPase subunit 9 is coded for by the mitochondrial genome in yeast and plants (Leaver and Gray, 1982; Leaver *et al.*, 1983), but by a nuclear gene in *N. crassa* and higher eukaryotes (Anderson *et al.*, 1981, 1982) (see also Section II,D,3). In the mitochondrial genome of *N. crassa*, a gene is present with strong homology to the nuclear gene of subunit 9 (excluding the presequence), but a translation product has not yet been found (van den Boogart *et al.*, 1982b). In plants subunit 1 of F_0F_1-ATPase is also synthesized within mitochondria (Boutry *et al.*, 1983; Hack and Leaver, 1983).

Subunit II of cytochrome oxidase from *N. crassa* and yeast is synthesized as a precursor protein with an amino-terminal extension (Machleidt and Werner, 1979; Sevarino and Poyton, 1980; van den Boogart *et al.*, 1982a). Pratje *et al.*, (1983) described a nuclear temperature-sensitive *pet* mutant of yeast in which the precursor accumulates. By comparing the amino-terminal protein sequences of subunit II in wild type and the mutant, an amino-terminal extension of 15 amino acid residues was found for

the precursor in the mutant strain. Interestingly, in this mutant the second processing step of cytochrome b_2, but not of cytochrome *c* peroxidase, is also blocked (Pratje and Guiard, 1986). At present, it cannot be decided whether this mutation inactivates a processing enzyme or a factor regulating this enzyme. The occurrence of a mitochondrially coded precursor form with a higher apparent molecular weight was also described for subunit I of cytochrome oxidase in *N. crassa* (Werner and Bertrand, 1979), but no evidence is available that a precursor with an additional sequence exists.

Translation of proteins in mitochondria appears to take place on membrane-associated ribosomes, although it has never been convincingly proven that membrane association and insertion of polypeptide chains are functionally linked. In *N. crassa,* the precursors of cytochrome oxidase subunits I and II can be accumulated when mitochondria are incubated in the absence of reducing agents (like NADH). The precursor of subunit II can be released from the membranes by treatment at alkaline pH (carbonate treatment, see Section II,D,1) suggesting that it is not integrated into the lipid phase of membranes. The translocation of mitochondrially synthesized proteins into the inner membrane appears to be independent of a membrane potential (Driever, Cook, and Neupert, unpublished).

Assembly of cytochrome oxidase and F_0F_1-ATPase will be discussed in Section V. In summary, little is known about the biogenesis or insertion into membranes of the mitochondrially synthesized proteins. This is primarily because all attempts to establish an *in vitro* submitochondrial translation system have failed so far.

IV. Coordination between Cytoplasmic and Mitochondrial Protein Synthesis

Most proteins coded for by exons of the mitochondrial genome are subunits of protein complexes which also contain cytoplasmically synthesized subunits (see Section III). Little is known about the regulation between the two translation systems.

Neurospora crassa cells grown in the presence of chloramphenicol are inhibited in mitochondrial protein synthesis and thus are deficient in cytochrome *b* of the bc_1 complex. Nevertheless, the cells synthesize normal amounts of the nuclear-coded cytochrome c_1 in the cytoplasm. Cytochrome c_1 is imported into the mitochondria, proteolytically processed, and inserted into a cytochrome *b*-deficient complex (Weiss and Kolb, 1979). Similarly, when cytoplasmic translation is inhibitied, subunits I–III of cytochrome oxidase (mitochondrial gene products) are still synthesized

for at least one doubling period of the cells. The proteolytic breakdown of mitochondrially synthesized proteins which are not assembled is increased (Neupert and von Rücker, 1976). Therefore, in *Neurospora* cells no stringent coupling between the two translation systems can be detected.

In the absence of mitochondrial protein synthesis, the cytoplasmically synthesized subunits of the F_1 part of yeast mitochondrial ATPase can still be assembled into a membrane-associated complex (see Section V,C). Subunits II (van Loon *et al.*, 1983b) and VIII (van Loon *et al.*, 1983a) of the bc_1 complex were overproduced by reintroducing the cloned genes on multicopy plasmids into yeast cells. This did not influence the synthesis or degradation of the remaining subunits of the complex. The overproduced subunits were transported into mitochondria, suggesting that import into mitochondria is not strictly coupled to synthesis of other subunits or to assembly of complexes.

On the other hand, coupling in yeast cells between both protein-synthesizing systems has been postulated at the level of transcription. Zitomer *et al.* (1979) compared the levels of messenger RNA for the nuclear-coded iso-1-cytochrome *c* under conditions of catabolic repression (growth on glucose) and derepression. The mRNA levels were decreased under repression. Similar results were obtained for the mRNAs of subunits IV–VII of cytochrome oxidase (Lustig *et al.*, 1982a) and of the nuclear-coded mitochondrial RNA polymerase (Lustig *et al.*, 1982b). The authors concluded that the control of nuclear transcription of the polymerase gene is important for modulating the expression of the mitochondrial genome.

Mutations in the yeast nuclear gene *PET 494* specifically block the mitochondrial synthesis of cytochrome oxidase subunit III. The *PET 494* gene codes for a mitochondrial protein which appears to interact with the leader region of the subunit III mRNA to promote its translation (Costanzo and Fox, 1986).

In summary, since components of the mitochondrial replication, transcription, and translation systems are coded by nuclear genes, long-term control of the mitochondrial protein-synthesizing system by the nucleus is likely. The question cannot be answered so far as to whether a short-term control also exists between the two systems.

V. Assembly of Protein Complexes

A. General Problems

The assembly of mitochondrial protein complexes raises a number of interesting questions. In which sequence are the subunits put together?

Which cofactors are required and what are their functions? Where are unassembled subunits localized? At what concentrations are they present? What are their properties (e.g., stability)? How are the cytoplasmic and mitochondrial protein-synthesizing systems coordinated (also see Section IV)? Can partially assembled complexes be accumulated and be used to provide information about the sequence and localization of assembly processes? Mutants lacking one or more subunits or deficient in the assembly process per se may be suitable subjects for these studies.

B. Assembly of Cytochrome Oxidase

Pulse-labeling experiments with *N. crassa* demonstrated that the assembly of cytochrome oxidase occurs from independent pools of precursor proteins. The pools range in size from 2% to 25% compared to the amounts of the respective subunits in cytochrome oxidase (Schwab *et al.*, 1972).

A considerable number of nuclear and extranuclear mutants deficient in cytochrome oxidase activity are known in *N. crassa* and yeast (Werner *et al.*, 1974; Ono *et al.*, 1975; Bertrand and Werner, 1977, 1979; Cabral *et al.*, 1978; Cabral and Schatz, 1978; Nargang *et al.*, 1978; Saltzgaber-Müller and Schatz, 1978; Werner and Bertrand, 1979; Pratje *et al.*, 1983; Dowhan *et al.*, 1985). Most of them are deficient in synthesis of one or more subunits; others contain precursor proteins with a higher apparent molecular weight (see Section III). Defects in the assembly of subunits are discussed below.

Copper-depleted cells of *N. crassa* have low levels of cytochrome oxidase and heme *a*. Labeling of the mitochondrial subunits, but not of the cytoplasmic subunits, is decreased in these cells (Werner *et al.*, 1974). Mitochondrial protein synthesis in *Neurospora* is diminished under conditions of heme deficiency. Thus Kumar and Padmanaban (1980) have concluded that heme is required for optimal translation on mitoribosomes. When a heme-deficient yeast mutant (lacking δ-aminolevulinic acid synthetase) was grown in the absence of heme or heme precursors, no cytochrome oxidase activity could be detected. The mitochondria still contained the subunits II, III, and VI, and in low amounts the subunits I and IV. Subunits V and VII, however, were undetectable. The residual subunits were not assembled with each other. When the mutant was grown in presence of heme or heme precursors, it showed the phenotype of wild-type cells. Thus, heme appeared to be required for the accumulation and assembly of cytochrome oxidase subunits in yeast (Saltzgaber-Müller and Schatz, 1978). Wielburski and Nelson (1984) demonstrated that the addi-

tion of heme *a* to isolated rat liver mitochondria induced the assembly of subunit I with subunits II and III. Assembly was not observed when isolated mitochondria were incubated in the presence of heme *b* or in the absence of heme. Heme *a* appeared predominantly to affect the assembly of subunit I with subunit III.

The formation of cytochrome oxidase in yeast depends on oxygen. Pulse-labeling experiments have suggested that oxygen exhibits no direct effect on protein synthesis. After yeast cells had been grown anaerobically, the addition of oxygen caused subunits I and II to form a complex with subunits VI and VII. Possible roles for oxygen in the assembly process include the induction of heme *a* formation or the maintenance of adequate oxidation–reduction states of heme *a* or copper (Woodrow and Schatz, 1979).

Wielburski *et al.* (1982) studied the assembly of the mitochondrially synthesized subunits of rat liver cytochrome oxidase. In pulse–chase experiments, labeled subunit II first associated with the holoenzyme (within minutes), then subunit III appeared in cytochrome oxidase. Subunit I associated with the holoenzyme only after a delay of ~2 hr, although it was detected in mitochondria after 10 min. Since no evidence for different pool sizes of the subunits could be found, the authors concluded that the assembly of rat liver cytochrome oxidase is an ordered and sequential event.

Dowhan *et al.* (1985) constructed a yeast mutant which lacked subunit IV. The mutant contained the other subunits of cytochrome oxidase, but these were not assembled into a stable and active complex. Introducing a wild-type subunit IV gene restored activity to cytochrome oxidase. Therefore, subunit IV appears to be necessary for assembly of cytochrome oxidase.

C. Assembly of ATPase-F_1 Complex

Petite mutants of yeast which lack protein synthesis within mitochondria contain mitochondrial ATPase activity. The ATPase activity is insensitive to oligomycin (which acts on the F_0 component) (Schatz, 1968). The enzyme complex was precipitated with antibodies directed against the $F_1\beta$ subunit. The cytoplasmically synthesized subunits of the F_1 part were found in the complex. Furthermore, the ATPase activity of the *petite* mitochondria was associated with mitochondrial membranes in a manner similar to that of respiratory-competent strains (Marzuki and Linnane, 1985). Similarly, inhibition of mitochondrial protein synthesis *in vivo* by thiamphenicol leads to the formation of an oligomycin-insensitive mem-

brane-bound ATPase in mitochondria of rat liver (De Jong *et al.*, 1979). This suggests that the assembly of a functional F_1 complex can occur independently of mitochondrial protein synthesis.

Lewin and Norman (1983) studied the import of *in vitro*-synthesized subunits of ATPase-F_1 complex into isolated yeast mitochondria. At least the three largest subunits assembled with each other. Incorporation of labeled subunits into the enzyme complex could be reduced by preincubation of mitochondria with unlabeled precursor proteins. They concluded that the assembly of F_1-ATPase occurred from a pool of subunits in mitochondria.

D. Assembly of ATPase-F_0 Complex

The assembly of the mitochondrially synthesized subunits of the F_0F_1-ATPase were studied with different *mit*$^-$ mutants of yeast which lack one mitochondrially synthesized subunit (i.e., subunit 6, 8, or 9, respectively). The ATPase complex of these mutants was relatively unstable but could be precipitated by an antibody directed against $F_1\beta$. In the mutant devoid of subunit 9, subunits 6 and 8 were not coprecipitated by this antibody. Subunit 9 appears to be the first mitochondrially synthesized subunit which is assembled into the ATPase. Subunit 8 seems to be assembled next since the ATPase in mutants lacking this subunit contained subunit 9 but not subunit 6. Subunit 6 is probably assembled in the final step (Marzuki and Linnane, 1985).

E. Concluding Remarks

Many questions about the assembly of mitochondrial protein complexes cannot as yet be answered. A number of aspects have only been studied with one complex or one species. Many results are contradictory and not without problems. For instance, the use of mutants raises the problem as to whether the residual assembly process reflects parts of the physiological assembly in wild-type cells or not.

Thus, the assembly of mitochondrial protein complexes remains unclear in many regards.

VI. Biogenesis Pathways

In earlier sections (II–V) we discussed general features of the biogenesis of mitochondrial energy-transducing complexes. Here we put together

findings and hypotheses on biogenesis pathways of specific relevant proteins. Although our knowledge about the pathways is limited, it seems clear that a number of different broad classes of assembly pathways exist. Within such groups individual proteins may represent variations of a common theme, i.e., they may use essentially similar mechanisms.

A. Subunit V of the bc_1 Complex

Subunit V (Fe-S protein) of the bc_1 complex is synthesized in the cytoplasm with a presequence (32 amino acid residues in *Neurospora*) (Harnisch *et al.*, 1985). In a receptor- and energy-dependent manner, it is completely transported into the matrix via translocation contact sites (Fig. 5). The processing peptidase cleaves after the first 24 amino acid residues of the presequence. The FeS cluster appears to be attached in the matrix space. Subunit V is then transported back to the outer face of the inner membrane (Hartl *et al.*, 1986; Hartl and Neupert, in preparation). It is not known so far whether the second proteolytic processing occurs before, during or after the retranslocation.

Cytochrome b_2, cytochrome-*c* peroxidase (two intermembrane space proteins), and perhaps also cytochrome c_1 may be imported and sorted by a similar mechanism. Hence, these proteins may be grouped together in

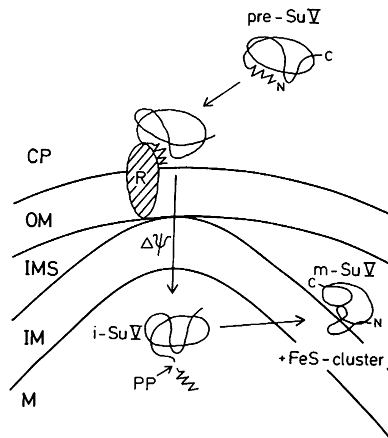

Fig. 5. Hypothetical import pathway for subunit V (Fe-S protein) of the bc_1 complex. Pre-Su V, i-Su V, and m-Su V are precursor-, intermediate-, and mature-sized subunit V, respectively; N, amino terminus; C, carboxy terminus; R, receptor; $\Delta\psi$, membrane potential; PP, processing peptidase. Other abbreviations are as in Fig. 1.

one "biogenesis pathway" in which they differ only in whether they are anchored to the inner membrane during the later stages of import.

B. Cytochrome c_1

Cytochrome c_1 is synthesized on free cytoplasmic polysomes as a larger precursor with an amino-terminal peptide extension (61 amino acid residues in yeast) (Sadler *et al.*, 1984). After transport through the cytosol, it probably binds to a specific receptor on the mitochondrial surface. The amino-terminal part of the protein appears to be transported into the matrix via translocation contact sites between outer and inner membranes. This step requires a membrane potential (inside negative) across the inner membrane. The processing peptidase removes part of the presequence (M_r ~3000), creating an intermediate form. The remaining portion of the presequence in the inner membrane could serve as a stop-transfer sequence [as suggested by Sadler *et al.* (1984), Hurt and van Loon (1986), and van Loon *et al.* (1986)]. It is cleaved off by a second protease which appears to be localized on the outside of the inner membrane. The second maturation step requires the presence of heme which is covalently attached to cytochrome c_1 (Gasser *et al.*, 1982b). The mature cytochrome c_1 is likely anchored in the inner membrane via a hydrophobic carboxy-terminal part (see Fig. 3).

An alternative possibility is that, as discussed above, the precursor protein of cytochrome c_1 is completely transported into the matrix. After cleavage to the intermediate form it would then be translocated back to the inner membrane (see Section II,E). Interestingly, cytochrome f of chloroplast b_6f complex, which is homologous to cytochrome c_1, is synthesized within the chloroplast as a larger precursor containing an amino-terminal sequence similar to the second portion of the presequence of cytochrome c_1 (Alt and Herrmann, 1984).

C. Cytochrome c

Apocytochrome c, the precursor of cytochrome c, is synthesized on free cytoplasmic polysomes without a presequence. It is transported through the cytosol in monomeric or dimeric forms, in contrast to most other precursor proteins which are transported in the form of high-molecular-weight aggregates. Apocytochrome c presumably spontaneously inserts into the outer membrane and then binds to its receptor, which may be a protein on the inside of the outer membrane (see Section II,D,1 and

Fig. 1). Heme is covalently attached (enzymatically); cytochrome *c* completely crosses the outer membrane and then moves to its final location on the outer face of the inner membrane. Apocytochrome *c* from a *Neurospora* mutant in which only the carboxyterminus is altered is imported into mitochondria at a very low efficiency (Stuart, *et al.*, 1987).

Import of cytochrome *c* appears to be relatively simple when compared to the import of most other proteins. Import of other proteins of the intermembrane space, such as cytochrome b_2 or cytochrome-*c* peroxidase, seems to be much more complicated: they probably bind to specific receptors on the mitochondrial surface. Then at least their amino-terminal portion is transported into the mitochondrial matrix in an energy-dependent manner, presumably via translocation contact sites. The first part of the presequence is cleaved off by the matrix-processing peptidase, the second part probably by a proteolytic activity on the outer face of the inner membrane (see Section II,G). In contrast, apocytochrome *c* only crosses the outer membrane and requires no $\Delta\psi$ for import. If one assumes that apocytochrome *c* has the ability to insert spontaneously into the outer membrane and that a protein in the intermembrane space has acquired the ability to bind apocytochrome *c*, then cytochrome *c* could have developed a "new" pathway which circumvents the complicated pathway typical for other heme proteins in the intermembrane space, such as cytochrome b_2 and cytochrome-*c* peroxidase.

Interestingly, it has been shown for protein transport in bacteria *(E. coli)* that certain proteins, such as M13 procoat, can insert into a lipid bilayer in the absence of proteinaceous components. Only phospholipids and the leader peptidase (which cleaves an amino-terminal peptide extension) appear to be required for assembly of procoat into the *E. coli* plasma membrane. Similar to the situation in mitochondria, the transport of several other proteins in *E. coli* is dependent on other specific components (such as the *sec* genes products) (Watts *et al.*, 1981; Michaelis and Beckwith, 1982; Beckwith and Silhavy, 1983; Ohno-Iwashita and Wickner, 1983; Ohno-Iwashita *et al.*, 1984; Ferro-Novick, 1985; Wolfe et al., 1985).

D. Subunit IV of Cytochrome Oxidase

Cytochrome oxidase subunit IV is cytoplasmically synthesized with an amino-terminal presequence (25 amino acid residues in yeast). The first 12 amino acid residues seem to contain sufficient information for its targeting to mitochondria and into the matrix (see Section II,B). Import requires an energized inner membrane. The presequence is cleaved off by the matrix-

processing peptidase. Subunit IV appears to be required for the assembly of a functional cytochrome oxidase (see Section V,B).

E. Subunit 2 of the F_0F_1-ATPase ($F_1\beta$)

The precursor of $F_1\beta$ is cytoplasmically synthesized with a presequence of M_r ~2,000. After transport through the cytosol it probably binds to a specific receptor on the mitochondrial surface. It appears to be transferred into the matrix through contact sites between outer and inner membranes. Thereby it enters the matrix space with the amino-terminal end first (see Section II,E and Fig. 2). Only the import of the amino-terminal portion needs a membrane potential. A major part of the protein can be translocated in the absence of $\Delta\psi$. After proteolytic processing, $F_1\beta$ is assembled into the F_1 complex of ATPase. In yeast, and maybe also in other organisms, the import of $F_1\beta$ requires a cytosolic protein with a molecular weight of ~40,000 ("cytosolic cofactor") (see Section II,C).

F. Subunit 9 of the F_0F_1-ATPase ("Proteolipid")

The proteolipid of yeast and plants is synthesized, without a presequence, inside mitochondria. In *N. crassa* and higher eukaryotes the proteolipid is synthesized in the cytoplasm with a presequence (66 amino acid residues in *Neurospora*). Subunit 9 is transported in the form of high-molecular-weight aggregates in the cytosol. It binds to a specific receptor on the mitochondrial surface. Translocation across the membranes is dependent on $\Delta\psi$. The presequence is removed in two steps (35 and 31 amino acid residues) by the matrix-located processing peptidase (Schmidt *et al.*, 1984). After import *in vitro*, the proteolipid can be precipitated by antibodies directed against the F_1 complex (Schmidt *et al.*, 1983b). This suggests that subunit 9 imported *in vitro* is assembled into the ATPase and thus is on the correct import pathway. Import of the proteolipid also seems to require a cytosolic cofactor.

Two different precursor proteins have been found for bovine proteolipid. The presequences are different in their primary structure, and are 61 and 68 amino acids long, whereas the amino acid sequences of the mature proteins are identical. The corresponding nuclear genes are expressed differently in various tissues (Gay and Walker, 1985). Translocation into mitochondria has not been studied with these precursor proteins.

G. ADP/ATP Carrier

The precursor of ADP/ATP carrier is synthesized on free cytoplasmic polysomes without a cleavable peptide extension. After transport through the cytosol in the form of high-molecular-weight aggregates (Zimmermann and Neupert, 1980), it binds specifically to a receptor protein on the mitochondrial surface, which could be related to, or identical to, the receptor for porin (an outer membrane protein) (Pfaller and Neupert, 1987). Then the precursor is inserted into the outer membrane. The amino-terminal 115-amino acid residues seem to contain sufficient information for the targeting to mitochondria (see Section II,B). Translocation into the inner membrane, which requires the membrane potential across the inner membrane, is mediated by translocation contact sites (Pfanner and Neupert, 1987). The functional form of the carrier in the inner membrane is a dimer of two identical subunits (Klingenberg *et al.*, 1978). The carrier imported *in vitro* acquires properties of the protein imported *in vivo*, such as binding of the specific inhibitor carboxyatractyloside.

Import of ADP/ATP carrier appears to require a cytosolic cofactor. The cofactor may be necessary for import-competent binding to the receptor sites.

VII. Perspectives

In face of the enormous complexity of mitochondrial biogenesis pathways, a large number of mechanistic details have to be elucidated.

1. What molecular signals are present on precursor proteins? A large body of evidence has accumulated that amino-terminal presequences contain sufficient information for the targeting of proteins to mitochondria. A major aim of future investigations is to understand the signaling functions of presequences and to characterize their common denominator. Presequences do not show striking homology to each other except the high content of basic amino acid residues and often serines and threonines. There could be important similarities, for instance, in their secondary structure. Are presequences also responsible for the sorting of imported mitochondrial proteins to their correct intramitochondrial location? What are the roles of sequences within the mature proteins for targeting, sorting, and efficiency of transport processes? Which signals are present in precursors without cleavable presequences?
2. What are the complementary structures in mitochondria for recognition and translocation of proteins? Proteinaceous receptors have been shown to recognize specifically mitochondrial precursor proteins. Studies

are underway to define the number of different receptors and to characterize them. What happens following binding of signals to receptors? So far it remains unclear how proteins with large hydrophilic domains are transported through the hydrophobic environment of a lipid bilayer. Presequences, for example, could influence the arrangement of mitochondrial lipids, leading to a destabilization of the lipid bilayer, and thereby facilitate the passage of a macromolecule. On the other hand, protein translocation could be mediated by a hydrophilic protein channel (Pfanner, Hartl, Guiard, and Neupert, submitted). (Un)folding of precursors seems to be an important prerequisite for their translocation across the mitochondrial membranes (Eilers and Schatz, 1986; Pfanner, *et al.*, 1987). How are translocation contact sites between outer and inner membranes structured, and how are they formed? What are the exact functions of cytosolic cofactors, nucleoside triphosphates, and the membrane potential for protein import? Further translocation intermediates have to be characterized to dissect the transport pathway into more resolved individual steps and to detect new components of the import machinery. The use of mutants of microorganisms can also be an important tool for the identification and characterization of components involved in transport processes.

3. Evidence is increasing that several precursor proteins are first transported completely into the matrix and are then sorted to their functional locations, an observation which agrees with the "endosymbiont theory" (see Section II,E). Considering models in such evolutionary terms may be helpful for understanding the complex process of mitochondrial biogenesis.

4. The biogenesis of mitochondrially synthesized proteins, the regulation between mitochondrial and cytoplasmic protein synthesis, and the assembly of subunits into protein complexes are undeveloped fields (discussed in Sections III–V) which will attract much interest in the near future.

5. An important objective in the study of protein import is the isolation and subsequent *in vitro* reconstitution of all components which are required for transport of proteins across membranes. This includes the precursor proteins, cytosolic cofactors, specific receptors, processing peptidases, other enzymes involved in the modification of precursor proteins, and certainly a number of other proteins (e.g., components of translocation contact sites) whose existence should become apparent during further analysis of transport pathways. The aim here is to understand all steps which take place during translocation of a protein through biological membranes at the molecular level.

As discussed above, the transport pathways of proteins across the membranes of mitochondria, chloroplasts, peroxisomes, glyoxysomes,

endoplasmic reticulum, and bacteria show similarities in various regards, but also several peculiarities (see also Wickner and Lodish, 1985). Thus, the mechanisms studied in the translocation of proteins across mitochondrial membranes are of general interest and relevance for the understanding of functions of biological membranes.

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