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Synthesis and Intracellular Transport of Mitochondrial Proteins

Matthew A. Harmey and Walter Neupert

I. INTRODUCTION

The biogenesis of the mitochondrion represents the result of a coordinated synergism between two distinct and spatially separate genetic systems. Not only are these two systems separate but they also have distinct modes of transcription and translation (Barrell *et al.*, 1979; Borst, 1981; Borst and Grivell, 1978, 1981). Genetic and inhibitor studies on the development of mitochondria established that the majority of mitochondrial proteins are coded for by the nuclear DNA and are the products of translation on the cytoplasmic ribosomes (Lamb *et al.*, 1968; Schatz and Mason, 1974; Neupert and Schatz, 1981; Heinrich, 1982). As the protein constituents of mitochondria are distinct from the bulk of cellular proteins, some mechanism must exist for the sorting of proteins destined for the mitochondria.

Transport of proteins into mitochondria and assembly of mitochondrial membranes is one example of the general phenomenon of intracellular protein sorting and protein insertion into and translocation across cellular membranes. Similar reactions must occur for the assembly of other cellular membranes and compartments such as the plasma membrane and the endomembrane system including the endoplasmic reticulum, Golgi apparatus, transport vesicles, and endocytic vesicles, for peroxisomes and glyoxysomes, and analogous to mitochondria, for chloroplasts. Furthermore, the transport of proteins out of the cell, i.e., secretion of proteins, requires basically the same reactions.

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The import of protein into organelles and organelle membranes is directly related to the problem of how membranes maintain their identity and their continuity in space and time. Since almost all proteins of the cell are made on cytoplasmic ribosomes but end up in a number of different compartments, specific mechanisms must exist to direct them into these compartments. The identity of a compartment is determined by the membrane surrounding this compartment. Thus, membranes must have devices to recognize not only newly made components for themselves but also for the compartment enclosed. Formation of cellular compartments or organelles, therefore, must entail as a first step recognition of new components or precursor proteins. This process must be highly specific since the compartment is believed to be absolutely unique. This recognition is, thus, the primary step in the formation of cellular membranes. Identity and continuity of membranes is determined by recognizing structures on their membranes. This explains at the same time why a membrane may undergo modulation but cannot arise *de novo,* since a membrane requires for its formation the continued presence of recognizing structures or "receptors" to maintain identity. Therefore, in a way, membranes are self-replicating structures. As in the replication of DNA, their formation entails an initial recognition. In the case of DNA replication, this is the base pairing step; in membrane replication, it is the binding of a newly made protein precursor to its receptor. The second step then is fixation; in the case of DNA replication, this is the formation of the diester bond between two nucleotides; in the case of membrane replication, this is the insertion into and or translocation across the membrane.

The details of these two basic steps are far from being understood. The second step, especially, appears to be particularly complex. We do not know how a polypeptide enters into and leaves a membrane. In the case of mitochondria, some proteins must traverse one membrane (the outer membrane) to reach another membrane (the inner membrane). Mitochondria have four clearly recognizable compartments (Ernster and Kuylenstierna, 1970) each with a distinctive protein complement related to the particular activities of each compartment, viz., outer membrane, intermembrane space, inner membrane, and matrix. Moreover, it is clear that the mechanisms involved in recognition of and transfer across membranes are not common to all the different sorting reactions. Two general mechanisms have been postulated, viz., cotranslational and posttranslational. In the case of secretory proteins which must pass into the lumen of the endoplasmic reticulum prior to secretion, a cotranslational mechanism has been shown to operate in all cases studied so far. The initiation of protein synthesis takes place on free polysomes. The emerging signal peptide of the nascent polypeptide chain is recognized by a signal recognition particle (SRP; Walter and Blobel, 1982) which arrests elongation until the complex of polysomes and SRP is bound to the microsomal membrane via the docking protein (Meyer and Dobberstein, 1980; Meyer *et al.*, 1982; Walter and Blobel, 1982). The complex may be further stabilized by interactions with the ribophorins (Kreibich *et al.*, 1978, Kreibich, 1982). The elongation recommences with the polypeptide being inserted into and across the membrane. A signal or leader peptidase on the luminal face of the ER cleaves the signal peptide to yield the mature polypeptide. This may already occur before the transmembrane journey is complete (Blobel *et al.*, 1979, Kreil, 1981). Proteins which do not contain a cleaved signal sequence have also been found. One example of a secreted protein is that of ovalbumin.

A number of proteins ending up in the plasma membrane which follow a very similar pathway are not initially synthesized with a cleavable sequence. One example is ovalbumin. A number of proteins ending up in the plasma membrane which follow a very similar pathway are not initially synthesized with a cleavable sequence (Kreil, 1981). This mechanism appears to obtain in the transfer of proteins into mitochondria, which will be discussed in detail. The weight of evidence indicates that proteins of these organelles are synthesized as precursors on free polysomes and are run off into the cytosol where they can be detected (Hallermayer *et al.*, 1977; Harmey *et al.*, 1977) and are subsequently imported into the mitochondria. It also appears that a posttranslational mechanism works in the transport of proteins into chloroplasts, peroxisomes, and glyoxysomes and of some proteins into the endoplasmic reticulum.

Furthermore, insertion and translocation of at least a few proteins into or across the plasma membrane of bacteria has been found to occur by a posttranslational mechanism. In particular, the insertion of the major coat protein of the bacteriophage M13 has been studied in great detail (Wickner, 1980). It has been proposed that, in this case, no specific recognizing structure in the recipient membrane is required. It remains to be shown whether this mechanism is of a general importance or restricted to this phage protein. It seems, however, already clear from genetic studies that secretion of periplasmic proteins in bacteria requires protein components associated with the membranes (Inouye and Beckwith, 1977; Emr and Silhavy, 1982). It has become apparent in recent years that there is not a clear demarcation between what is regarded as cotranslational and posttranslational transport. These two types may represent extremes of a system with graded intermediate conditions. For instance, it is not clear whether secretory proteins are translocated across the membranes in a linear fashion as the chain elongates or whether stretches of the nascent chain fold on the ribosomal side of the membrane and these folded "domains" are translocated across in a discontinuous fashion (Randall, 1983).

The transfer of proteins from cytosol to mitochondria is generally regarded as a one-way process, so that the ingress pathway is not available for exit. The mitochondria appear to be impermeable to added mature mitochondrial proteins (Neupert and Schatz, 1981). If the internalized proteins behave in a similar manner, then the cleavage and ensuing conformational changes provide a mechanism for the containment of mature mitochondrial proteins and provide a logic for proteolytic cleavage. There have been a number of reports that mitochondria take up mature aspartate amino transferase (Marra *et al.*, 1977) and more recently, the same group reported a similar uptake of malate dehydrogenase (Passarella *et al.*, 1980). This process was considered as a model system for protein translocation. The described phenomenon cannot be clearly identified with precursor uptake; this is apparent from large number of differences between the characteristics of precursor uptake *in vivo* and the described systems (Sonderegger *et al.*, 1980; Sakakibara *et al.*, 1980; Aziz *et al.*, 1981).

In approaching the problem of how mitochondrial proteins are transported into mitochondria, a number of questions may be posed, some of which can be answered while others can only be partly answered.

- How do precursor proteins travel through the cytosol
- How are precursor proteins recognized by mitochondria?
- How are proteins translocated across the mitochondrial membranes?

- How do proteins reach their specific compartment and how are they integrated into their functional locations?
- How are protein subunits assembled into multimeric complexes?

We shall attempt to review the available answers to these questions.

II. SYNTHESIS OF NUCLEAR CODED PROTEINS

Of the total mitochondrial proteins, almost all are synthesized on cytoribosomes. This can be clearly demonstrated by the effect of cycloheximide on the incorporation of labeled amino acids into mitochondrial proteins. As this is a most effective inhibitor of cytoplasmic protein synthesis, it causes a striking decrease in the synthesis of mitochondrial proteins. It does not, however, prevent the import of proteins *per se* into the mitochondria and this is one of the observations that led to the proposal that import of mitochondrial proteins was a posttranslational phenomenon (Hallermayer and Neupert, 1977).

The biosynthesis of mitochondrial proteins *in vivo* has been followed principally by kinetic studies involving dual labeling and pulse- and chase-labeling studies carried out at low temperture (5–8°C). These studies have been particularly useful in following the synthesis of mitochondrial proteins in organisms such as *Neurospora* and yeast, but have also been applied to vertebrate cells. When coupled with the use of specific antibodies, kinetic studies have allowed investigators to locate and follow the movement of individual proteins from their sites of synthesis to their final location. Hallermayer and Neupert (1977) using *Neurospora* cells, and Schatz (1979) using yeast cells, demonstrated that newly synthesized mitochondrial proteins could first be detected in the cytosol of the cell and were subsequently imported into the mitochondria. A distinct time lag could be demonstrated between the two phenomena. Newly synthesized material could be recognized in the case of dual label studies such as those shown in Figure 1. Different lag times were found with different proteins suggesting that the various extramitochondrial precursor proteins have different extramitochondrial concentrations, or pool sizes. The transfer from the cytosol could be readily observed either by the application of a chase of cold amino acid or after additon of cycloheximide to stop translation.

The pool of precursor proteins, at least of those investigated, in the cytosol is very small; in fact, at temperatures of 20–30°C in both yeast and *Neurospora* cells, the size of the pool and the dwell time of the precursors is so small as to make it difficult to detect the precursors (Morita *et al.*, 1982; Schatz and Butow, 1983; Hallermayer *et al.*, 1977; Schmidt *et al.*, 1983a,b). However, as will be discussed later, incorporation at low temperatures or in the presence of uncouplers has made the detection of many precursors possible.

In vitro synthesis of mitochondrial proteins has been shown by translation of extracted mRNA in either the rabbit reticulocyte lysate (Hunt and Jackson, 1974) or the wheat germ system (Roberts and Paterson, 1973). These translation systems have been used extensively in the synthesis of mitochondrial proteins as attested to in the data presented in Table 1. The list given is one to which new proteins are continually being added as the proteins and their precursor forms are identified.

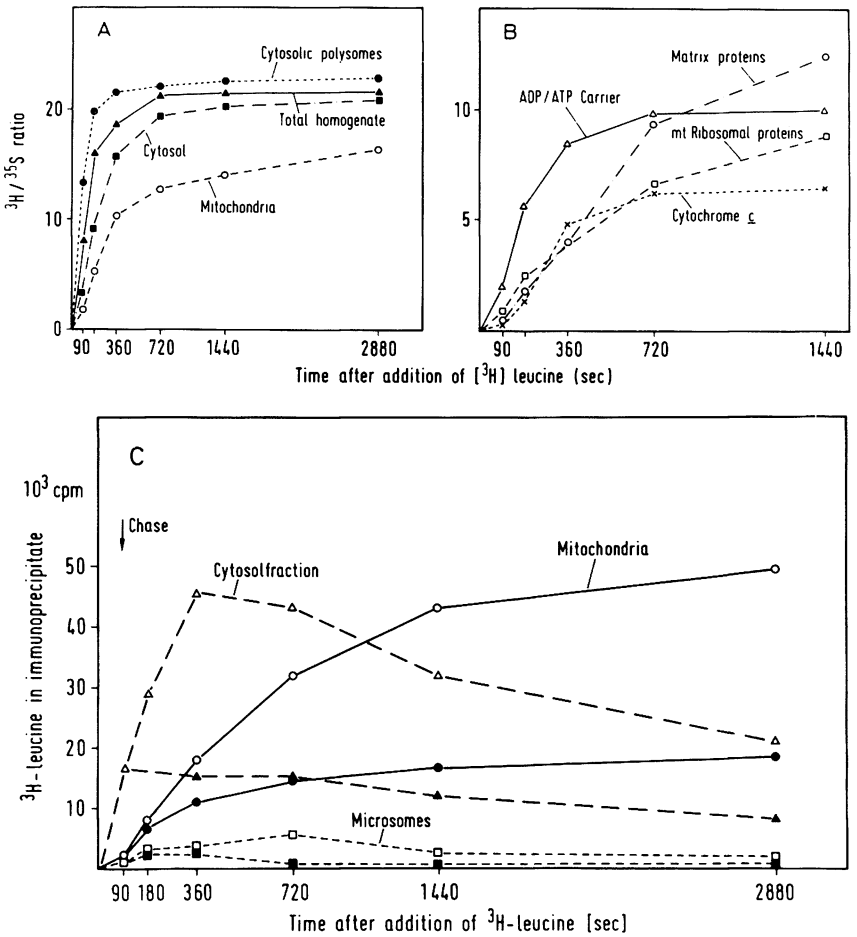

Figure 1. Pulse-chase kinetics of incorporation of leucine into subcellular fractions and into selected mitochondrial proteins. *Neurospora* cells prelabeled with 35 S sulphate were pulse labeled with 3H leucine at 8°C and the cells fractionated. The distribution of radioactivities in the different cell fractions was determined at intervals following the addition of the leucine (A). Radioactivities in a number of proteins immunoprecipitated from mitochondria were also determined (B). The distribution of matrix proteins in the different cell fractions was also determined by immunoprecipitation and the radioactivities are plotted (C).

A large number of mitochondrial proteins have been shown to be translated on free but not membrane-bound polyribosomes (Zimmermann and Neupert, 1980; Suissa and Schatz, 1982; Neupert and Schatz, 1981). This was done either by read-out systems using isolated ribosomes or by translation of message extracted from free and bound polysomes in heterologous cell-free systems (Zimmermann and Neupert, 1980; Suissa and Schatz, 1982; Freitag *et al.*, 1982). This is consistent with the above described passage of precursor proteins through the cytosol. It should, however, be mentioned that particularly with yeast cells more complicated situations have been described (Kellems and Butow, 1974; Ades and Butow, 1980). Suissa and Schatz (1982) studied

Table 1. Cytoplasmic Precursors of Mitochondrial Proteins

Mitochondrial compartment	Protein	Tissue	Apparent molecular weight in K: precursor	Apparent molecular weight in K: mature protein	Post-translational import demonstrated	Energy or membrane potential dependence	References
Outer membrane	Porin	*Neurospora crassa*	30	30	+	None	Freitag *et al.* (1982)
	Porin	Yeast	29	29	+	None	Mihara *et al.* (1982)
	35K protein	Rat liver	35	35	+	None	Shore *et al.* (1981)
	Monoamine oxidase	Rat liver	59	59	ND[a]	ND	Sagara and Ito (1982)
Intermembrane space	Cytochrome *c* peroxidase	Yeast	39.5	33.5	+	+	Maccechini *et al.* (1979a,b)
	Cytochrome b_2	Yeast	67	57	+	+	Gasser *et al.* (1982a,b)
	Adenylate kinase	Chick liver	28	28	ND	None	Watanabe and Kubo (1982)
	Sulfite oxidase	Rat liver	59	55	ND	ND	Mihara *et al.* (1982)
Inner membrane	Cytochrome *c*	*Neurospora crassa*	12	12	+	None	Korb and Neupert (1978)
		Rat liver	12	12	+	None	Matsuura *et al.* (1981)
	ADP/ATP carrier	*Neurospora crassa*	32	32	+	+	Zimmermann *et al.* (1979a,b)
	F_0F_1 ATPase subunit 9	*Neurospora crassa*	12	8	+	+	Michel *et al.* (1979)
	bc_1 complex subunit I	*Neurospora crassa*	51	50	+	+	Teintze *et al.* (1982)
	Cytochrome c_1	Yeast	44.5	44	+	+	Cote *et al.* (1979)
	Subunit II	*Neurospora crassa*	47.5	45	+	+	Teintze *et al.* (1982)
		Yeast	40.5	40	+	+	Cote *et al.* (1979)

	Cytochrome c_1	*Neurospora crassa*	38	31	+	+	Teintze *et al.* (1982)
		Yeast	37	31	+	+	Nelson and Schatz (1979)
	Subunit V	*Neurospora crassa*	28	25	+	+	Teintze *et al.* (1982)
		Yeast	27	25	+	+	Cote *et al.* (1979)
	Subunit VI	*Neurospora crassa*	14	14	ND	ND	Teintze *et al.* (1982)
		Yeast	25	17	+	ND	Cote *et al.* (1979)
	Subunit VII	*Neurospora crassa*	12	11.5	+	+	Teintze *et al.* (1982)
		Yeast	14	14	ND	ND	Cote *et al.* (1979)
	Subunit VIII	*Neurospora crassa*	11.6	11.2	ND	ND	Teintze *et al.* (1982)
		Yeast	11	11	ND	ND	Cote *et al.* (1979)
	Cytochrome oxidase						
	Subunit IV	Rat liver	19.5	16.5	ND	ND	Heinrich (1982)
		Yeast	17	14	ND	ND	Mihara and Blobel (1980)
	Subunit V	Rat liver	15.5	12.5	ND	ND	Heinrich (1982)
		Yeast	15	12.5	ND	ND	Mihara and Blobel (1980)
	Subunit VI	Yeast	14	12.5	ND	ND	Mihara and Blobel (1980)
	Subunit VII	Yeast	5	5	ND	ND	Mihara and Blobel (1980)
Matrix	F_1 ATPase						
	Subunit α	Yeast	64	58	+	+	Maccechini *et al.* (1979)
	Subunit β	Yeast	56	54	+	+	Nelson and Schatz (1979)
	Subunit γ	Yeast	40	34	+	+	Suissa and Schatz (1982)
	Subunit β	*Neurospora crassa*			+	+	Zwizinski *et al.* (1983)
	F_1 Inhibitor	Yeast	12	10	+	+	Yoshida *et al.* (1983)
	Citrate synthase	*Neurospora crassa*	47	45	ND	ND	Harmey and Neupert (1979)

(Continued)

Table 1. (Continued)

Mitochondrial compartment	Protein	Tissue	Apparent molecular weight in K: precursor	Apparent molecular weight in K: mature protein	Post-translational import demonstrated	Energy or membrane potential dependence	References
	Malate dehydrogenase	Rat liver	38	36	ND	ND	Mihara *et al.* (1982) Aziz *et al.* (1981)
	Isopropyl malate synthase	Yeast	70	70	+	+	Suissa and Schatz (1982)
		Yeast	65	65	+	+	Hampsey *et al.* (1983)
	δ-Amino levulinate synthase	Rat liver	75	66	ND	ND	Yamamoto *et al.* (1982)
			75	65	ND	ND	Ades and Harpe (1981)
		Chick liver	76	68	ND	ND	Srivastava *et al.* (1982)
	Glutamate dehydrogenase	Rat liver	60	54	ND	ND	Mihara *et al.* (1982)
	Superoxide dismutase	Yeast	26	24	+	+	Autor (1982)
	Ornithine carbamyl transferase	Rat liver	39.4	36	+	+	Mori *et al.* (1980)
			43	39	+	+	Conboy and Rosenberg (1981) Morita *et al.* (1982)
	Carbamyl phosphate synthase	Rat liver	165	160	+	+	Mori *et al.* (1979) Raymond and Shore (1979) Shore *et al.* (1979)
	Serine pyruvate amino transferase	Rat liver	42	40	ND	ND	Oda *et al.* (1981)
	RNA polymerase	Rat liver	47	45	ND	ND	Lustig *et al.* (1982a,b)
	Aspartate amino transferase	Chick heart	47.5	44.5	ND	ND	Sonderegger *et al.* (1980)
		Rat liver	47	45	+	ND	Sakakibara *et al.* (1980)
	Ornithine amino transferase		49	46	+	ND	Mueckler *et al.* (1982)
	Adrenodoxin	Bovine adrenal cortex	20	12	+	+	Nabi and Omura (1980)

[a] ND = not determined.

the synthesis of some 12 mitochondrial proteins with respect to the location of the messenger RNA for these proteins. They showed that polysomes bound to mitochondria contain messenger RNA for mitochondrial proteins, which is in accord with the findings of Kellems and Butow (1974). However, the bulk of the translatable RNA for these proteins was found to be associated with free cytosolic ribosomes. The occurrence of the mitochondrial bound polyribosomes has been explained by the fact that, in the presence of cycloheximide, the cytosolic precursor pools are so reduced that the mitochondrial capacity for import is far from saturated, a situation which could lead to incomplete nascent chains still attached to polysomes being bound to the mitochondria (Schatz and Butow, 1983).

The majority of the proteins listed in Table 1 have been shown to be synthesized in a form that is different to the mature mitochondrial protein (Figure 2). The commonest feature is that the initial translation product is larger by anything up to 10K daltons than the mature protein. These additional pieces are all amino terminal additions as far as have been determined (Viebrock *et al.*, 1982; Kaput *et al.*, 1982); none have yet been discovered on the carboxy terminus. A number of exceptions to this behavior have been found. Cytochrome *c* is synthesized as apocytochrome *c* which essentially is not any different to the holoprotein in terms of amino acid composition (Korb and Neupert, 1978). A further exception is the ADP/ATP carrier protein. The mature protein is an integral membrane protein of the inner membrane and the precursor is made on cytoribosomes in a form that has the same apparent molecular weight as the mature protein (Zimmermann *et al.*, 1979a,b). Both of these proteins were shown not to have an amino terminal extension by synthesis in a heterologous system in the presence of ^{35}S labeled formylmethionyl tRNA (Figure 3). If the protein retains a ^{35}S labeled amino terminus on being imported into the mitochondria then the precursor retained its initiating methionine, and can be adjudged to have no cleavable amino terminal extension piece. This has been shown to be the case for cytochrome *c* and

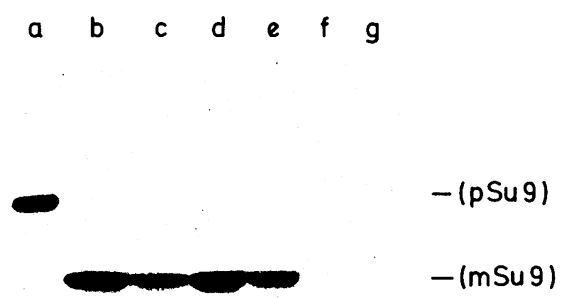

Figure 2. Transfer of the *Neurospora* precursor to subunit 9 of the F_1 ATPase into *Neurospora* mitochondria (a–c) and into yeast mitochondria (d–g). Precursor was synthesized by translation of polyA RNA in a rabbit reticulocyte lysate. Following synthesis, a postribosomal supernatant was prepared and isolated mitochondria incubated in this supernatant at 25°C for 30 min after which subunit 9 was immunoprecipitated from the mitochondria. Immunoprecipitates were analyzed by SDS–gel electrophoresis and autoradiography. (a) Precursor from reticulocyte lysate supernatent. (b) Transfer of precursor into *Neurospora* mitochondria. (c) Mature subunit 9 immunoprecipitated from 35 S labeled mitochondria. Transfer into yeast mitochondria. (d) Control transfer. (e) After transfer, mitochondria were suspended in isotonic medium and treated with proteinase K for 60 min at 0°C. (f) Transfer in the presence of 2 μM valinomycin. (g) Transfer in the presence of 10 μM oligomycin, 4 μM antimycin A, and 1 mM KCN. pSu9, precursor protein; mSu9, mature protein.

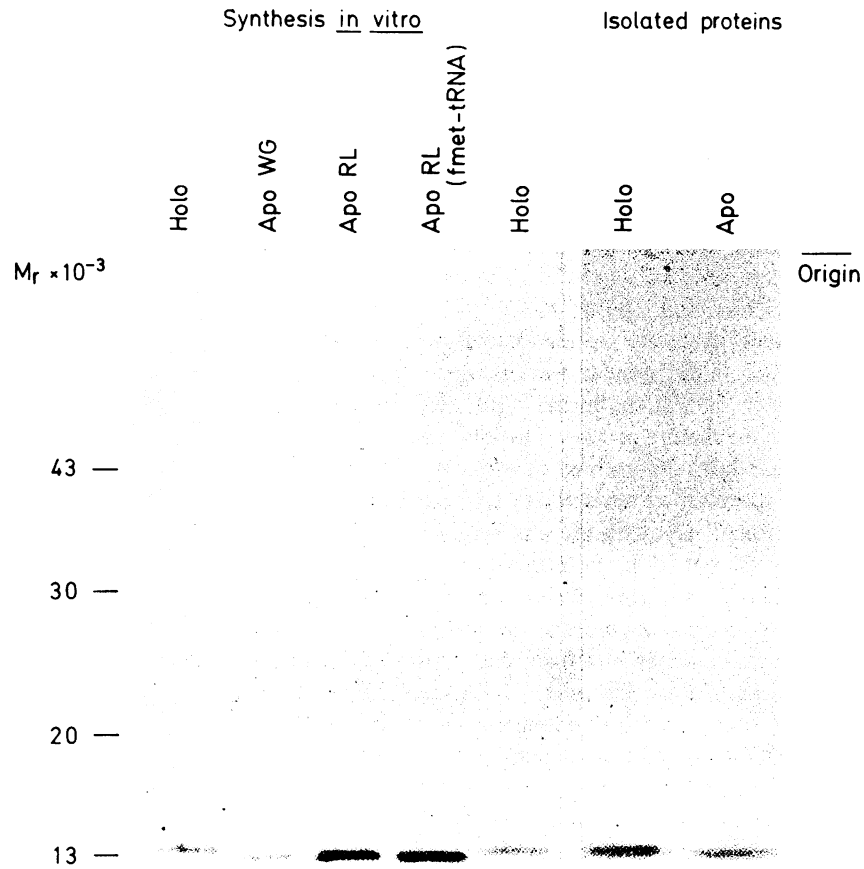

Figure 3. *In vitro* synthesis of apocytochrome *c*. Apocytochrome *c* was immunoprecipitated from rabbit reticulocyte (RL) and wheat germ (WG) translation systems programmed with *Neurospora* polyA RNA in the presence of ^{35}S methionine or ^{35}S formylmethionyl-tRNA. The immunoprecipitates were subjected to SDS–gel electrophoresis and the dried gels autoradiographed. Holocytochrome *c* was immunoprecipitated from labeled mitochondria. Coomassie stained isolated proteins are included.

ADP/ATP carrier (Zimmermann *et al.*, 1979a,b). This does not rule out the possibility of an unstable carboxy terminal extension, but there is no evidence for such an extension nor any precedent on any of the proteins studied to date. The outer membrane protein porin from *Neurospora* (Freitag *et al.*, 1982) and from yeast (Mihara *et al.*, 1982) has been found on translation to give a product which has the same molecular weight as the mature protein. In addition, Schatz has used labeling with ^{35}S-fmet-tRNA to show that this protein in yeast does not have an additional amino terminal sequence (Gasser and Schatz, 1983). Adenylate kinase and monoamine oxidase from rat liver have been shown to behave in a like manner (Watanabe and Kubo, 1982; Sagara and Ito, 1982). The matrix protein isopropyl malate synthase has also been shown to be made without an amino terminal extension (Hampsey *et al.*, 1983). No correlation

between the presence and absence of an additional sequence and its final location can thus be made. Perhaps one exception is that, until now, no proteolytic processing has been found to be involved in the assembly of outer membrane proteins.

Structural characteristics of precursor proteins have proved quite difficult to determine, principally because it is quite difficult to procure sufficient quantities of most precursors to carry out such studies. Nonetheless, it has been possible in a few instances to obtain reasonable amounts of precursor. The apo form of cytochrome *c* prepared by the removal of the heme moiety is not distinguishable from the native precursor (Korb and Neupert, 1978). A water soluble porin has been prepared which will bind to mitochondria and insert into the outer membrane in a manner identical to the native precursor (Freitag and Neupert, unpublished). Transport studies which will be discussed below have also provided a means of accumulating precursor proteins in the cytosol in yeast cells (Reid and Schatz, 1982). Notwithstanding the problems of acquiring sufficient amounts of the precursors, a number of general features have been ascribed to these precursors. All appear to be quite soluble in a hydrophilic medium, i.e., the cytosol, although the mature proteins are in many cases extremely hydrophobic intrinsic membrane proteins (Klingenberg, 1976). All appear to have different conformations from their mature counterparts, e.g., the antibody to apocytochrome *c* does not react with holocytochrome *c* (Korb and Neupert, 1978). Kraus *et al.* (1981) have also described differences in reaction to antibodies in the case of ornithine transcarbamylase. The precursor to the ADP/ATP carrier does not bind carboxyatractyloside, a characteristic inhibitor of the functional carrier (Schleyer *et al.*, 1982). In those precursors having amino terminal extensions, one can predict an influence on the conformation depending on the size and nature of the extension piece. In the small number of extensions which have been sequenced so far, they appear to be basically hydrophilic and may assist in the maintenance of the precursors in a soluble form in their aqueous milieu (Viebrock *et al.*, 1982; Adolphus *et al.*, 1982; Kaput *et al.*, 1982). A further feature of many of the precursors is a marked tendency to form aggregates (Zimmermann and Neupert, 1980; Schmidt *et al.*, 1983a,b; Mori *et al.*, 1979; Lewin *et al.*, 1980). This may be a means of retaining solubility by the masking of hydrophobic regions of the proteins by hydrophobic interactions between precursor subunits.

One characteristic of the precursors is that they are selectively recognized by the mitochondria. Korb and Neupert (1978) demonstrated that, in the presence of a large excess of holocytochrome *c*, the apo form was selectively taken up by the mitochondria. The interpretation of this is that the configuration of the precursor is sufficiently different to allow the mitochondrial recognition system to differentiate between the precursor and mature forms and that, in effect, these two forms do not compete for uptake by the mitochondria. Differential stability of precursors to proteolytic enzymes has also been described (Harmey and Neupert, 1979; Jaussi *et al.*, 1981).

The genes coding for mitochondrial precursor proteins have recently been isolated in a number of laboratories. Viebrock *et al.* (1982) described the cloning and sequencing of the messenger RNA for subunit 9 of the ATPase ("proteolipid" or "DCCD-binding protein") and so the entire amino acid sequence could be deduced. The amino terminal extension of 66 amino acids has a high percentage of polar and basic amino acids while the mature protein of 81 amino acids is rich in hydrophobic components. Kaput

et al. (1982) have cloned and sequenced the gene for cytochrome *c* peroxidase and consequently have been able to propose a model for the shape and insertion of this protein in the mitochondrial membrane prior to proteolytic processing. The amino terminal extension of 68 amino acids contains a stretch of 23 nonpolar residues, including ten consecutive alanines, and is basic in character. These 23 amino acids have been suggested to span the inner membrane as an α-helix, with the more polar stretch of the first 18 amino acids protruding into the matrix. There appears to be no similarity between the presequence of the proteolipid and the cytochrome *c* peroxidase. It has not been possible to detect common sequences, nor any size uniformity between the presequences of the various precursor proteins. There is currently a burgeoning interest in the cloning approach to the study of precursor proteins as it allows an amino sequence determination readily and it is hoped that it may be exploited to provide increased amounts of precursors to allow the construction of simplified reconstitution systems to study precursor transport and also to allow conformational considerations with a view to understanding the molecular details of precursor movement into and across membranes.

III. TRANSPORT OF MITOCHONDRIAL PRECURSOR PROTEINS FROM CYTOSOL INTO MITOCHONDRIA

The transport process is really a composite of a number of integrated processes. In order to dissect the steps involved in transport *in vitro,* assays of import were devised (Harmey *et al.*, 1977; Maccechini *et al.*, 1979a; Zimmermann and Neupert, 1980). These assays were based on the provision of a pool of precursor proteins either by read-out or by translation of isolated mRNA. Incubation of mitochondria in postribosomal supernatants of such systems led to uptake of precursors into the mitochondria. While the precursors were external to the mitochondria, they were sensitive to added proteases; however, when internalized, the proteins became inaccessible to such proteases. Termination of the proteolysis allowed immunoprecipitation and localization of the imported proteins (Figure 2). These assays allowed a comparison of newly converted proteins with the external precursor forms (Neupert and Schatz, 1981).

That the proteins have reached their final destination and have been integrated into their functional location is difficult to demonstrate in an unequivocal manner and can only be deduced from alterations in the characteristics of the precursors en route. Apocytochrome *c* is antigenically different to its holo counterparts (Korb and Neupert, 1978). The transport of the apo molecule into the mitochondria results in the covalent attachment of heme and results in a change in conformation such that the antibody to the apo enzyme no longer recognizes the imported protein. The ADP/ATP carrier on transfer to the mitochondria acquires the ability to bind carboxyatractyloside, a specific inhibitor of the carrier, and to reorient its subtrate binding sites in the same manner as the functional carrier molecule (Zimmerman and Neupert, 1980; Schleyer and Neupert, unpublished). Furthermore, the mature protein binds both ATP and carboxyatracytloside, while the precursor does not (Zimmermann and Neupert, 1980). In the case of subunit 9 of the F_1 part of the F_0F_1 ATPase, the imported subunit can be

immunoprecipitated from the mitochondria subsequent to import, by antibody to the F_1 ATPase, while the precursor is not recognized by this antibody (Schmidt *et al.*, 1983a,b). Lewin and Norman (1983) have observed that subunits of F_1 ATPase imported into isolated yeast mitochondria become assembled into F complexes. Gasser *et al.* (1982a,b) and Daum *et al.*, (1982a) studied uptake of a number of proteins into yeast mitochondria and demonstrated by fractionation of the mitochondria that the imported proteins were now located in the same compartment as their mature counterparts. They also were able to show by nondestructive criteria that preproteins requiring proteolytic processing had reached the matrix. These cited instances suggest that the imported proteins reach and at least in some instances are integrated into their functional locations.

It was found that the import of most proteins into mitochondria was inhibited *in vivo* and *in vitro* by treatment with uncouplers of oxidative phosphorylation (Hallermayer and Neupert, 1976; Harmey *et al.*, 1977; Nelson and Schatz, 1979; Zimmermann *et al.*, 1981; Reid and Schatz, 1982; Schleyer *et al.*, 1982; Mori *et al.*, 1980; Kolansky *et al.*, 1982; Jaussi *et al.*, 1981; Daum *et al.*, 1982b). There are, however, proteins whose import is not energy dependent; these exceptions will be considered later. Table 1 lists many proteins whose import has been shown to be energy dependent (see also Figure 2). The one common feature that is shown by the precursors whose transport requires coupling of oxidation and phosphorylation is that at some time they are either inserted into or actually cross the inner mitochondrial membrane (Gasser *et al.*, 1982a&b). Which form of energy is required for this transport of proteins? The application of uncouplers leads to a depletion of ATP due to the continuing action and stimulation of the oligomycin-sensitive ATPase (Stigall *et al.*, 1979) and also to discharge of the membrane potential. It became important to diagnose which of these events, viz., the dissipation of the membrane potential or the depletion of ATP is responsible for the inhibition of the transport process. A series of experiments reported by Schleyer *et al.* (1982) demonstrated that in *Neurospora* it is the electrochemical potential across the inner membrane that is required for the import of proteins into or across this membrane. They studied the transport of the ADP/ATP carrier under conditions where (1) the ATP was high and the membrane potential depleted, and (2) ATP was low and membrane potential high. In the first instance, *in vitro* transfer of proteins into mitochondria was performed in the presence of CCCP and oligomycin. The oligomycin inhibits the ATPase while the CCCP dissipates the membrane potential. Under these conditions, the ATP level in the mitochondria was high due to the fact that in uncoupled mitochondria the ADP/ATP carrier system tends to equilibrate the ATP inside and outside the mitochondria and the incubation medium had a high level of ATP. The transport of all proteins studied except porin and cytochrome *c* was inhibited (Schleyer *et al.*, 1982; Teintze *et al.*, 1982). On the other hand, in the second instance when the mitochondria were first treated with oligomycin and carboxyatractyloside to deplete their ATP pool but to leave the membrane potential intact, transport of the proteins proceeded uninhibited. Similar results have been obtained for yeast cells by Schatz and his group (Gasser *et al.*, 1982a,b) and for rat liver by Conboy and Rosenberg (1981).

In a further series of experiments (Zwizinski *et al.*, 1983), mitochondria were incubated in the presence of antimycin A and oligomycin and incubated in a reticulocyte

lysate supernatant. This treatment prevents the generation of a membrane potential and so inhibits the transport of the precursors into the mitochondria. It does not, however, prevent the binding of the precursors to the outer surface of the mitochondria where they remain sensitive to added proteases. The membrane potential could be restored by the addition of ascorbate and TMPD which funnels electrons through cytochrome *c* to cytochrome oxidase (Wikstrom and Krab, 1982). The reestablishment of a membrane potential restored the transport of the precursor proteins. This experimental procedure also differentiates between binding and transport. On washing, the mitochondria retained the bound precursors and, on restoration of the membrane potential, the bound precursors were directly imported from their bound location. These results not only differentiate between binding and transport but they further show that the membrane potential is necessary only for the transfer across or insertion into the inner membrane.

Schatz (1979) and Nelson and Schatz (1979) have observed that the transfer of a number of mitochondrial precursor proteins does occur in *rho-* mutants of yeast. These cells lack a functional oligomycin-sensitive ATPase and also some essential components of the respiratory chain and, thus, cannot produce a membrane potential either by respiration or ATP hydrolysis (Nelson and Schatz, 1979). They do, however, have an ATP/ADP carrier which can internalize ATP in an electrogenic manner (Klingenberg and Rottenberg, 1977) and so may generate a membrane potential. More recent reports by Gasser *et al.* (1982a) based on the use of CCCP, oligomycin, and valinomycin have indicated that the transport of precursor proteins into yeast mitochondria may be driven by either a pH gradient, electrical potential, or both. In this context, it is interesting to note that Schleyer *et al.* (1982) found that addition of nigericin, which dissipates the proton gradient by exchanging K^+ for H^+ did not inhibit the uptake of the ADP/ATP carrier precursor. This finding would argue against a proton gradient being involved in the transport phenomenon. However, much more detailed studies are necessary to understand the exact role of the membrane potential in the transport process.

Outer membrane proteins typified by mitochondrial porin have been shown in *Neurospora* and in yeast to be posttranslationally incorporated into a protease-insensitive location in an energy-independent manner (Freitag *et al.*, 1982; Mihara *et al.*, 1982). Apocytochrome *c* has also been shown to be transported into mitochondria without dependence on a membrane potential as reflected by responses to added uncouplers or ATP levels. Only proteins whose transport is governed by the inner membrane appear to require a membrane potential for their uptake. Different proteins, therefore, appear to use different pathways to reach their final functional destination subsequent to recognition by the mitochondria.

IV. MITOCHONDRIAL RECOGNITION OF PRECURSORS

In all cases of transport, one common feature applies, viz., the precursors are initially bound tightly (Hennig *et al.*, 1983; Zwizinski *et al.*, 1983) to the outer membrane surface. The bound precursors while resistant to washing can be readily

removed by added proteases or can be exchanged for added precursor. Pretreatment of mitochondria with trypsin destroys their ability to import precursors and to specifically bind them on their surface (Gasser *et al.*, 1982a; Zwizinski, unpublished). These findings clearly indicate the presence of recognition proteins on the outer mitochondrial surface which mediate the binding of the precursors.

The mitochondria only bind the precursor protein form and the binding has been clearly shown to be selective. When mitochondria were incubated in the presence of labeled apocytochrome *c* and a large excess of unlabeled holocytochrome *c*, the apo form was selectively bound and the binding was not inhibited by the presence of a large excess of holocytochrome *c* (Korb and Neupert, 1978). Well-defined binding studies have been few because of the problem of unavailability of reasonable quantities of purified precursor proteins. Two proteins exist, however, whose precursors can be produced in reasonable quantities, viz., cytochrome *c* and porin.

Hennig *et al.* (1983) have studied the binding of apocytochrome *c* to mitochondria in the presence of the protoheme analogue deuterohemin. Under these conditions, the conversion of the apo to holo form was inhibited and import of the apocytochrome *c* did not take place. They found that binding takes place in a saturable manner. Using Scatchard plots, high-affinity binding sites with a frequency of 60–90 *p*moles/mg mitochondrial protein were demonstrated and a Ka of $2.2 \times 10^7\ M^{-1}$ was obtained. A low-affinity component was also detected; however, only the high-affinity sites were sensitive to trypsin (Koehler *et al.*, 1983). Apocytochrome *c* did not compete with other mitochondrial preproteins for the binding sites. The only proteins which were found to displace bound apocytochrome *c* from *Neurospora* mitochondria were apocytochromes from other species (Figure 4). The apocytochrome *c* from *Paracoccus* was exceptional in that it had no effect on the binding behavior of the homologous apocytochrome *c*. This may be due to the fact that *Paracoccus* is a prokaryote and that its apocytochrome *c* lacks the highly conserved sequence at amino acid positions 60–80 present in all eukaryotic cytochromes *c* and which was implicated in the binding of apocytochrome *c* (Matsuura *et al.*, 1981; Hennig and Neupert, unpublished). The other apocytochromes inhibited the binding process to a varying degree; the extent of inhibition may be related to their phylogenetic affinity to *Neurospora* cytochrome *c*, a behavior which emphasizes the specificity of the binding reaction. The possibility that a nonspecific charge interaction is involved in the apocytochrome *c* binding can be discounted in view of the fact that neither holocytochrome *c* nor amino terminal fragments with similar charge to the whole apomolecule could displace the bound apo molecules. Polylysine and apocytochrome *c* denatured by repeated freezing and thawing were also ineffective in displacing the bound apocytochrome (Hennig *et al.*, 1983). It appears that apocytochrome is recognized by some surface component of the outer mitochondrial membrane with a high degree of specificity. The exact nature of the surface component is presently under investigation.

Freitag and Neupert (unpublished) have recently isolated porin from *Neurospora* mitochondria and transformed it into a water soluble form which is very similar in behavior to the precursor protein synthesized *in vitro*. This water soluble form of porin binds to mitochondria at low temperature in a rapid and saturable manner, while the conversion to the mature form proceeds slowly at the low temperature. The water-

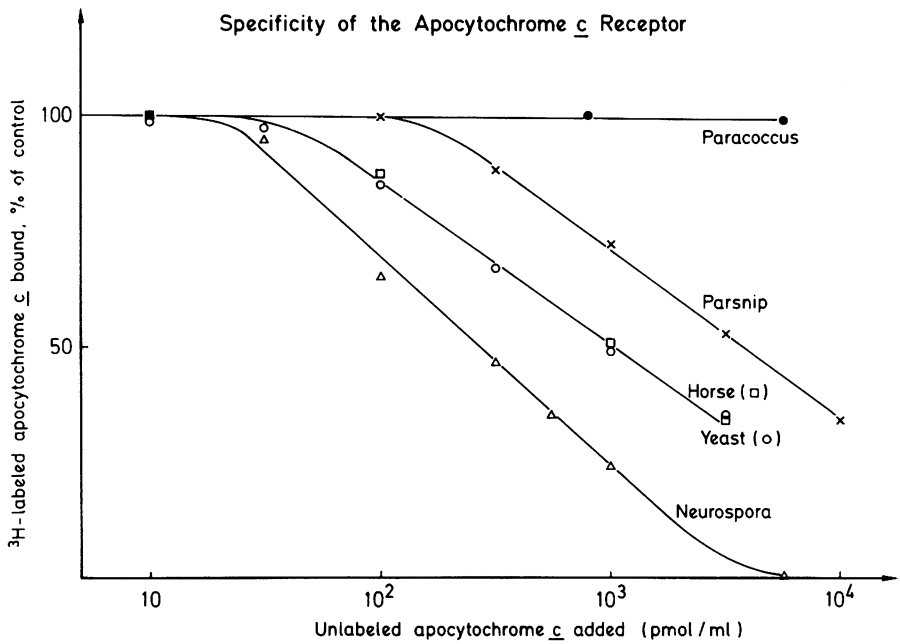

Figure 4. Competition between *Neurospora* apocytochrome *c* and apocytochrome *c* from various species for binding to *Neurospora* mitochondria. *Neurospora* mitochondria were incubated in the presence of deuterohemin (10 nmoles mg mitochondrial protein) for 5 min at 25°C. Apocytochrome was synthesized in a cell-free homogenate in the presence of 3H leucine for 10 min, after which time further protein synthesis was blocked by the addition of cycloheximide. A postribosomal supernatant was prepared and the deuterohemin-treated mitochondria were incubated in this supernatant for 15 min at 25°C. Following incubation, the mitochondria were reisolated and washed in a sucrose-MOPS buffer. The mitochondria containing the bound apocytochrome *c* were then incubated in an unlabeled postribosomal supernatant containing varying amounts of apocytochrome from different sources. After equilibration of free and bound apocytochrome *c*, the mitochondria were reisolated, twice washed, and lysed with 1% Triton X-100. Apocytochrome was immunoprecipitated from the lysates and analyzed by SDS–gel electrophoresis. Radioactivity was determined in the sliced gels by scintillation counting (From Koehler *et al.*, 1983).

soluble porin, therefore, may be used as a second system to study mitochondrial precursor recognition. One interesting feature emerged from the binding studies with porin and that was that increasing amounts of water-soluble porin when bound to mitochondria inhibited the transport of a fraction of the bulk precursor proteins into the mitochondria. This would infer that porin and some other precursor proteins share the same recognition sites.

A further feature of the specificity of the mitochondrial receptors is that precursor proteins from one species are recognized by mitochondria from another phylogenetically far-removed species. Not only are such proteins specifically recognized but they are also processed to the correct mature form. For instance, it has been observed that rat liver and yeast mitochondria import several *Neurospora* precursor proteins such as the ADP/ATP carrier, porin, or the F_1 ATPase subunit 9 (Zimmermann and Neupert,

1980, Schmidt *et al.*, 1983a; Freitag *et al.*, 1982; Hennig *et al.*, 1983). Furthermore, mitochondria from rat kidney were found to import the precursor of rat liver ornithine transcarbamylase, an enzyme only present in liver mitochondria (Mori *et al.*, 1980).

Subunit 9 of the F_1 ATPase is an especially interesting protein in that, in yeast, this protein is coded by the mitochondrial genome and is not translated as a larger precursor (Macino and Tzagoloff, 1979), whereas, in *Neurospora crassa*, it is coded by a nuclear gene and translated as a larger molecular weight precursor (Michel *et al.*, 1979; Schmidt *et al.*, 1983b). The yeast mitochondria can selectively bind this precursor and cleave off the prepiece in the same manner as *Neurospora*. It would appear, therefore, that the receptor proteins are highly conserved in evolution. With respect to the number of different receptor proteins on the mitochondria, it would appear that more than one type exists, as apocytochrome *c* does not compete for the binding sites of other precursor proteins (Zimmermann *et al.*, 1981). However, it is not clear how many recognition proteins are involved in the binding of precursors. It is clear, on the other hand, that each mitochondrial protein cannot have its own specific receptor. A detailed analysis has to wait until more precursor proteins are available on such scale that the ligand receptor interaction can be studied in the same manner as in the case, e.g., of hormone receptors of the plasma membrane.

V. INSERTION OF PRECURSORS INTO AND TRANSPORT ACROSS MEMBRANES

Subsequent to binding to the outer membrane, the precursors must either insert into a membrane as in the case of porin or else be translocated across a single membrane as apocytochrome *c* or be translocated across the outer membrane and inserted into the inner membrane, e.g., ADP/ATP carrier protein. For some proteins, transport across both the outer and the inner membrane is necessary. How can the transition from receptor-bound precursor to translocated precursor be analyzed? As outlined above, the mitochondrial proteins can be divided into two general groups in respect of transport: (1) those requiring no membrane potential, and (2) those which require a membrane potential. Apocytochrome *c* which belongs to the first of these groups is bound to mitochondria but not translocated in the presence of deuterohemin. If an excess of protohemin is added to the mitochondria, the inhibition of heme addition is reversed and the mitochondria import the bound apocytochrome *c* directly from its bound location on the mitochondrial outer surface. In studies on the import of the outer membrane porin by *Neurospora* mitochondria, Freitag *et al.* (1982) have shown that binding of the precursor protein can take place at 0°C but the insertion of the proteins into the outer membrane is slow at this temperature. The bound protein can be distinguished from the inserted protein on the basis of its sensitivity to added protease. The precursor form of porin is tightly bound to the mitochondria and is not readily washed off. On transferring mitochondria with precursor porin bound to the outer membrane to a higher temperature, the porin is immediately inserted into the outer membrane (Freitag *et al.*, 1982). In this instance, the insertion of the bound molecules takes place without any apparent detachment of the bound molecules. Mihara

et al. (1982) have also found that yeast porin synthesized in rabbit reticulocyte lysate was bound and inserted into yeast mitochondria. However, attempts to bind it to or insert it into other cell membranes failed. Recently, Gasser and Schatz (1983) showed that yeast porin inserted into isolated outer mitochondrial membrane.

It is possible that the recognition of binding sites may play a role in positioning the precursors in such a way that the next step, membrane insertion or transmembrane transport, can occur. Zwizinski *et al.* (1983) showed that ADP/ATP carrier binds to mitochondria in the presence of antimycin A and oligomycin. Subsequent restoration of a membrane potential resulted in the translocation and insertion of the bound carrier directly into a protease-resistant carboxyactractyloside binding location.

The majority of the mitochondrial precursor proteins carry an additional polypeptide sequence and must, therefore, be processed proteolytically before they can be integrated into their final location. In view of the fact that all reports to date indicate that the initial proteolytic processing takes place in the matrix (Boehni *et al.*, 1980; Schatz and Butow, 1983), then it follows that all larger molecular weight precursors must be exposed in total or in part to the matrix protease at some stage during processing. The import of all of the precursor proteins destined for the inner membrane and beyond has been found to require a membrane potential. A question immediately springs to mind, viz., which of the two functions, transport across the membrane or proteolytic processing requires the membrane potential or do both processes require a membrane potential? Two lines of experimental evidence can be offered to provide the answer. Zwizinski *et al.* (1983) inhibited the processing of the B subunit of F_1 ATPase by *Neurospora* mitochondria in a reticulocyte lysate postribosomal supernatant by EDTA and *o*-phenanthroline (Figure 5). The unprocessed protein was transported into the mitochondria, clearly showing that import and processing are independent phenomena. The second line of evidence comes from the results of the Schatz group. They have shown that the simultaneous import and processing of the β-subunit of

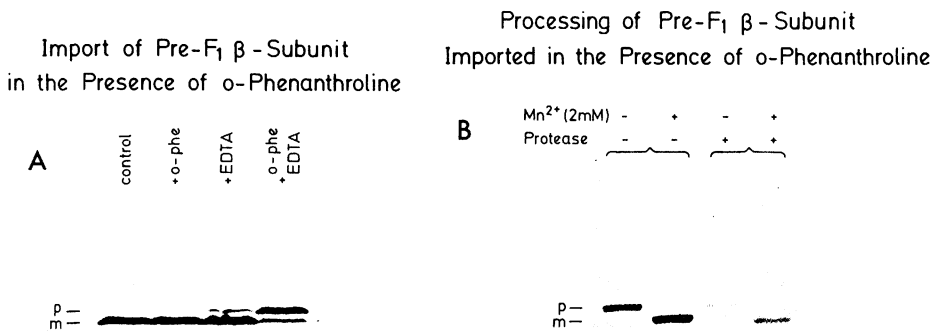

Figure 5. Import of precursor to β-subunit of the F_1 ATPase in the presence of *o*-phenanthroline and EDTA. Isolated mitochondria were incubated with 35 S labeled reticulocyte lysate supernatant. Transfer was performed in the presence of EDTA 7.5 mM and 100 μM *o*-phenanthroline (A). Mitochondria were allowed import pre β in the presence of *o*-phenanthroline and EDTA and were incubated in the presence and absence of MnCl (B). Protease sensitivity was assessed by treatment of the mitochondria at 0°C with proteinase K (Zwizinski *et al.*, 1983).

ATPase required a membrane potential. Proteolytic processing of the precursor could, on the other hand, be carried out by hypotonic extract of the mitochondria (Gasser *et al.*, 1982a). Kolansky *et al.* (1982) have found similar behavior in the case of the translocation of the precursor of ornithine transcarbamylase across the inner membrane of rat liver mitochondria. The presence of uncoupler was without effect on processing by hypotonic extracts. Furthermore, Reid and Schatz (1982) found uncleaved precursors in yeast mitochondria, the further processing of which was independent of added CCCP. It may, therefore, be concluded that it is the transport across the inner membrane that requires the membrane potential.

In yeast, the import of cytochrome *b*2, an intermembrane space enzyme, has been shown to require a membrane potential. Similarly, import of cytochrome *c*1 whose functional location is on the outer side of the inner membrane is energy dependent. The processing of the precursors of both of these proteins requires exposure to the matrix. Therefore, it is necessary for them to cross the inner membrane, hence, the requirement of a membrane potential (Gasser *et al.*, 1982a,b).

The details of how precursor proteins cross the mitochondrial membranes are not known. In fact, the same can equally be said about proteins crossing cellular membranes in general (Wickner, 1980; Randall, 1983). In her studies on protein translocation in *E. coli.*, Randall produced evidence more in favor of the idea of domains of protein crossing the membranes stepwise than of the progressive linear insertion of the emergent nascent chain. A similar type of situation could be envisaged for the translocation of mitochondrial proteins where, at least *in vitro,* whole polypeptides can cross the membranes. Some precursor proteins have a quite large molecular weight such as precarbamyl phosphate synthase (165 K) and a folded diameter at least equal to that of the membrane bilayer. The problem is how does such a molecule with a predominantly hydrophilic exterior pass through both mitochondrial membranes. Are there translocator proteins which combine with these precursors and carry them across the membrane? The presence of surface receptor proteins is generally agreed but the question of translocators cannot yet be answered. One could envisage some combination of the hydrophilic precursor being necessary to modulate their hydrophilic character and thus facilitate their passage through the lipid bilayer. Little attention has been paid to the role of the membrane lipids in the process of translocation. Tanner and his group (Komar *et al.*, 1979) have shown that the energy status of the cells has a marked effect on the sensitivity of the membranes to added detergents. When the membranes are energized, they are susceptible to damage by detergents such as Triton X-100. On the other hand, if the cells are treated with uncouplers to deplete their membrane potential, they become insensitive to the detergents. This would suggest that the membrane potential makes the lipid bilayer more labile or penetrable and may go some way towards explaining the need for a membrane potential in protein translocation. Curiously, the magnitude of the potential does not appear to be related to uptake or the threshold potential required may be rather low. Energized membranes have a definite polarity. In mitochondria and in bacteria, the membranes are negatively charged on the inside, yet the direction of protein translocation is opposite in both. It would not appear that translocation is a simple electrophoretic phenomenon. For some of the precursors investigated (Viebrock *et al.*, 1982; Kaput *et al.*, 1982), the pre-

sequences were predominantly basic and would therefore hardly be attracted to the positively-charged face of the membrane which faces out. However, one could envisage the binding of the precursor to the receptor leading to the positioning of the precursor in such a way that positively-charged domains could now respond to the negatively-charged inside of the membrane. Not all of the precursors have been shown to be more basic than the mature proteins. Wada and his group have described the precursor to aspartate amino transferase as being more acidic (Kamisaki *et al.*, 1982). It remains to be shown whether the presequences play a direct role in translocation across membranes or whether their role is to alter the conformation of the proteins so that recognition, or receptor attractive, domains are exposed which are hidden or masked in the mature proteins. The whole question of how the actual translocation takes place is one of the challenging mysteries of cell biology today.

A further unresolved problem is how polypeptides destined for the inner membrane or the matrix space traverse the outer and inner membranes. Are there two individual steps which can be experimentally separated or does translocation occur in a single step across both membranes? In the case of the second alternative, the two membranes would have to come into intimate contact or even fuse at certain points. The answers to these questions are really not known. Contact sites of outer and inner membranes have repeatedly been reported on the basis of electron microscopic studies. Kellems *et al.* (1975) have described the preferential association of cytoplasmic polyribosomes at sites where outer and inner membranes were in close proximity. This could be taken as an indication that precursor proteins cross both outer and inner membranes at specific sites in a single step.

VI. MITOCHONDRIAL PROTEASES AND THE PROCESSING OF PRECURSORS

The processing of imported precursors involves, in most cases, a proteolytic cleavage of a larger molecular weight precursor. In a number of instances, proteolysis may involve two proteolytic steps (Neupert and Schatz, 1981). In the case of cytochrome c_1 and cytochrome b_2 of yeast, two separate proteases are involved; other phenomena such as heme addition may attend this process (Gasser *et al.*, 1982b).

In all cases studied so far, the initial proteolytic event appears to be carried out by a metal-dependent enzyme located in the mitochondrial matrix first described by Boehni *et al.* (1980). They found that a hypotonic extract of rat liver and of yeast mitochondria could cleave the precursors of the β- and γ-subunits of the F_1 ATPase to the correct mature size. Fractionation of the mitochondria indicated that the enzyme was located in the matrix. They also found that a similar enzyme was present in the matrix of rat liver mitochondria. The proteolytic activity was inhibited by chelating agents such as *o*-phenanthroline and EDTA, but unaffected by inhibitors such as TLCK/TPCK and PMSF. Gasser *et al.* (1982a,b) showed that hypotonic extracts of yeast mitochondria could process the precursors of cytochrome c_1 and cytochrome b_2. In this instance, however, the matrix enzyme cleaved the precursors to the intermediate form but not to the mature form. The final proteolytic step was heme dependent. The

assay of these proteases presents problems in that it is extremely difficult to set up standard assays using precursor proteins as substrates.

A partially purified enzyme preparation from rat liver has been described by Miura *et al.* (1982) which cleaved the precursor of ornithine transcarbamylase to a size intermediate between that of precursor and mature size. It remains to be shown that this activity is actually involved in the *in vivo* processing since the significance of the intermediate form is not known. McAda and Douglas (1982) suggested that the metalloprotease from yeast is a dimeric protein with a molecular weight of 105,000 since, in partially purified preparations, a 59000 M_r polypeptide was prominent. However, from the data by others (Boehni *et al.*, 1983; Miura *et al.*, 1982), the question of the enzyme being monomeric or dimeric remains open. McAda and Douglas (1982) found that low levels of the detergents Triton X-100 and deoxycholate inactivated the enzyme, but this inactivation could be reversed by the addition of phospholipids. Recently Schmidt *et al.* (1983b) have partially purified an enzyme from whole *Neurospora* cells which can process a number of precursors. The precursor to cytochrome c_1 was cleaved to the intermediate form and the precursor of subunit 9 of the F_1 ATPase through an intermediate to the mature protein. Attempts to separate this activity into two separate entities have not yet been successful.

The only firm consensus on the nature of the protease responsible for the proteolytic cleavage of the mitochondrial precursor protein is that it is a soluble matrix located enzyme which has a requirement for divalent metals such as Zn^{2+}, Mn^{2+}, or Co^{2+} and can be inhibited by chelating agents. Reports on the reversibility of inhibition are contradictory.

A second protease has been postulated for the final processing of precursors of proteins such as cytochrome b_2, cytochrome *c* peroxidase, and cytochrome c_1 (Gasser *et al.*, 1982b). Cytochrome *c* peroxidase is an intermembrane space protein as is cytochrome b_2 (Daum *et al.*, 1982a,b)and their processing by a matrix enzyme appears to involve a detour of all or part of the molecule across the inner mitochondrial membrane. This is consistent with the need for a membrane potential in the processing of these proteins by whole mitochondria as discussed above. In the case of cytochrome c_1, a second heme-dependent cleavage has been postulated to take place at the outer face of the inner membrane (Ohashi *et al.*, 1982). A similar location has been suggested for the second proteolytic step in cytochrome b_2 (Gasser *et al.*, 1982b). This would locate the second hypothetical protease outside the inner membrane. However, no active extracts showing the proteolytic activity have yet been described so it remains a hypothetical entity.

One interesting feature of the matrix protease is its apparently widespread distribution and its specificity for mitochondrial precursor proteins. It can apparently recognize mitochondrial proteins from other cellular proteins. However, in most cases, it remains to be demonstrated that the proteolytic cleavage affected by this enzyme extract takes place at the correct molecular site. This latter point is particularly relevant in view of the number of instances where addition of partially purified enzyme to precursor containing translation supernatants results in processing to intermediate forms. It has been shown, however, that the processing of subunit 9 of the F_1 ATPase in *Neurospora* results in the generation of the correct amino terminus (Schmidt *et al.*,

1983a,b). It has also been shown that, in yeast, the protease generates the correct amino terminus of subunit 5 of cytochrome *c* oxidase (Cerletti *et al.*, 1983).

VII. GENERAL AND SPECIFIC TRANSPORT FEATURES OF INDIVIDUAL PROTEINS DESTINED FOR DIFFERENT COMPARTMENTS

The protein precursors have varied itineraries depending on their final location (see Figures 6–9). In general, proteins of the outer membrane appear to be made not as larger precursors but as proteins of the same molecular weight as the mature proteins; a typical example is porin (Freitag *et al.*, 1982; see Figure 6). Other outer membrane proteins of unidentified function have been analyzed in yeast and have been found to be made without extensions (Riezman *et al.*, 1983). Neither the binding nor the insertion show an energy or membrane potential requirement. The two processes show different temperature requirements; insertion may depend to a greater extent on membrane fluidity than does binding. Since insertion of porin into the outer membrane was inhibited by treatment with trypsin, receptor proteins on the outer membrane surface appear to be involved. It is not possible to make valid generalizations on the outer membrane proteins as Shore *et al.* (1981) have reported a slightly larger precursor for an outer membrane protein from rat liver. However, proteolytic cleavage of this protein remains to be demonstrated.

Intermembrane proteins have been variably reported as having polypeptide ex-

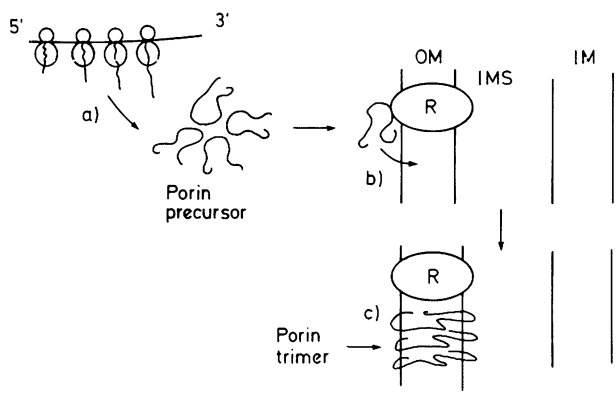

Figure 6. Hypothetical import pathway of an outer membrane protein, porin. (a) The water-soluble precursor is released from the polysomes to give a cytosolic pool. The precursor is here shown as monomers but aggregation may occur, the extent to which this happens *in vivo* remains to be clarified. (b) Precursor molecules are recognized by a surface receptor which results in the precursors being firmly bound to the protease accessible exterior surface of the mitochondria. (c) The bound precursors are inserted into the outer membrane and now become inaccessible to external proteases and undergo conformational changes which result in changes in the solubility characteristics and in the formation of oligomeric complexes, probably a trimer in the case of porin.

tensions as in cytochrome b_2 (Gasser *et al.*, 1982a) and as not in the case of adenylate kinase (Watanabe and Kubo, 1982). In the case of cytochrome b_2, the first processing appears to be performed by the matrix enzyme (Gasser and Schatz, 1983), and so one must postulate that this protein traverses the inner membrane until perhaps its precursor extension protrudes into the matrix space where it is cleaved to the intermediate form. This initial membrane traversal would require a membrane potential. Cytochrome c_1 which is really an inner membrane component is also processed in a two-step fashion. The location of the second protease is, however, not established. It appears to be membrane bound and may be located on the outer face of the inner membrane. The latter view is based on the fact that the carboxy terminus of cytochrome c_1 is highly hydrophobic and is firmly inserted into the outer face of the inner membrane and the second heme-induced cleavage of the precursor is believed to take place here (see Figure 7). The assembly of cytochrome *c* which can be regarded as an intermembrane protein or as a peripheral inner membrane protein has been discussed in detail already. The uptake of apocytochrome *c* appears to follow a rather unique pathway as it is linked to heme addition but is independent of a potential across the inner membrane

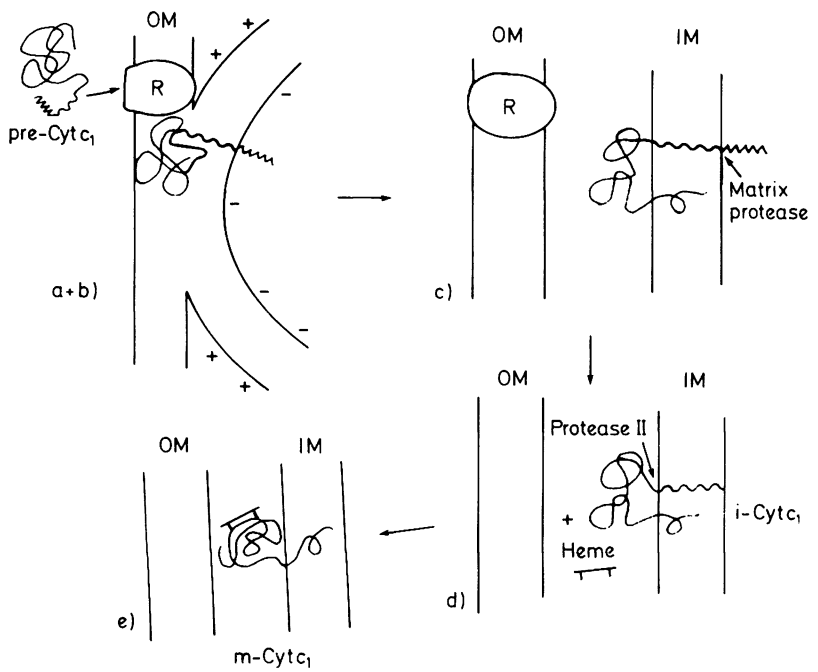

Figure 7. Hypothetical steps in the import of cytochrome c_1. (a) Synthesis and release of precursor. (b) Binding to the outer surface receptor, coupled to the insertion of the amino terminal portion of the precursor across the inner membrane. This latter step may involve contact sites between the inner and outer membranes. (c) Insertion of the carboxy terminus of the precursor into the inner membrane and cleavage of the presequence by the matrix protease to yield the intermediate form of the precursor. (d) Heme addition and second proteolytic cleavage. (e) Folding to mature form of cytochrome *c*.

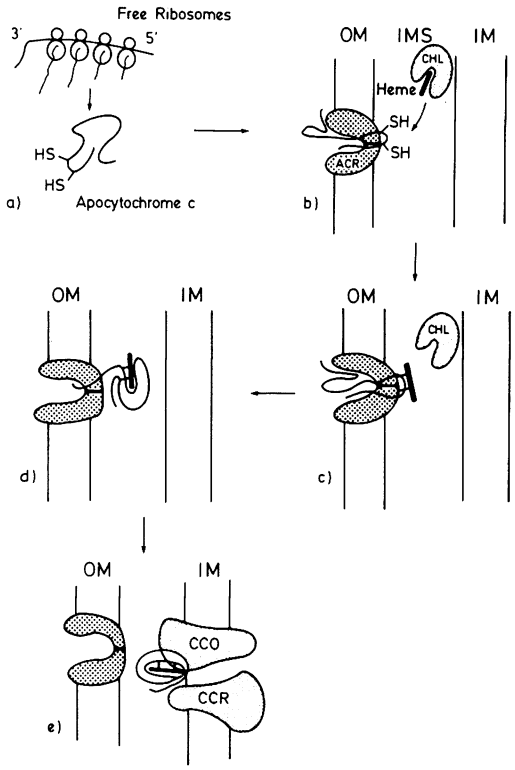

Figure 8. Hypothetical import pathway of cytochrome *c*. (A) Synthesis and release of apocytochrome *c* into the cytosol. (B) Recognition by a specific apocytochrome *c* receptor. (C) Heme addition to the receptor-bound apocytochrome *c* by cytochrome *c* heme lyase (CHL). (D) Internalization and refolding of the cytochrome *c*. (E) Binding of the completed and internalized cytochrome to the outer surface of the inner membrane (CCO, cytochrome *c* oxidase; CCR, cytochrome *c* reductase).

(Figure 8). It is also characterized by having an apparently separate or unique receptor protein (Hennig and Neupert, 1981). It may be speculated that the apocytochrome *c* is positioned by the receptor in such a way that addition of the heme group can take place. The enzyme catalyzing the formation of the thioether bonds between the vinyl groups of the heme and the thiols of cysteines 14 and 17 of the apoprotein may be located in the intermembrane space. It can be further speculated that the free energy of refolding of the polypeptide chain occurring after heme linkage provides the energy to completely pull the molecule through the outer membrane.

ADP/ATP carrier, on the other hand, inserts into the inner membrane (Zimmermann and Neupert, 1980) and traverses it. In common with other proteins studied, it binds to the outer membrane as a protease-sensitive precursor which does not have the ability to bind carboxyatractyloside nor ATP, as the mature protein does. The evidence presented by Zimmermann and Neupert (1980) and Schleyer *et al.* (1982) indicate that there is a change of conformation from a relatively hydrophilic form of the protein to a highly hydrophobic one. This transformation requires the presence of an energized inner membrane. It has recently been shown that the imported precursor binds carboxyatractyloside in a manner identical to the mature protein (Schleyer, unpublished). The possible pathways of two processed precursors, β subunit and subunit 9 of the F_0F_1 ATPase are outlined in Figure 9A. Obviously, most of the steps proposed are quite hypothetical and may have to be amended in the future.

The majority of matrix proteins described so far are synthesized as larger molecular weight precursors and their transport behavior accords with the belief that passage across their inner membrane requires a membrane potential (Figure 9b). The fact that in the matrix both peripheral membrane and soluble matrix proteins require both proteolytic processing and a membrane potential has led in some cases to the belief that the two processes were obligately linked. Import of precursors into the matrix

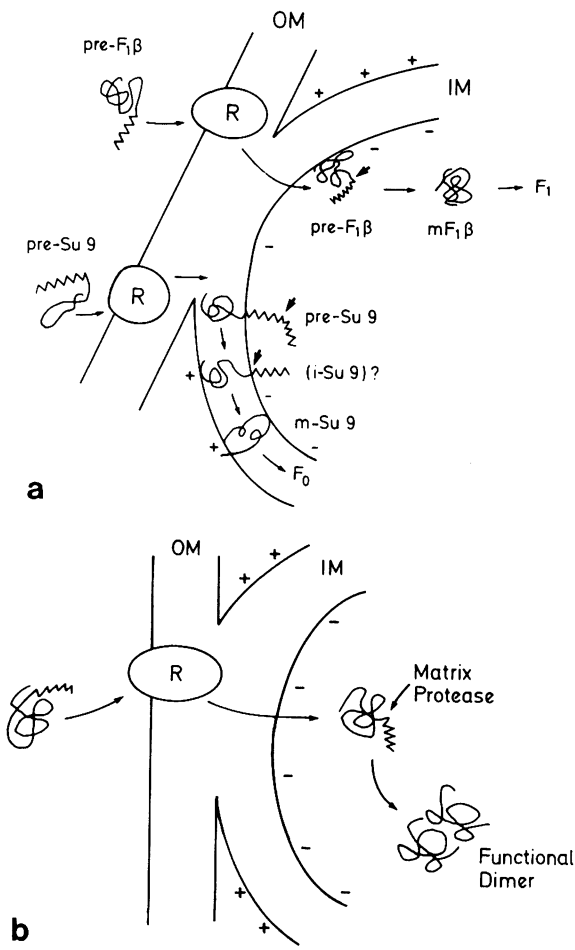

Figure 9. Hypothetical import pathways of (a) subunits of the F_1 ATPase. Receptors on the outer membrane channel; the proteins across the inner membrane. The β-subunit precursor has been located in the matrix while the precursor to subunit 9 remains membrane associated (Zwizinski and Neupert, unpublished). The presequence of su9 must protrude into the matrix to allow the protease access to the cleavage site. Following cleavage the mature protein is incorporated into the F_1 ATPase complex. Su9 undergoes a change of conformation and acquires its characteristic hydrophobic properties and is firmly embedded in the inner membrane. Post cleavage, both proteins are precipitable by antibody to the F_1 ATPase complex. (b) Citrate synthase, a soluble matrix protein. Binding and transfer is similar to above. Following transfer, the precursor is cleaved and the cleaved precursors can now dimerize to yield the mature dimeric protein.

occurs in the absence of proteolytic cleavage. Thus, proteolytic processing is apparently not an essential step for translocation. In agreement with this view, the precursor to isopropylmalate synthase, a matrix enzyme in yeast, does not differ in molecular weight from the mature protein and was reported to be taken up by yeast mitochondria in a membrane potential-dependent manner without a change in its apparent molecular weight (Hampsey *et al.*, 1983).

VIII. PROTEINS CODED FOR BY THE MITOCHONDRIAL GENOME

The mitochondrial genome has been extensively studied in a few organisms. The whole genome has been sequenced in man and mouse while in species such as yeast it has been only partly sequenced (Attardi, 1981). At the other end of the scale, the genome of plant mitochondria which appears to be relatively large (Leaver and Grey, 1982) has had little sequence analysis applied to it. The mitochondrial genetic system shows a number of unique common features. The code usage is at variance with that in the nucleocytoplasmic system in that the same codons do not code for the same amino acids in both systems, e.g., mammalian mitochondria read AGA and AGG as stop signals rather than as codons for the amino acid arginine and translate AUA as methionine rather than as isoleucine (Grivell, 1983). The differences between the two systems make it unlikely that messages from the nucleocytoplasmic system would be imported into the mitochondria for translation.

Despite a wide range of variation in the sizes of mitochondrial DNA, as measured by contour length and restriction analyses, from a range of species, the products of translation appear limited to much the same repertoire in most cases. *In vitro* studies on the mitochondrial translation system have yielded various estimates on the number of mitochondrial products. Bhat *et al.* (1981) quote the number of translation products from mitochondria from some mouse cell lines as 18, ranging in size from 3.8–67.0K. The former appear somewhat small for a functional polypeptide. The proteins synthesized by all mitochondria studied so far are cytochrome oxidase subunits I, II, and III, subunit VI of the ATPase, and the apocytochrome *b*. Subunit 9 of ATPase is coded by the mitochondrial DNA in yeast but by nuclear DNA in *Neurospora* and in higher organisms. Recently, a 10K mitochondrial translation product has been found in yeast which appears to be associated with the ATPase; however, no function could be assigned to this component (Macreadie *et al.*, 1983). There is also in yeast and *Neurospora* mitochondrial ribosomes one protein which appears to be essential for proper ribosomal function (varl protein in yeast) which is a mitochondrial translation product. There is experimental evidence (Leaver and Gray, 1982) that plant mitochondria do synthesize a greater number of proteins than do their fungal or mammalian counterparts. One of the additional translation products in plants has been identified as the α-subunit of the F_1 ATPase. Apart from the identified translation products there are variable numbers of unassigned reading frames depending on the source of the mitochondria (Borst and Grivell, 1981). The translation system of mitochondria resembles that of prokaryotes with respect to antibiotic sensitivity, as typified by its sensitivity to chloramphenicol and tetracyclines. The exact nature of the mitochondrial

translation products from many species remains to be classified. Studies on the synthesis of cytochrome oxidase subunits has shown that in some cases they may be synthesized as large precursors such as subunit I in *Neurospora* (Werner and Bertrand, 1979) and subunit II in yeast (Poyton and McKemmie, 1979; Pratje *et al.*, 1983). Little is known about the exact location of the translation, i.e., free or bound polysomes. Mitoribosomes have been reported by many to be associated with the inner face of the inner membrane; however, no clear evidence in favor of either a post- or cotranslational mechanism of precursors has been presented so far, nor is there any definitive data on the nature of the protease involved in the processing of mitochondrially synthesized precursors.

An interesting feature has been observed in a comparison of a subunit of the ATPase in yeast and *Neurospora*. In yeast, subunit 9 (proteolipid) is synthesized without any detectable polypeptide extension and is coded for by the mitochondrial DNA. In *Neurospora*, the same protein is coded by the nuclear genome and is synthesized as a larger molecular weight precursor on cytoplasmic ribosomes as discussed above. Interestingly, a gene with striking homology to those of either yeast or *Neurospora* subunit 9 has been detected in the mitochondrial genome of *Neurospora crassa*. However, expression of this gene has not been observed although it carries the characteristics of a duplication rather than a transposition (van den Boogart *et al.*, 1982).

A general feature of all functionally identified mitochondrial gene products is that they are all constituents of multisubunit complexes and occur combined with nuclear gene products. Therefore, a problem of mitochondrial biogenesis is that of understanding how the assembly of the translation products of the two systems, i.e., nucleocytoplasmic and mitochondrial takes place and how it is regulated. In yeast, there is evidence of a degree of coupling between the activities of the cytoplasmic translation system and that of the mitochondria. Lustig *et al.* (1982a,b) followed the levels of mRNA for the cytoplasmically synthesized subunits of cytochrome *c* oxidase under conditions of catabolite repression and derepression of mitochondria. The levels of mRNA for the subunits were low under conditions of repression and gradually increased with derepression. They also studied the level of transcripts for the nuclear coded mitochondrial RNA polymerase and found that the changes in messenger activity for the subunits of the oxidase were mirrored by those for the RNA polymerase. This result would suggest that the mitochondrial transcription increases to accommodate the influx of precursors from the cytoplasm. It may of course also be argued that this increase in the polymerase was merely due to a general increase in the synthesis of cytoplasmically synthesized mitochondrial protein precursors and, as such, does not represent a coordination of the two systems. In *Neurospora* cells, no tight coupling of the two translation systems has been found. Weiss and Kolb (1979) grew cells in the presence of chloramphenicol; these cells were deficient in cytochrome *b*, nevertheless, they continued to synthesize normal amounts of cytochrome c_1. This was taken up and processed by the mitochondria and inserted into a cytochrome *b*-deficient complex. Rucker and Neupert (1976) showed that inhibition of cytoplasmic translation led to continued production of subunits I–III of cytochrome oxidase at least for the duration of a doubling period of the *Neurospora* cells. However, increased breakdown was observed of the mitochondrially synthesized subunits which could not be assembled

into functional enzyme complexes. Poyton and McKemmie (1979) suggested from their studies on the biosynthesis of cytochrome oxidase that the synthesis of subunits I, II, and III by mitochondria was stimulated by a precursor to subunits IV–VII. The data on which this suggestion was made is open to question as they also suggested that the precursor was a polyprotein which is not in accordance with recent findings by other groups (Schmelzer and Heinrich, 1980; Lewin *et al.*, 1980; Mihara and Blobel, 1980).

IX. CONCLUSIONS

A. Precursors

Mitochondria import precursor proteins from the cytosol in a posttranslational manner. Precursors are mostly translated with amino terminal extensions on free ribosomes. A number of exceptions to this rule exist, e.g., ADP/ATP carrier, adenylate kinase, apocytochrome *c*, porin, and isopropylmalate synthase. Many precursors have a tendency to aggregate. It is not yet known whether there is an equilibrium between monomeric and oligomeric forms or whether there is selective recognition or uptake of one form vs. the other.

B. Recognition

Specific receptor sites exist on the outer mitochondrial surface. Competition experiments indicate the existence of more than one type. Treatment with proteases indicate that recognition is mediated by proteins. Requirements for binding and translocation across the membrane are different. Precursors can be bound at low temperature in the absence of insertion or translocation. On elevating the temperature to 25°C, the proteins are inserted or translocated without intervening detachment from the binding sites. Binding of precursor proteins is independent of the energy status of the mitochondria. Mitoplasts have been described as importing precursors; it is unclear, however, whether uptake is occurring via remnants of outer membrane or via specific receptors on the mitoplast surface as distinct from those on the outer membrane.

C. Translocation

Translocation across or insertion into the inner membrane requires a membrane potential. The nature of the process of transfer or translocation across the inner membrane remains a mystery. The role of the membrane potential is equally obscure, the magnitude of the potential does not seem to be directly related to the extent of import. Interactions of outer and inner membranes may play a role in the transfer of proteins into inner membrane and beyond but this is essentially a speculation. It appears that there are different pathways of translocation for different precursors depending on both the final location and solubility characteristics of the mature protein.

D. Proteolytic Processing

A matrix located metalloenzyme is responsible for proteolysis of the precursor protein to their mature form. The process is inhibited by chelating agents and is independent of membrane potential or ATP. The processing is indirectly influenced by these factors as they control translocation of the substrates to the site of action. In a number of cases, a second, as yet uncharacterized, protease is required to fully process the precursors.

The current practice of cloning and sequencing the genes for individual precursors will hopefully lead to the availability of sufficient amounts of individual precursors to allow investigation of binding and transport of individual proteins under defined conditions.

As stated at the outset we would attempt to provide answers to a number of questions posed. It is perhaps appropriate also to finish this chapter by outlining a number of open questions which remain to be answered.

How do precursors of insoluble membrane proteins move through the predominantly aqueous milieu of the cytosol? What is the nature of the receptors which mediate the recognition of mitochondrial precursor proteins? How many types of recognition proteins exist and how are they arranged in the membranes? Are there specific receptors on the inner membrane? How do polypeptides cross the membranes, i.e., as compact folded entities or do extended polypeptide chains move through the membrane? Are other membrane components besides receptors (translocators) required for translocating polypeptides across membranes? What exactly is the role of the membrane potential in polypeptide chain translocation? What is the role of the additional sequence; do they play a role in recognition or are the recognition domains associated with the mature position of the protein?

Are cytosolic pools of precursors involved in the regulation of synthesis of mitochondrial proteins?

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