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on

HOW MITOCHONDRIA IMPORT PROTEINS FROM THE CYTOPLASM

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1. Introduction

How are mitochondria formed? This question, first posed almost 90 years ago [1] is now partially answered. Mitochondria are formed by an interaction between two genetic systems: the nucleo–cytoplasmic genetic system and the mitochondrial genetic system [2]. The nucleo–cytoplasmic system makes about 90% of the proteins and all of the lipids and carbohydrates of mitochondria. The mitochondrial system manufactures most (and probably all) of the mitochondrial RNAs and roughly one dozen rather hydrophobic polypeptides; these include three subunits of cytochrome *c* oxidase, the apoprotein of cytochrome *b*, at least two subunits of the oligomycin-sensitive ATPase complex and a polypeptide (termed *var 1*) associated with the small subunit of mitochondrial ribosomes [2,3].

During the past 25 years, most of the research in mitochondrial biogenesis has focussed on the mitochondrial genetic system, particularly that of the yeast *Saccharomyces cerevisiae* (see [3] for a recent survey). Many of the genes located on yeast mitochondrial DNA have been identified and mapped and several of them are currently being sequenced. It is already clear that mitochondrial genes may contain intervening sequences and are thus not at all 'bacteria-like' as had been supposed before (e.g., [4]). For example, the mitochondrial gene for cytochrome *b* contains at least two intervening sequences; mutations in the coding regions often cause the formation of shorter fragments of apocytochrome *b* whose apparent molecular weight is directly related to the map position of the mutation; mutations in the intervening sequences affect not only cytochrome *b* but subunit I of cytochrome *c* oxidase as well [5–8]. Since mitochondrial DNA is relatively small, this opens up the possibility of studying phenotypic effects of mutations in intervening sequences of a functionally defined gene. Work in this area is now progressing quite rapidly and it is reasonable to expect that the complete nucleotide sequence of a mitochondrial DNA will become available in the not too distant future.

More recently, increasing attention is being focussed on the interaction between the two genetic systems in mitochondrial biogenesis. As already mentioned, most of the mitochondrial polypeptides are made by the nucleo–cytoplasmic system on extramitochondrial 80 S-type ribosomes. How do these polypeptides get into the mitochondria? For several years it was speculated that they might be made on a special subclass of 80 S ribosomes which would be bound to the mitochondrial outer membrane and discharge their nascent polypeptide products into the mitochondrial interior by 'vectorial translation' [9]. This was a reasonable hypothesis since vectorial translation is the mechanism by which polypeptides are transferred across the plasma membrane in prokaryotes and the endoplasmic reticulum in eukaryotes [10]. If cytoplasmically-made proteins are indeed imported into mitochondria by vectorial translation, one can make the following predictions:

(i) The cytosol should not contain any pools of mitochondrial proteins since these are imported into the mitochondrion as they are being synthesized;
(ii) Import of polypeptides into mitochondria should be strictly coupled to protein synthesis;
(iii) Since vectorial translation usually proceeds via transient formation of an N-terminal 'signal sequence' which is usually removed before transla-

tion of the polypeptide is completed, no discrete larger precursors to cytoplasmically-made mitochondrial polypeptides should accumulate in intact cells.

We have tested these predictions and found them to be incorrect. In this brief survey I hope to show that mitochondria import cytoplasmically-made polypeptides by a process which is not directly coupled to protein synthesis.

2. Results

2.1. *The experimental system*

Our initial studies on mitochondrial protein import concentrated on the three largest subunits (α, β and γ) of the F_1-ATPase of yeast mitochondria. Yeast F_1 consists of five non-identical subunits which are coded by nuclear genes, synthesized on extramitochondrial 80 S ribosomes and transported across both mitochondrial membranes to the matrix-side of the mitochondrial inner membrane [2,12]. Yeast F_1 has been studied in our laboratory for many years and monospecific antisera against the enzyme and its three largest subunits were already available [13].

2.2. *Newly-synthesized F_1-subunits are not recovered in mitochondria, but in a high-speed supernatant*

Max Schär and Heiner Grüninger, two undergraduate students, grew yeast cells overnight in $^{35}SO_4^{2-}$ to label all yeast proteins. These cells were then pulse-labeled for various time periods with [^{3}H]leucine and fractionated into mitochondria and $10^5 \times g$ supernatant. The two largest F_1-subunits (α and β; app. mol. wt ~58 000 and 54 000) were then isolated from these two subcellular fractions by immunoprecipitation and analyzed for their $^3H/^{35}S$ ratio. The result (fig.1, round symbols) shows that F_1-subunits recovered from the high-speed supernatant are much more heavily pulse-labeled than the corresponding F_1-subunits isolated from mitochondria. For example, after a 6 min pulse, the $^3H/^{35}S$ ratio of α-subunit recovered from the supernatant is $\geq$ 10-times that of the α-subunit recovered from mitochondria (fig.1A). If the [^{3}H]leucine pulse is followed by a chase with unlabeled leucine as well as unlabeled sulfate, the $^3H/^{35}S$ ratios of mitochondrial F_1-subunits increase whereas those of soluble F_1-subunits remain almost

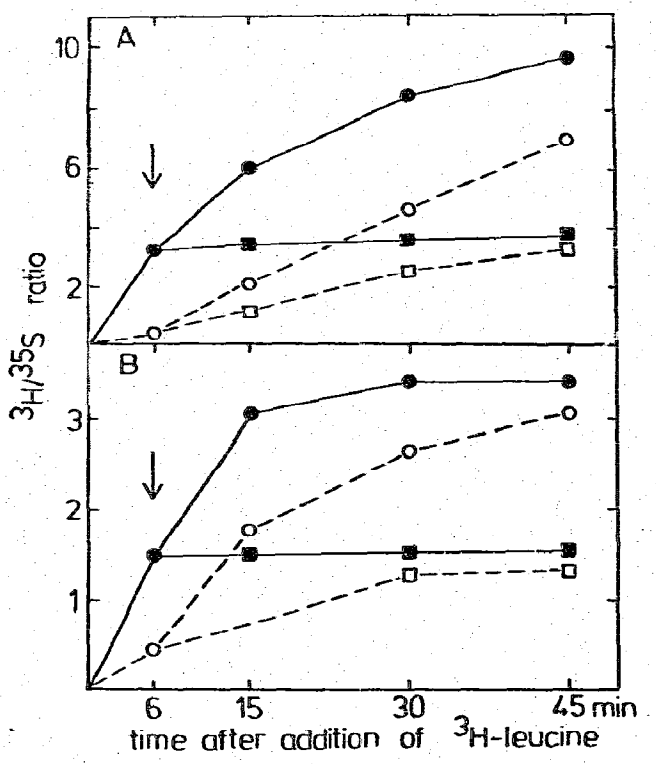


Fig.1. Soluble F_1-subunits are pulse-labeled more rapidly than mitochondrial F_1-subunits. Wild-type yeast cells (strain D273-10B) were grown in the presence of $^{35}SO_4^{2-}$ and pulse-labeled with [^{3}H]leucine for the indicated times. Aliquots were also chased with unlabeled leucine and unlabeled sulfate. The cells were homogenized with glass beads. Mitochondria and highspeed supernatant were analyzed for the presence of labeled F_1-subunits by immunoprecipitation, electrophoretic separation of the recovered F_1-subunits and counting of each subunit peak for ^{3}H and ^{35}S. See [14]. (A) α-subunit; (B) β-subunit. Closed symbols (● - ●, ■ - ■): subunits isolated from high-speed supernatant. Open symbols (○ - - ○, □ - - □): subunits isolated from mitochondria. Round symbols (○ - - ○, ● - - ●): pulsed with [^{3}H]leucine. Squares (□ - - □, □ - □): pulsed and then chased with unlabeled leucine at 6 min (arrow).

constant (fig.1, squares). Control experiments verified that the chase completely stopped the further incorporation of ^{3}H into total yeast protein. Additional experiments (not shown) demonstrated that a chase with unlabeled leucine was accompanied by a loss of *total* ^{3}H-labeled F_1-subunits from the supernatant.

This result suggests that the α- and β-subunits of F_1 are first made outside the mitochondria and only subsequently incorporated into these organelles.

Similar observations have been reported for *Neurospora crassa* cells [15]. However, since the electrophoretic properties of the 'soluble' and mitochondrial F_1-subunits recovered by us were identical, we could not exclude the possibility that the newly-made (i.e., ^3H-labeled) F_1-subunits had been selectively solubilized from mitochondria by the homogenization procedure.

2.3. *F_1-subunits are initially made as larger precursors in vitro and in vivo*

In a remarkable Ph.D. thesis which was done in collaboration with Günter Blobel, Maria-Luisa Maccecchini studied the formation of the three largest F_1-subunits in a reticulocyte lysate programmed with yeast RNA (i.e., in vitro) and in pulse-labeled yeast spheroplasts (i.e., in vivo). In contrast to our earlier experiments with intact yeast cells, pulse-labeling of the spheroplasts was stopped by adding a mixture of protease inhibitors and dropping the spheroplast suspension directly into a 20% SDS solution kept at 100°C [16]. This technique had been worked out by Claude Côté in our laboratory in an attempt to minimize proteolytic artefacts in the study of polypeptide precursors in yeast [17]. It was found that each of the three largest F_1-subunits was made as a larger precursor, both in vitro as well as in vivo. Moreover, much of the larger precursors made in vivo disappeared if the pulse-labeled spheroplast were subsequently chased. The result with the F_1 β-subunit is shown in fig.2. Analogous results were obtained for the α- and γ-subunit.

In parallel studies, Claude Côté and Marc Solioz showed that several cytoplasmically-made subunits of the cytochrome bc_1 complex are likewise made as larger precursors. These subunits include cytochrome c_1 and the non-heme iron protein (mol. wt 25 000) [17,18]. Although the intramitochondrial location of these subunits is not as firmly established as that of F_1, some of them appear to be partially embedded in the mitochondrial inner membrane or associated with the outer face of that membrane [19]. Figure 3 depicts the precursor to the mol. wt 25 000 subunit of the cytochrome bc_1 complex. The mature form and its larger precursor are not only immunologically related, but also yield closely similar proteolytic fingerprints (not shown).

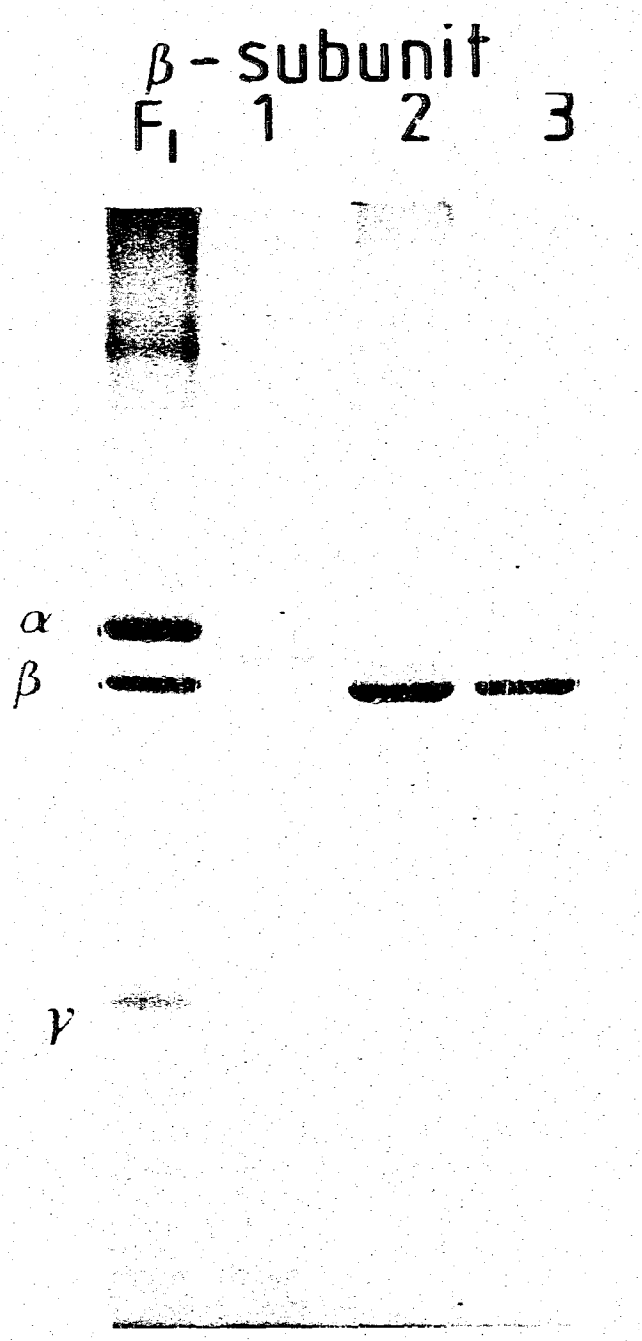


Fig.2. F_1 β-subunit labeled in vitro (track 1), in pulse-labeled yeast spheroplasts (track 2) and in pulse-chased yeast spheroplasts (track 3). Labeling was with [^{35}S]methionine and the immunoprecipitated subunits were resolved on 10% polyacrylamide gel slabs in the presence of SDS and visualized by radioautography. The track labeled 'F_1' displays the three largest subunits (α, β, γ) of 'mature' F_1 that had been immunoprecipitated from cells grown for 10 generations in $^{35}SO_4^{2-}$. From [16].

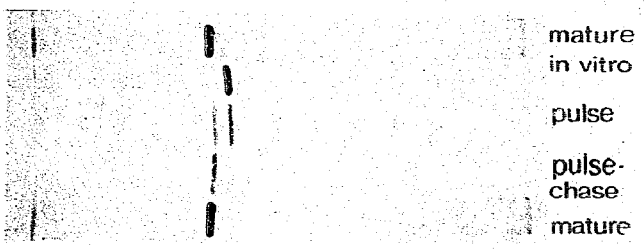


Fig.3. The cytoplasmically-made mol. wt 25 000 subunit (subunit V) of the mitochondrial cytochrome bc_1 complex in yeast is made as a larger precursor in vitro as well as in pulse-labeled spheroplasts. The experiment was analogous to that described in fig.2. See [17] for details.

2.4. *The F_1-subunit precursors are processed and imported into mitochondria in the absence of protein synthesis*

When the in vitro-synthesized precursors to F_1-subunits were incubated with isolated yeast mitochondria under conditions excluding protein synthesis, they were converted to the mature polypeptides. Moreover, when the mitochondria were isolated by centrifugation, they contained the radioactive mature forms in a state which was resistant to externally-added protease. In contrast, any precursors remaining in the supernatant or adhering to the sedimented mitochondria were fully protease-sensitive (fig.4). Apparently, processing of the precursors was accompanied by the uptake of the mature subunit into the mitochondria. This uptake appears to be specific: when ^{35}S-labeled mature F_1-subunits or in vitro synthesized ^{35}S-labeled glyceraldehyde-3-P-dehydrogenase were incubated with isolated mitochondria, they remained fully protease-sensitive.

2.5. *Import of cytochrome c peroxidase into the intermembrane space*

All the polypeptides discussed so far are transported not only across the outer membrane, but also completely or partially across the inner membrane. How about a protein that is transported only across the mitochondrial outer membrane? Maria-Luisa Maccecchini and Yvonne Rudin have approached this question by studying the import of cytochrome *c* peroxidase. This mitochondrial hemoprotein consists of a single subunit of mol. wt 33 500 [20] which is

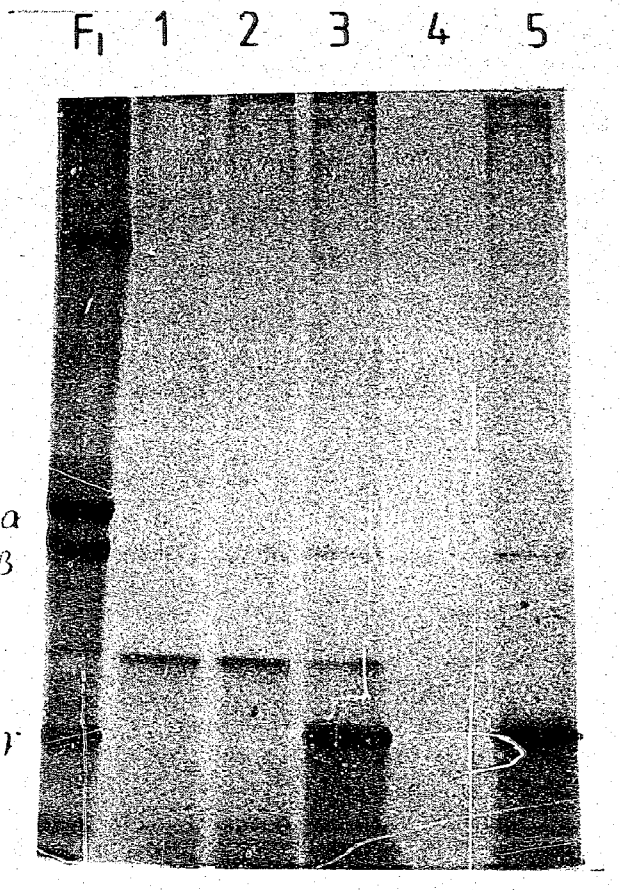
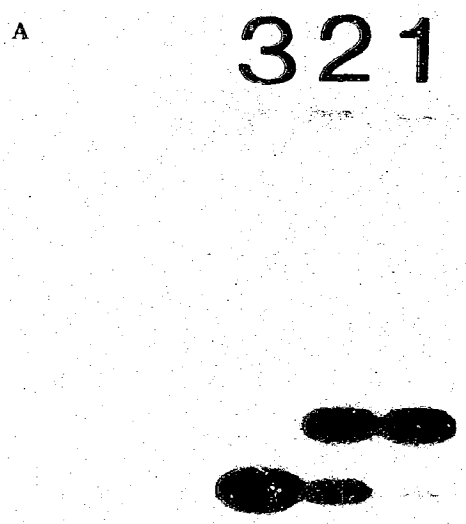


Fig.4. The in vitro synthesized F_1 γ-subunit precursor is processed and imported into mitochondria in vitro. (F_1) mature F_1-ATPase standard (see fig.2); (1) γ-subunit synthesized in vitro; (2) after labeling in vitro, the reticulocyte lysate was inhibited with cycloheximide and incubated for 60 min at 29°C with intact yeast mitochondria (3–5 mg/ml), freed from mitochondria by centrifugation and subjected to immunoprecipitation with an anti-γ subunit serum; (3) same as (2) except that the mitochondrial pellet was analyzed; (4) same as (2) except that the lysate-mitochondria mixture was incubated for 20 min at 23°C with trypsin and chymotrypsin before removal of the mitochondria; the mitochondria-free supernatant was then subjected to immunoprecipitation; (5) same as (4) except that the mitochondrial pellet was analyzed.

synthesized in the cytoplasm and, in the intact cell, imported into the space between the outer and inner membrane [22,23]. Figure 5 shows that this polypeptide, too, is initially made as a larger precursor in vitro and in vivo. Upon addition of isolated mitochondria to the in vitro-synthesized precursor, 40–50%



of it are converted to the mature form and ~1/3rd of the resulting mature form is associated with mitochondria in a form that is resistant to externally-added proteases. Apparently the mature form has been imported into the mitochondria [24].

Does the movement of proteins into mitochondria require energy? The answer seems to be yes. Nathan Nelson, one of our sabbatical visitors, has specifically depleted the ATP level in the mitochondrial matrix

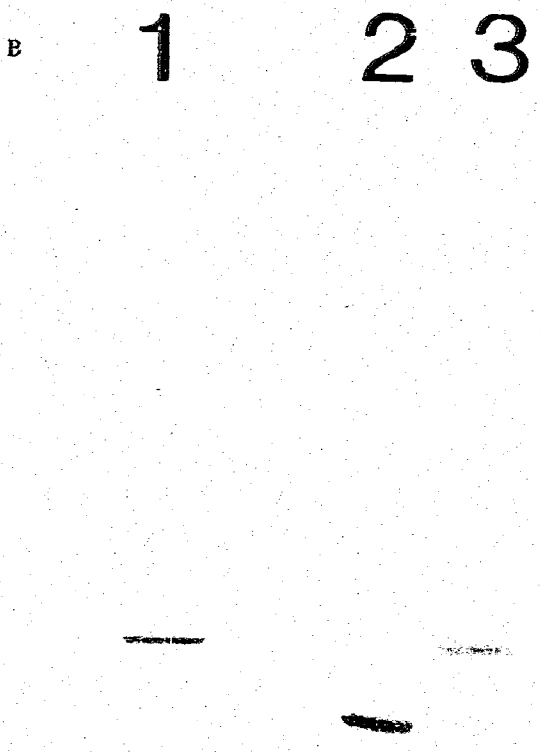


Fig.5. Yeast apo-cytochrome *c* peroxidase is made as a larger precursor in vitro and in vivo.

(A) Cytochrome *c* peroxidase was immunoprecipitated from: track 1, yeast spheroplasts pulse-labeled for 5 min with [^{35}S]methionine; track 2, as track 1, but then chased for 20 min with unlabelled methionine; track 3, yeast cells grown for 18 h with $^{35}SO_4^{2-}$. A 12.5% SDS–polyacrylamide gel was used. See [24].

(B) Cytochrome *c* peroxidase immunoprecipitated from: track 1, a reticulocyte lysate programmed with yeast RNA; track 2, continuously labeled yeast cells as track 3 in fig.5A; track 3, yeast spheroplasts pulse-labeled for 10 min with [^{35}S]methionine. A 10% SDS–polyacrylamide gel was used. See [24].

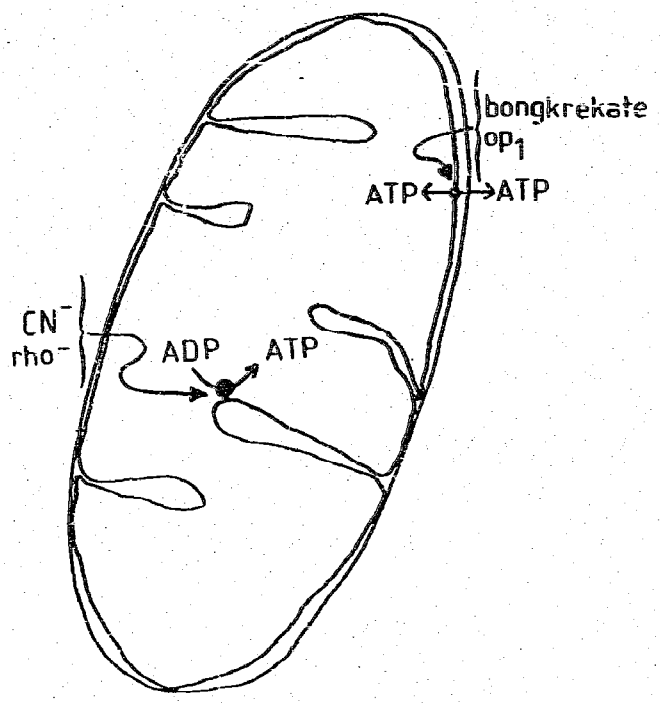


Fig.6. The two major pathways for supplying ATP to the mitochondrial matrix and experimental possibilities for specifically blocking each of these two pathways in yeast cells. Oxidative phosphorylation can be inhibited by respiratory inhibitors (KCN or antimycin A) or by the extrachromosomal *rho*$^-$ mutation [2,21]. Import of glycolytically-generated ATP from the cytoplasm via the adenine nucleotide transporter is inhibited by bongkrekic acid [25] or the nuclear *op*$_1$ mutation [26–28].

of intact yeast spheroplast by blocking both oxidative phosphorylation and the adenine nucleotide carrier of the mitochondrial inner membrane (fig.6). When these inhibited spheroplasts were pulse-labeled with [^{35}S]methionine, they no longer processed the precursors to those polypeptides that are normally transported into the matrix or inserted into the mitochondrial inner membrane [29] (fig.7). In contrast, no such effect was seen with the precursor to cytochrome *c* peroxidase; this is to be expected since the mitochondrial outer membrane (in contrast to the inner one) is freely permeable to ATP made by glycolysis in the cytosol [30]. Control experiments showed that ATP-depletion of the mitochondrial matrix did not affect overall protein turnover in the yeast cells (not shown). Nelson's experiments show that processing and/or the import of the precursors requires energy. In addition, we now have a very simple system for preparing large amounts of precursors to cytoplasmically-made mitochondrial proteins.

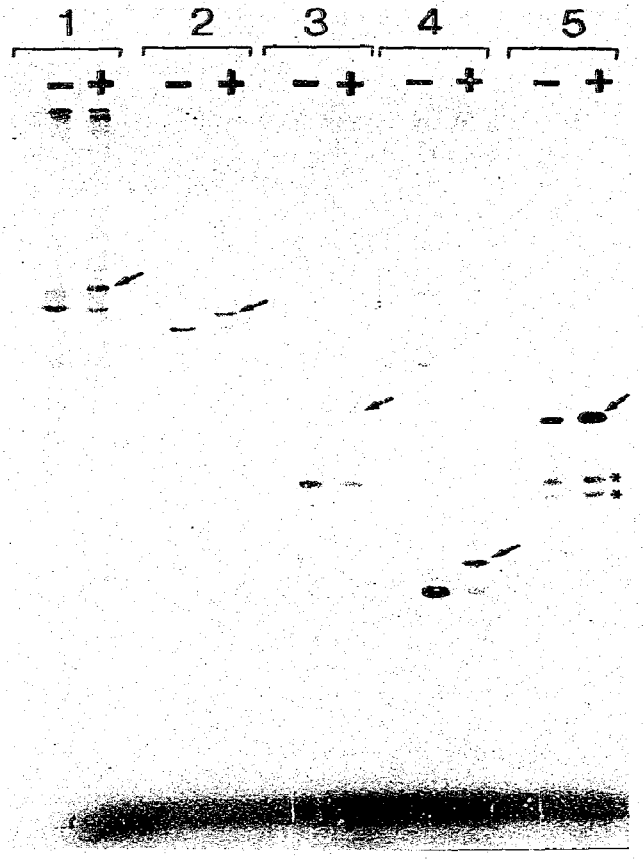


Fig.7. ATP depletion of the mitochondrial matrix inhibits the in vivo maturation of cytoplasmically-made proteins located within, or on the matrix side of the mitochondrial inner membrane. Spheroplasts of a *rho*$^-$ yeast mutant were labeled with [^{35}S]methionine for 20 min in the absence (–) or presence (+) of 14.6 μM bongkrekic acid. SDS-extracts of the spheroplasts were subjected to immunoprecipitation with antisera directed against the following components: (1) F_1 α-subunit; (2) F_1 β-subunit; (3) F_1 γ-subunit; (4) subunit V of the cytochrome bc_1 complex; (5) cytochrome *c* peroxidase. In contrast to the experiments of fig.5, the immunoprecipitates were dissociated and electrophoresed in the absence of 2-mercaptoethanol; under these conditions, mature cytochrome *c* peroxidase forms two bands (asterisks) rather than the single band shown in fig.5. Precursors are marked by arrows.

Table 1
Precursors to cytoplasmically-made mitochondrial polypeptides

Polypeptide	Intramitochondrial location	Molecular weight		Ref.
		Mature form	Precursor	
F_1-ATPase α-subunit	Matrix	58 000	64 000	[16]
F_1-ATPase β-subunit	Matrix	54 000	56 000	[16]
F_1-ATPase γ-subunit	Matrix	34 000	40 000	[16]
Subunit V of cytochrome bc_1 complex	Inner membrane	25 000	27 000	[17]
Cytochrome c_1	Inner membrane	31 000	37 000	[17,18]
Cytochrome c peroxidase	Intermembrane space	33 500	39 500	[24]

3. Concluding remarks

What do these experiments tell us? It seems clear that mitochondria import proteins from the cytoplasm by a process that is dependent on energy and independent of translation. In those cases studied by us, import is coupled to the processing of larger precursors (table 1) but, as we shall see in a moment, processing is not always necessary. Processing of the precursors listed in table 1 is almost certainly a proteolytic event and this may also explain why our initial experiments with conventionally prepared cell homogenates had failed to detect these larger precursors. A similar import mechanism appears to operate during the transport of polypeptides into chloroplasts [31,32] and perhaps also during the transport of bacterial toxins across the plasma membrane of prokaryotes and eukaryotes [33].

What drives these transport processes? In 'vectorial translation' it might be the ribosomal elongation machinery which is energized by energy-rich phosphate bonds. Such a mechanism cannot, of course, explain protein transport into mitochondria since, as we have seen, this transport is not coupled to translation. It is my current prejudice that movement of a cytoplasmically-made mitochondrial polypeptide from one aqueous compartment *across* a membrane into a second aqueous compartment is driven by the covalent modification of a precursor polypeptide. This modification could be a proteolytic cleavage (as with the precursors listed in table 1), the reduction of a disulfide bridge (as perhaps with some toxins) or even the covalent addition of a heme group (as with cytochrome *c*; see [34]). By analogy to 'vectorial translation', such a transmembrane movement of proteins might be termed 'vectorial processing'. This term indicates that a basically scalar processing step can drive a vectorial event by operating on only one side of a membrane. No processing might be required if a polypeptide is simply inserted *into* one of the mitochondrial membranes since the driving force could then be provided by a partitioning phenomenon or specific binding sites. Such a mechanism might govern the import of a hydrophobic transmembrane protein such as the adenine nucleotide transporter which is not made as a larger precursor (N. Nelson, unpublished; W. Neupert, personal communication; G. Blobel, personal communication). How 'vectorial processing' works is still a mystery but, in closing, I would like to present a simple hypothesis (fig.8). A polypeptide destined to be imported into the mitochondrial matrix is first made on 80 S ribosomes in the cytosol with a (presumably N-terminal) extension of 20–60 amino acids. The precursor is then released into the cytoplasm (A) and migrates to a point on the mitochondrial surface where the outer and inner membrane are in close contact. This point contains a receptor recognizing the N-terminal extension of the precursor (B). The interaction of the precursor with the receptor opens up a pore across both membranes which allows the polypeptide to equilibrate across the membrane barrier. Unidirectional transport could then be most simply driven by the irreversible proteolytic processing of

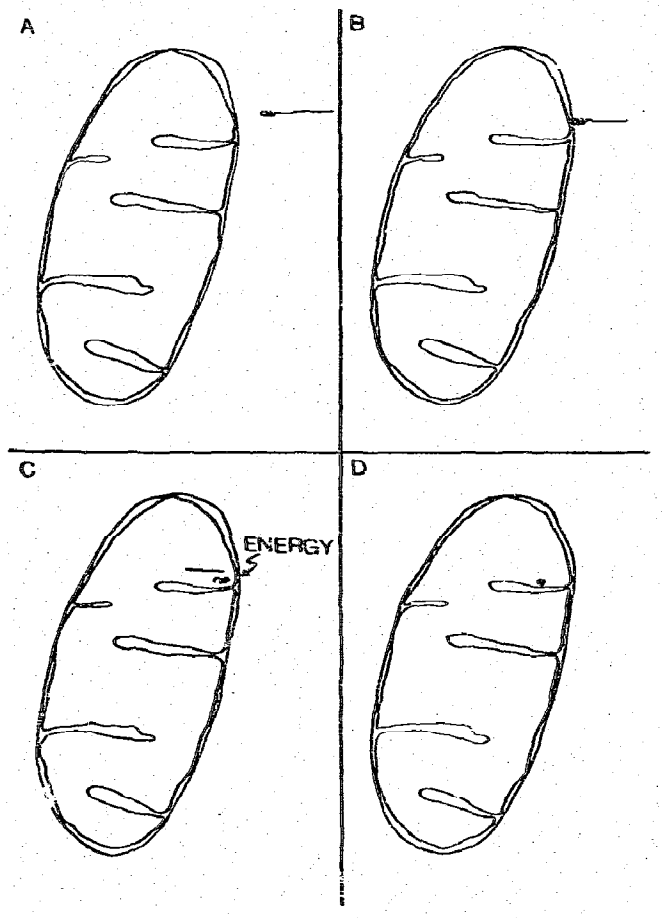


Fig.8. A 'minimal' hypothesis on how cytoplasmically-made polypeptides are transported into the mitochondrial matrix. See text for details. For the sake of clarity, the polypeptide to be imported is depicted as a straight line and the transient 'pre-piece' as a spiral.

the precursor in the matrix space (C). This transmembrane movement and/or the proteolytic processing requires energy. In the case of polypeptides such as F_1-subunits, an additional driving force could be supplied by the assembly of the mature subunits to the oligomeric enzyme (D). It will be of great interest to learn whether precursors imported into different mitochondrial compartments interact with distinct receptors on the mitochondrial surface or whether there exists only a single type of surface receptor coupled to distinct, specific proteases in different mitochondrial compartments.

The present studies also raise intriguing questions about the possible evolution of mitochondria and chloroplasts from free-living prokaryotes. It is well documented that the polarity of the bacterial plasma membrane is opposite to that of the mitochondrial inner membrane; for example, the F_1-ATPase of bacteria faces the cytoplasm and is thus made on the same side of the cell membrane where it is located. We must therefore assume that, during the evolution of mitochondria from prokaryotes, each of the individual F_1-subunits was equipped with suitable 'pre-pieces'. Did the 'pre-pieces' which are now operative in 'vectorial processing' evolve from those operative in vectorial translation and, if so, how have the nuclear genes for the subunits of mitochondrial F_1 acquired the extra nucleotide sequences? These questions should remind us that some of the most exciting problems of mitochondrial biogenesis are only now coming into view.

Acknowledgements

In reviewing recent work from my laboratory I feel deeply indebted to my colleagues mentioned in this article. Their talents and dedication have not only uncovered new information but have also generated an atmosphere of excitement and fun in our laboratory. I also wish to thank Dr G. Blobel for the hospitality extended to Dr M.-L. Maccecchini and to Drs P. M. and P. V. Vignais for a valuable sample of bongkrekic acid. This research was supported by grants 3.172-0.77 and 3.212-0.77 from the Swiss National Science Foundation.

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