H^+-ATPase (ATP SYNTHASE): STRUCTURE, FUNCTION, BIOGENESIS THE F_0 F_1 COMPLEX OF COUPLING MEMBRANES

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The F_0F_1 complex of coupling membranes

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TRANSPORT OF SUBUNIT POLYPEPTIDES OF F_0F_1-ATPase INTO MITOCHONDRIA

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ABSTRACT

The majority of mitochondrial proteins are coded for by nuclear genes and are synthesized as precursor proteins on cytoplasmic ribosomes. These precursor proteins are then transported to their sites of function within the mitochondrion. We have analysed the transport of the precursors to F_1F_0-ATPase subunit 2 (F1β) and subunit 9 (proteolipid, DCCD binding protein, Su9) into the mitochondria of Neurospora crassa. Transport appears to involve at least two types of specific components of the mitochondria: i. receptors which serve in the recognition and binding of the precursor proteins by mitochondria and ii. a proteolytic enzyme which catalyses the processing of the precursor proteins within the mitochondria.
The recognition step is shown to be mediated by protease sensitive components on the mitochondrial surface. **Neurospora** mitochondria lost the ability to bind and transfer **in vitro** the precursors to F1β and Su9 after mild trypsin treatment, but not after elastase treatment.
The processing step is catalysed by a processing peptidase which is a water soluble enzyme located in the mitochondrial matrix. Processing appears not to be necessary for translocation across the inner membrane. It can occur after the translocation has been completed. Hypothetical pathways for the import of ATPase subunits are proposed.

INTRODUCTION

Most of the subunits of the mitochondrial F_1F_0-ATPase are coded for by nuclear genes and are synthesized as precursor proteins on cytoplasmic ribosomes. These precursor proteins are imported into the mitochondria in a highly specific and rather complex process (Neupert and Schatz 1981, Teintze and Neupert 1982). The precursor proteins are synthesized on free cytoplasmic ribosomes and released as water soluble polypeptides into an extramitochondrial pool (Hallermayer et al. 1977, Suissa and Schatz 1982). Many but not all of these precursor proteins carry aminoterminal additional sequences of essentially unknown function (Zimmermann 1984). The precursors bind to the mitochondrial surface apparently via specific receptor sites (Hennig et al. 1983, Zwizinski et al. 1983). The bound precursor proteins are then translocated to their functional sites within the mitochondrion. With most, but not with all precursors a membrane potential across the inner mitochondrial membrane is required for import (Schleyer et al. 1982, Gasser et al. 1982a). Among the

precursors which do require a potential are all those that are inserted into the inner membrane and all those that have to cross partially or completely the inner membrane. The import of outer membrane proteins (Freitag et al. 1982, Gasser and Schatz 1983) and of apocytochrome c (Zimmermann et al. 1981) does not require a membrane potential. Up to now it is not known whether or not the translocation occurs at contact sites between inner and outer membrane (see Zimmermann 1984 for discussion).
During or shortly after the translocation the aminoterminal peptide extensions present on many precursors are removed (Teintze and Neupert 1982, Zimmermann 1984). In the case of yeast mitochondria, the processing enzyme was further characterized (Böhni et al. 1983). It is a water soluble protein located in the mitochondrial matrix. It requires divalent metal ions such as zinc or manganese for activity.
The last step in the translocation process is the assembly of newly transferred polypeptides into functional complexes. Very little is known about these latter reactions.
In this report we will focus on two aspects of the biogenesis of mitochondrial ATPase. These are (a) the recognition of the precursor proteins by mitochondria and (b) the proteolytic processing of the precursor proteins.

RECOGNITION

The first interaction between the precursor polypeptides and the mitochondrial transport machinery is binding of precursors to the mitochondrial surface. Earlier **in vitro** experiments employing isolated mitochondria and precursor proteins synthesized in a reticulocyte lysate showed that in the absence of translocation across the mitochondrial membranes, precursors were still bound to mitochondria (Zimmermann and Neupert 1980, Freitag et al. 1982, Gasser et al. 1982a). Transport can be inhibited in such an **in vitro** system by dissipating the electrical potential across the inner mitochondrial membrane (Schleyer et al. 1982). The binding of the precursors meets the following criteria: (i) the bound precursor is very sensitive to proteolytic digestion, i.e. it is exposed on the mitochondrial surface (Zimmermann and Neupert 1980, Schleyer and Neupert 1984), (ii) binding is tight, the precursors cannot be removed from the binding sites by washing of the mitochondria (Zwizinski et al. 1983), (iii) the binding sites are saturable (Hennig and Neupert 1981), (iv) binding is specific as judged from the observation that binding of precursors cannot be competed by the respective mature proteins (Hennig and Neupert 1981) and (v) transfer occurs directly from the binding sites upon reestablishment of a membrane potential (Zwizinski et al. 1983). The conclusions drawn from these experiments are that the binding sites are located on the mitochondrial surface and that binding is an essential step in the transport pathway of each precursor protein investigated so far.

In order to further characterize the nature of the binding sites, mitochondria were exposed to mild protease treatment before they were assayed for their ability to bind precursor proteins. It turned out that very low doses of trypsin led to the complete loss of the binding activity (Zwizinski et al. 1984). Fig. 1 shows that treatment of the

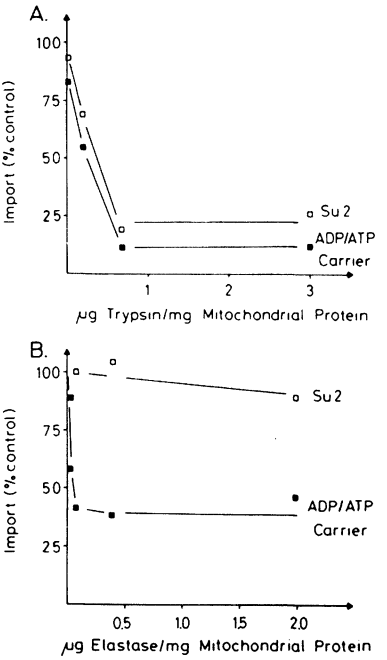

Figure 1. Protease pretreatment of mitochondria differentially affects the import of ATPase subunit 2 ($F_1\beta$) and ADP/ATP carrier. Mitochondria were pretreated with various amounts of trpysin or elastase. They were then incubated with radiolabelled precursors synthesized in a rabbit reticulocyte lysate. After incubation the reactions were divided in two and the mitochondria in each portion recovered by centrifugation. One portion was immunoprecipitated for $F_1\beta$. Import was taken to be the amount of mature $F_1\beta$ formed. The second portion was subjected to hydroxylapatite chromotography and the pass-through fraction immunoprecipitated for ADP/ATP carrier. This fraction of the carrier was taken to reflect ADP/ATP carrier imported into mitochondria.
Su2: ATPase subunit 2 ($F_1\beta$)
A: Trypsin treatment
B: Elastase treatment

mitochondria with about 500 ng trypsin/mg mitochondrial protein leads to a 80% decrease of the import of $F_1\beta$ and of the ADP/ATP carrier. With the latter protein it was demonstrated that it is actually the binding of the precursor that is affected by the protease treatment. A different response was observed when mitochondria were treated with elastase. In this case, mitochondria were still able to import the precursors to Su9 and $F_1\beta$ but a considerable loss of binding sites

for the precursors to porin and ADP/ATP carrier was observed. Elastase concentrations as low as 10-20 ng/mg mitochondrial protein were sufficient to lead to this destruction of binding sites. Apparently proteinaceous elements on the mitochondrial surface act as receptor molecules. They expose trypsin sensitive parts of their structure to the cytoplasmic face of mitochondria. More than one receptor may exist for the different precursor proteins to be transported.

PROTEOLYTIC PROCESSING

It is clear from the fact that many precursor proteins are synthesized with aminoterminal peptide extensions that these precursors have to be proteolytically processed during or shortly after their transport into mitochondria. Using an **in vitro** processing assay it was shown that the processing activity referred to as processing peptidase is located in the mitochondrial matrix (Böhni et al. 1980, Conboy et al. 1980, Schmidt et al. 1984). It is a water soluble enzyme that can be inhibited by chelating agents such as EDTA and o-phenanthroline (Fig. 2). On the basis of these observations conditions were found

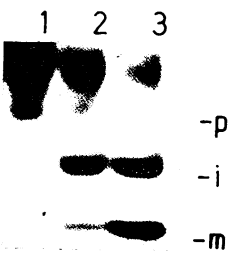

Figure 2. Processing peptidase activity depends on the presence of divalent metal ions. Precursor proteins were synthesized in reticulocyte lysates and incubated with mitochondrial membrane free extracts in the absence and presence of additions as outlined below. The samples were immunoprecipitated for ATPase subunit 9. A fluorogram of an SDS-gel is shown
(1) + 2mM Mn^{2+}, + 2mM o-phenanthroline
(2) - no additions
(3) + 2mM Mn^{2+}
p = precursor, i = intermediate, m = mature Su9

under which transfer of the precursors to Su9 and F1β into mitochondria occurred in the absence of processing (Fig. 3). This was achieved by adding EDTA and o-phenanthroline to a reconstituted system including precursor proteins synthesized in rabbit reticulocyte lysates and isolated Neurospora mitochondria. The former chelator cannot penetrate the mitochondria but binds metal ions in the extramitochondrial space. Low concentrations of the membrane permeable o-phenanthroline are then able to enter the mitochondria and inhibit the matrix processing peptidase. In this system precursor proteins were transported into the mitochondria and were no longer accessible

Transport of F_1F_o-ATPase subunits

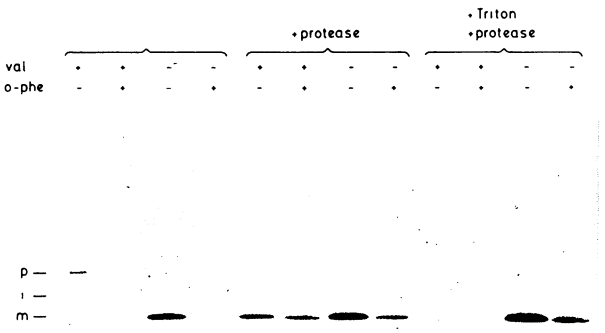

Figure 3. Import of the precursor to ATPase subunit 9 in the presence of EDTA and o-phenanthroline. Precursor proteins were synthesized in reticulocyte lysates. The lysates were adjusted to a EDTA concentration of 5 mM and incubated in the absence and presence of 2 µM valinomycin and 50 µM o-phenanthroline with isolated mitochondria for 30 min at 25°C. Then mitochondria were reisolated and either directly immunoprecipitated for Su9 or treated with proteinase K in the absence and presence of Triton X-100 and then immunoprecipitated.
p = precursor, i = intermediate form, m = mature Su9.
The antibody used in this experiment was raised against the mature Su9. It reacts only incompletely with the precursor form, which carries a prepeptide of 66 amino acids in addition to the 81 amino acids of the mature protein (Viebrock et al. 1982). Therefore, the intensities of the precursor bands in this figure do not represent the actual amounts of precursor associated with the mitochondria.

to added protease. They were associated with the mitochondrial membranes. Upon addition of excess metal ions, the transported precursors were processed to mature sized polypeptides in the absence of a membrane potential. The conclusion drawn from these experiments is that processing can occur after translocation of precursor proteins into the mitochondria. A membrane potential is apparently required only for translocation, but not for processing (Zwizinski and Neupert, 1984).
Another interesting feature of processing peptidase is that it cleaves the precursor to Su9 in two steps (Fig. 4) (Schmidt et al. 1984). The two cleavage sites display striking similarities as deduced from radiosequencing experiments and from the primary structure of the pre-peptide determined by sequencing of the cloned c-DNA (TABLE 1) (Viebrock et al. 1982). These cleavage sites are similar to putative cleavage sites in yeast pre-cytochrome c peroxidase (Kaput et al. 1982). It remains to be determined whether these sequences are really the complete recognition sites for the processing peptidase. It is furthermore not clear whether there is only one metal ion dependent processing peptidase in the mitochondrial matrix. This question can only be answered by purification of the(se) enzyme(s) to homogeneity. This has not yet been achieved.

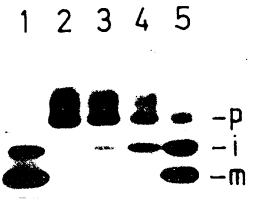

Figure 4. Time course of processing of the precursor to ATPase subunit 9 **in vitro.** Precursor proteins were synthesized in rabbit reticulocyte lysates and incubated for various time periods with a membrane free extract of mitochondria. After incubation the samples were immunoprecipitated for subunit 9. A fluorograph of an SDS-gel is shown.
(1) 60 min, (2) 5 min, (3) 10 min, (4) 15 min, (5) 30 min,
p: precursor; i: intermediate; m: mature subunit.

CONCLUSIONS

The results presented in this report give some insights into the mechanism by which precursor proteins to the F_1F_o-ATPase are transported into mitochondria. A hypothetical sheme for the assembly pathway of ATPase subunit 9 as derived from these results is presented in Fig. 5.

The binding of soluble precursor proteins is mediated by proteins on the mitochondrial surface with receptor-like function. It will be a considerable effort to purify these receptors because the ligands are not available in chemical quantities. In contrast, the precursor form is available in the case of cytochrome c and it was therefore possible to purify a protein with the characteristics of a receptor for apocytochrome c (Köhler et al. 1984). The receptor for apocytochrome c, however, is not functioning in the uptake of precursors of ATPase subunits, or of any other precursor analysed so far. It remains an open question, how many different receptors are involved in the recognition of mitochondrial precursor proteins.

TABLE 1: Comparison of the amino acid sequence at the two cleavage sites of the precursor to ATPase subunit 9.

```
                                  -1 ↓+1
a. -gln-ala-phe-gln-lys-arg-ala-tyr-ser-ser-glu

                                  ↓
                              -32 -31
b. -ala-gln-val-ser-lys-arg-thr-ile-gln-thr-gly
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The cleavage sites are indicated by the arrow. The second cleavage site is shown in a., the first one in b..

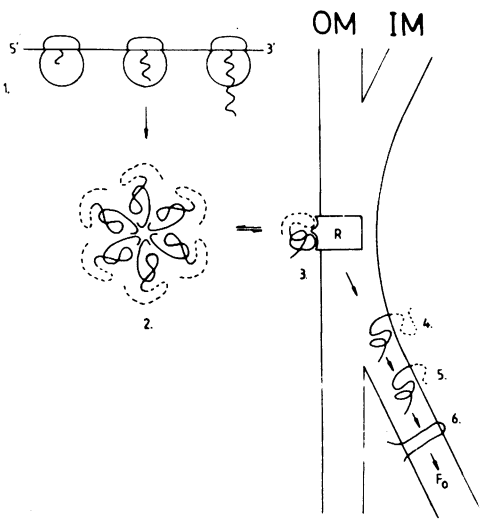

Figure 5. Hypothetical pathway of the biogenesis of ATPase subunit 9 in **Neurospora**. The precursor is synthesized on free cytoplasmic ribosomes (1.) and released as water soluble species into the cytoplasm (2.). After binding to a receptor on the mitochondrial surface (3.), the precursor is translocated into the inner membrane(4.). Processing occurs in two steps (5., 6.) in the mitochondrial matrix. Subsequently the protein is assembled into F_o. OM: outer membrane, IM: inner membrane, R: receptor

Recognition and binding are followed by the translocation of the precursor polypeptides into or across the inner mitochondrial membrane. The aminoterminal portions of the precursors thereby become accessible to the processing peptidase. The processing of subunit 9 of ATPase appears to be of particular interest since it occurs in (at least) two steps. Both steps seem to be catalysed by the same enzyme as judged from the striking similarities of the cleavage sites and the inhibitor sensitivity. Two step processing has been described before for the precursors of cytochrome c_1 (yeast and Neurospora) and cytochrme b_2 (yeast) (Gasser et al. 1982b, Teintze et al. 1982). However, in these cases the second processing step is not performed by the metal dependent matrix enzyme, but by an enzyme with rather different properties which is probably located on the outer face of the inner mitochondrial membrane.

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