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Transport of the Precursor to *Neurospora* ATPase Subunit 9 into Yeast Mitochondria

IMPLICATIONS ON THE DIVERSITY OF THE TRANSPORT MECHANISM*

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Isolated yeast mitochondria were able to take up *Neurospora* ATPase subunit 9 *in vitro* although the homologous yeast protein is synthesized within the mitochondria and inserted into the membrane from the matrix side (Tzagoloff, A., and Meagher, P. (1972) *J. Biol. Chem.* 247, 594-603). The transfer of the protein was dependent on an energized mitochondrial inner membrane. It was accompanied by proteolytic processing of the precursor to the mature protein with the correct NH_2 terminus as determined by Edman degradation of the transferred protein. The possibility is discussed that there are common features in the uptake machinery neither specific for one species nor specific for individual precursor proteins in the same species.

Import of proteins of mitochondria occurs by a post-translational mechanism (1-3). Precursor proteins are synthesized on free cytoplasmic polysomes and released into the cytosol (3-5). They are then imported into the mitochondrion in a step which is, with most but not all proteins, dependent on an electrical potential across the inner mitochondrial membrane (6, 7). With the majority of precursor proteins, this transfer is accompanied by proteolytic processing (3).

Several hundred different proteins are transported into the mitochondria by this mechanism. Studies with a number of mitochondrial proteins have clearly indicated that more than one pathway exists. Cytochrome *c* appears to use a receptor and a mechanism for transmembrane transfer which is not shared with any other protein tested so far (6, 8). Mitochondrial porin, a protein of the outer mitochondrial membrane, seems also to have an assembly pathway different from other mitochondrial proteins (5). On the other hand, we consider it unlikely that every precursor protein has a specific receptor and uptake mechanism; rather, we suggest that groups of different proteins may be transported by a common pathway. A number of different precursor proteins may then have a common structure required for the interaction with the components of that pathway. One indication that this is actually true is the observation that precursor proteins of *Neurospora* can be imported *in vitro* into mitochondria from organisms phylogenetically as distant as rat and yeast (7, 9).

Here, we address the question of common recognition structures on different precursors and a limited number of receptor structures on mitochondria by investigating the special case of intracellular transport of subunit 9 of the mitochondrial ATPase.

In yeast, subunit 9 of the mitochondrial ATPase is coded for by mitochondrial DNA and is translated on mitochondrial ribosomes (10, 11). It is inserted into the inner membrane from the matrix side. No cleavage of a presequence is involved in this process; in fact, the initiating formylmethionine is retained in the mature protein (12, 13). In contrast, in *Neurospora* and in higher organisms, subunit 9 is coded for by a nuclear gene (14), is synthesized by cytoplasmic polysomes as a larger precursor (15), and is post-translationally transferred into the inner membrane with concomitant proteolytic processing (8). Yeast and *Neurospora* subunit 9 are closely related in their amino acid sequences with some 40% homology (16). The question then is: can the *Neurospora* precursor be imported into yeast mitochondria and processed, although this is clearly not the assembly pathway *in vivo*? We show here that indeed the heterologous precursor is imported by yeast mitochondria and correctly processed to the mature size.

MATERIALS AND METHODS

Growth of *Neurospora crassa* wild type 74A, radioactive labeling of cells, and isolation of mitochondria was carried out as described (4, 17). *Saccharomyces cerevisiae* diploid wild type strain 211-1b was grown according to Maccecchini *et al.* (18). Mitochondria were isolated after converting cells to spheroplasts according to the following procedure. After washing in distilled water, cells were incubated in 0.1 M Tris, 10 mM dithioerythritol, pH 9.2, for 5 min at 30 °C. Then, cells were harvested again and resuspended in 1.3 M sorbitol, 10 mM MOPS,[1] pH 7.2, at 300 mg (wet weight)/ml and incubated with 3 mg/ml of Zymolase 5000 (Seikagaku Kogyo Co., Ltd., Tokyo, Japan) for 20 min at 30 °C. Cells were washed once in sorbitol/MOPS buffer and resuspended in 250 mM sucrose, 1 mM EDTA, 10 mM MOPS, pH 7.2, at a concentration of 1 g of spheroplasts (wet weight)/10 ml. After addition of PMSF (final concentration 1 mM), they were homogenized in a Dounce homogenizer by 10 strokes. After a low speed spin at 1,000 × *g* for 5 min, mitochondria were pelleted at 17,300 × *g* for 10 min.

Synthesis of *Neurospora* precursor proteins in rabbit reticulocyte lysates and transfer into isolated mitochondria was performed according to published procedures (7, 17).

Isolation of *Neurospora* ATPase subunit 9 and preparation of antibodies was carried out as described (7). Immunoprecipitation from mitochondria was performed under the following conditions. Mitochondria (250 μg of protein) were dissolved in 50 μl of 2% SDS, 5% 2-mercaptoethanol, 60 mM Tris-HCl, pH 6.8, and boiled for 3 min. 1 ml of Triton buffer (1% Triton X-100, 0.3 M NaCl, 5 mM EDTA, 10 mM Tris-HCl, pH 7.5) and 10 μl of a 0.1 M solution of PMSF and *o*-phenanthroline in ethanol was added. The solution was freed of insoluble material by centrifugation for 15 min at 27,000 × *g*. Protein A-agarose (Sigma) with bound immunoglobulins against subunit 9 was added to the supernatant and the mixture was shaken for 30 min at 4 °C. The protein A-agarose immune complex was washed three times in Triton buffer and once in 10 mM Tris-HCl, pH 7.5. The

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[1] The abbreviations used are: MOPS, 4-morpholinepropanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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immunoprecipitates were dissolved in 100 μl of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 60 mM Tris-HCl, pH 6.8, by shaking for 30 min at 4 °C and then heating to 95 °C for 2 min.

Precipitation of precursor to subunit 9 from reticulocyte lysate supernatants was carried out as follows. The supernatant was adjusted to 1% Triton, 0.3 M NaCl, 5 mM EDTA and centrifuged for 15 min at 27,000 × *g*. Antiserum was added to the supernatant, and the mixture was incubated for 16 h at 4 °C. Then, the sample was mixed with preswollen protein A-agarose for 30 min. Washing and dilution of immunoprecipitates was carried out as described above.

For microscale sequencing experiments, the precursor was synthesized in a reticulocyte lysate in the presence of 0.08 mCi/ml of [^{3}H] tyrosine (76 Ci/mmol, New England Nuclear). The postribosomal supernatant was subjected to immunoprecipitation. The mature protein was obtained by labeling intact cells with [^{3}H]tyrosine (100 μCi/100 ml of culture) for 15 min. Mitochondria were isolated and subunit 9 was immunoprecipitated. *In vitro* transferred subunit 9 was obtained by carrying out a transfer experiment with yeast mitochondria as described in the legend to Fig. 1. Subunit 9 was immunoprecipitated after reisolating the mitochondria.

The immunoprecipitates were dissolved and subjected to electrophoresis. The gels were fluorographed (19). The radioactive bands were cut out; the gel slices were washed in water, and extracted with 80% (v/v) formic acid. After removal of the formic acid, the proteins were subjected to manual Edman degradation (20).

Electrophoresis on 16% polyacrylamide gels was performed according to Laemmli (21). After fluorography (19) dried gels were analyzed as described (17).

RESULTS

Neurospora Subunit 9 Is Transferred into Yeast Mitochondria—The precursor to subunit 9 was synthesized in a reticulocyte lysate programmed with *Neurospora* RNA in the presence of [^{35}S]methionine. It could be immunoprecipitated from the postribosomal supernatant (Fig. 1, *track 1*) with the antibody against the mature subunit 9. The postribosomal supernatant was incubated either with mitochondria isolated from *Neurospora* or yeast. In both cases, the mitochondria, which were reisolated after incubation, contained processed subunit 9 (Fig. 1, *tracks 2* and *4*). Subunit 9 processed *in vitro* had the same apparent molecular weight as mature subunit 9 immunoprecipitated from *Neurospora* mitochondria labeled *in vivo* (Fig. 1, *track 3*). *Neurospora* subunit 9, transferred into yeast mitochondria *in vitro*, is largely resistant to Proteinase K (Fig. 1, *track 5*). Breakdown of the membrane potential of the yeast mitochondria by addition of valinomycin (Fig. 1, *track 6*) or carbonyl cyanide *p*-chlorophenylhydrazone (not shown) leads to a complete elimination of import and processing. The same has been found for the import of the *Neurospora* precursor into *Neurospora* and rat liver mitochondria (7). Transfer into *Neurospora* mitochondria was completely inhibited by a combination of oligomycin and antimycin A plus KCN which inhibit the formation of a membrane potential either through electron transport or through the oligomycin-sensitive ATPase (7). The import of *Neurospora* subunit 9 into yeast mitochondria was also largely inhibited by these agents (Fig. 1, *track 7*).

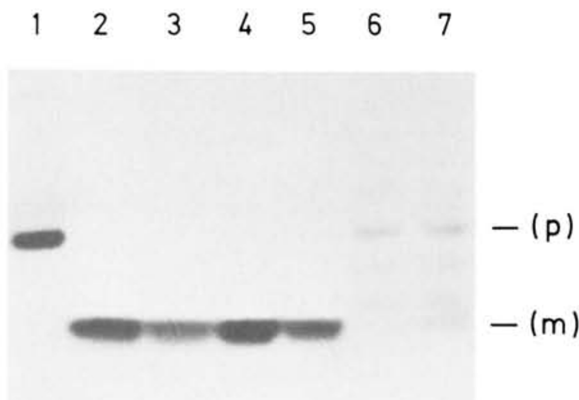

FIG. 1. **Transfer of the *Neurospora* precursor to subunit 9 into yeast mitochondria.** *1*, Precursor immunoprecipitated from reticulocyte lysate supernatant; *2*, transfer into *Neurospora* mitochondria; *3*, mature subunit 9 immunoprecipitated from [^{35}S]sulfate labeled mitochondria; *4–7*, transfer into yeast mitochondria; *4*, control transfer; *5*, after transfer mitochondria were suspended in isotonic medium and then treated with 300 μg/ml of Proteinase K for 1 h at 0 °C; *6*, transfer in the presence of 2 μM valinomycin; *7*, transfer in the presence of 10 μM oligomycin, 4 μM antimycin A, 1 mM KCN. (*p*), precursor; (*m*), mature subunit 9.

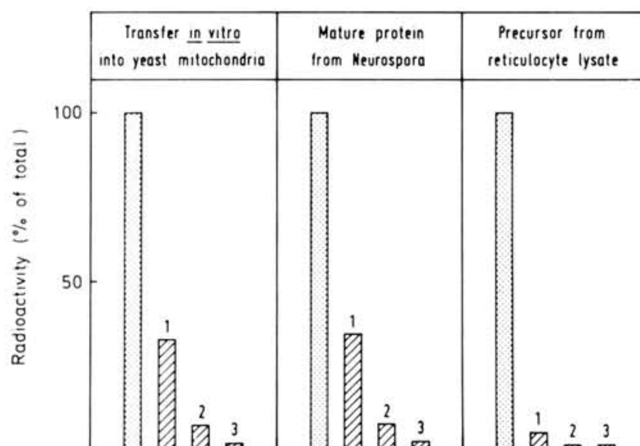

FIG. 2. **Edman degradation of different forms of subunit 9 labeled with [^{3}H]tyrosine.** The total activities employed were: subunit 9 transferred into yeast mitochondria, 1160 cpm; mature subunit 9 from *Neurospora* mitochondria, 1640 cpm; and precursor protein, 2800 cpm. Total radioactivities in each case were set to 100% (*dotted bars*) and the radioactivities released in the first three Edman cycles are expressed as % of total radioactivities (*hatched bars*).

The Precursor to Neurospora Subunit 9 Is Correctly Processed by Yeast Mitochondria—Neurospora subunit 9 was isolated from yeast mitochondria subsequent to transfer and processing *in vitro*. The precursor in this experiment was synthesized in the presence of [^{3}H]tyrosine, which is the NH_2-terminal amino acid in the mature protein. A total of two tyrosine residues are present in the *Neurospora* protein (16). Edman degradation was performed, and the radioactivity released during the first three steps was compared to that obtained when the precursor was analyzed. Also, mature subunit 9, labeled *in vivo* by adding [^{3}H]tyrosine to the culture medium, was subjected to Edman degradation. As shown in Fig. 2, about 30% of total radioactivity appeared in the first Edman step when both mature subunit 9 and subunit 9 transferred into yeast mitochondria were sequenced. In contrast, no significant radioactivity was released in the first three steps when the precursor protein was analyzed (Fig. 2). These data show that *Neurospora* subunit 9 is correctly processed when transferred into yeast mitochondria *in vitro*.

DISCUSSION

We have shown that yeast mitochondria have the ability to import and correctly process a protein that in yeast cells is synthesized within mitochondria and inserted from the matrix side. The most likely explanation of this phenomenon is that the import mechanism is not constructed in such a way that each precursor protein has its own uptake device. Rather, recognition on the mitochondrial surface must involve structures not specific for individual mitochondrial precursor proteins. Also, further steps in the transfer process such as translocation across the outer membrane and proteolytic processing appear to be common for different mitochondrial pro-

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teins. The observation that the yeast mitochondrial processing enzyme(s) correctly process a precursor protein from a different organism, which in yeast is assembled via a quite different pathway, suggests that there must be signals common to at least classes of mitochondrial precursors. On the other hand, it is clear that there is not one unique pathway. Subunit 9 of ATPase belongs to that class of proteins which requires an energized inner membrane for transfer (7). Within this class it falls into the group of precursors which are proteolytically processed during or after membrane translocation. This group contains the majority of proteins studied so far. Precursor proteins which belong to a second class, not requiring a membrane potential, are cytochrome *c* and the porin of the outer mitochondrial membrane (6, 5). These two proteins do not have to be inserted into or translocated across the inner membrane. Both of these proteins are not proteolytically cleaved during assembly. However, they do use different pathways, as demonstrated by the observation that excess unlabeled apocytochrome *c* does not compete with porin for assembly.[2] Furthermore, cytochrome b_2 (22) in yeast and cytochrome c_1 (9, 22) in yeast and *Neurospora* are transported and assembled in a two-step processing event which seems to involve a "detour" of the precursors to the matrix before they reach their functional site in the intermembrane space or at the outer face of the inner membrane (22).

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