

DEVELOPMENTS IN GENETICS

Volume 2

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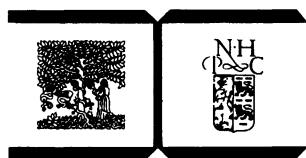
Volume 2 The Organization and Expression of the Mitochondrial Genome
A.M. Kroon and C. Saccone editors

THE ORGANIZATION AND EXPRESSION OF THE MITOCHONDRIAL GENOME

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Editors

A.M. KROON
and
C. SACCOME



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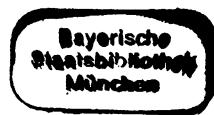
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POSTTRANSLATIONAL TRANSPORT OF PROTEINS IN THE ASSEMBLY OF MITOCHONDRIAL MEMBRANES

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INTRODUCTION

Assembly of the mitochondrion involves the transfer of a large number of proteins from the cytosol to the various subcompartments of this organelle. During the last years we have accumulated a body of evidence that this transfer occurs by a posttranslational mechanism ¹⁻⁹. Such a mechanism implies the existence of extramitochondrial pools of precursor proteins. In order to understand the transfer on a molecular basis, the overall reaction must be dissected into a number of individual steps. Furthermore, the signals on the precursors and the complementary structures on mitochondria which are responsible for the specificity of intracellular traffic must be investigated. A number of representative proteins should be studied to assess whether differences in the assembly pathway exist for the various proteins, e.g. soluble vs. integral membrane proteins, matrix vs. intermembrane proteins. We report here on the transport of cytochrome c, a peripheral membrane protein at the c-side of the inner membrane, on that of the ADP/ATP carrier, an integral transmembrane protein of the inner membrane ¹⁰, and on that of "subunit 9" of the ATPase complex, also an integral protein of the inner membrane.

RESULTS AND DISCUSSION

CYTOCHROME C. A peculiar step in the biosynthesis of cytochrome c is the covalent attachment of the haem group to the apoprotein. The question whether the haem group is added before or after completion of the polypeptide chain, was answered in the following way. *Neurospora* cells were first labelled with ³H leucine and were then pulse labelled with ³⁵S methionine at 8°C. At various times after the pulse cells were frozen in liquid N₂, disrupted and extracted. Antibodies specific for *Neurospora* apocytochrome c and holocytochrome c, respectively, were employed to immunoprecipitate the two components from each of the samples. Fig. 1 shows the labelling kinetics of apo- and holocytochrome c. This data demonstrates that apocytochrome c is present in the cells and suggests a precursor-product relationship between apo- and holocytochrome c.

The haem group is linked in a reaction with occurs posttranslationally.

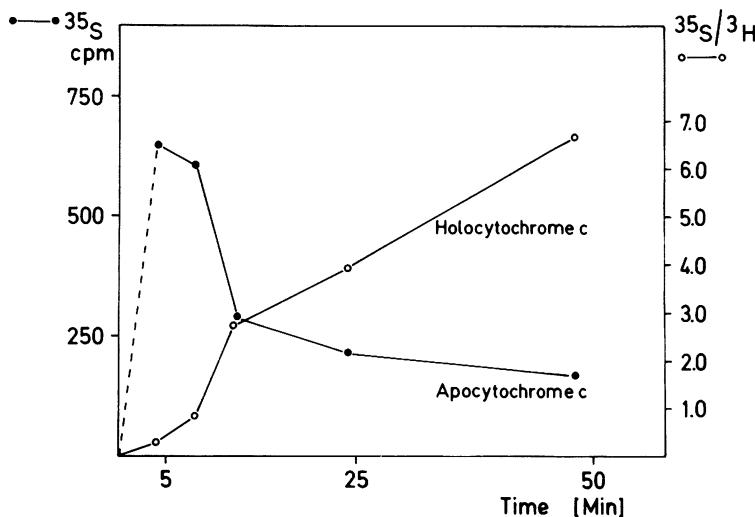


Fig. 1. Kinetics of labelling of apo- and holocytochrome c in *Neurospora* cells.

Neurospora cells were labelled with ^3H leucine after 12h growth. After further 2h cells were cooled to 8°C and after further 1h ^{35}S methionine was added to the culture. At the time points indicated, aliquots were withdrawn, the cells rapidly harvested, frozen in liquid N_2 , broken by grinding and extracted with Triton containing buffer. Then from each sample apo- and holocytochrome c were immunoprecipitated with specific antibodies. Immunoprecipitates were analysed by SDS gel electrophoresis and radioactivities in the cytochrome c peaks determined.

In order to decide whether the apocytochrome c found in this experimental system is the primary translation product or a component already processed, apocytochrome c was translated in cell free heterologous systems. The *in vitro* product had the same size as the isolated apocytochrome c, and the amino terminal sequence was identical to that of holocytochrome c⁵. This is in agreement with recent results on the DNA sequence of the coding region for iso-1-cytochrome c from yeast¹¹.

To elucidate the intracellular pathway of apocytochrome c, the relation between synthesis and haem incorporation was investigated in reconstituted systems. For this purpose protein synthesis was carried out in homologous and heterologous cell free systems, then postribosomal supernatants were prepared and incubated with isolated mitochondria. Fig. 2 shows that apocytochrome c,



Fig. 2. Protease resistance of cytochrome c transferred into mitochondria in vitro.

Neurospora poly(A)RNA was translated in a reticulocyte lysate in the presence of ^{35}S methionine and the postribosomal supernatant prepared. Two samples of this supernatant were incubated with mitochondria (0.5 and 1 mg protein per ml, respectively) for 60 min. Then mitochondria were reisolated by centrifugation and resuspended in sucrose buffer. One half of each sample was treated with proteinase K for 60 min at 0-4°. Then PMSF was added to all samples and they were lysed with 1% Triton. Immunoprecipitation was carried out with antibody against holocytochrome c. Immunoprecipitates were subjected to SDS gel electrophoresis and autoradiography. Arrow indicates position of stained holocytochrome c. Proteinase K was shown in a separate experiment to digest holocytochrome c in solution under the conditions applied here.

Lanes 1 and 3: 0.5 mg mitochondrial protein per ml; lanes 2 and 4: 1 mg mitochondrial protein per ml; lanes 1 and 2: control; lanes 3 and 4: treated with proteinase K.

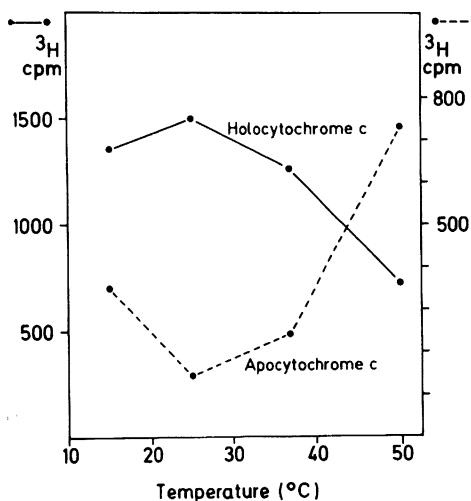


Fig. 3. Temperature dependence of the formation of holocytochrome c from apocytochrome c in a reconstituted system.

A postmitochondrial supernatant of a Neurospora homogenate was incubated with ^3H leucine for 10 min. Then a 1 h 150 000 x g supernatant was prepared and incubated for 30 min with mitochondria isolated from unlabelled cells at the temperatures indicated. After incubation, the mixtures were lysed with Triton and divided into two equal portions. Immunoprecipitation with antibodies specific for apo- and holocytochrome c was performed, immunoprecipitates were analysed by SDS gel electrophoresis and radioactivities in the cytochrome c peaks determined.

present in the supernatant of a reticulocyte lysate programmed with *Neurospora* poly(A)RNA is transferred into added isolated *Neurospora* mitochondria and converted to holocytochrome c. The newly formed holocytochrome c is resistant to added protease. This suggests translocation across the outer mitochondrial membrane. The same observation was made when a supernatant of a homologous cell free system was employed.

Fig. 3 shows the temperature dependence of the apo- to holocytochrome c conversion. There is an optimum at about 25°C. Furthermore, this experiment gives a quantitative evaluation, indicating that the conversion occurs with a high efficiency. More than 90% of the apo form is converted to the holoprotein at optimal temperature.

Intact mitochondria are a prerequisite for linkage (incorporation) of the haem moiety with the apoprotein. Addition to the supernatant of haemin chloride, of detergent-lysed mitochondria, sonicated mitochondria or hypotonically pre-swollen mitochondria does not lead to the formation of holocytochrome c (Fig. 4). Excess apocytochrome c from *Neurospora* but not excess holocytochrome c can compete for the transfer and conversion of apocytochrome c synthesized in the cell free system.

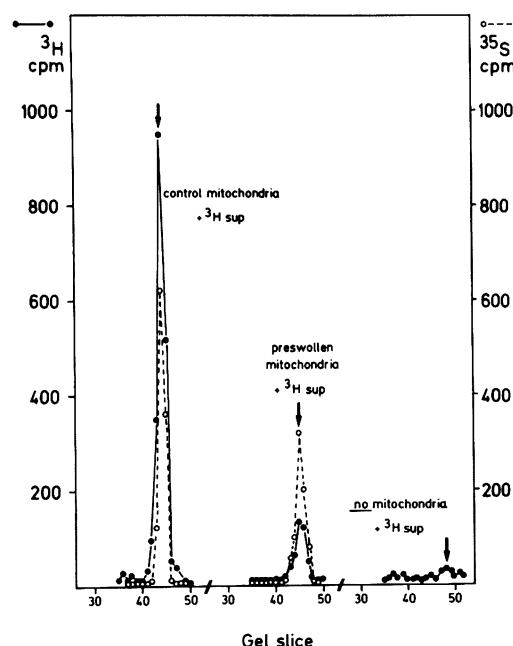


Fig. 4. Dependence of transfer in vitro of cytochrome c on intactness of isolated mitochondria.

A postmitochondrial supernatant of a *Neurospora* homogenate was incubated with ^3H leucine for 10 min. Then a 1 h 150 000 \times g supernatant was prepared and incubated a) with mitochondria isolated from cells grown on ^{35}S sulfate, b) with mitochondria as in a, but preswollen with 10 mM Tris HCl, c) without added mitochondria, but with haemin chloride (30 μM). After incubation for 30 min, Triton was added, immunoprecipitation and SDS gel electrophoresis of immunoprecipitates were carried out. Gels were sliced and ^3H and ^{35}S radioactivities determined. Arrow indicates position of co-electrophoresed holocytochrome c on the gel.

On the basis of this data we propose the following assembly pathway for cytochrome c. Apocytochrome c, synthesized on free polysomes is released into the cytosolic compartment. Its conformation is such that it penetrates the outer membrane with part of the molecule through a pore. An enzyme which catalyses the formation of the thioether bridge between apocytochrome c and haem acts in the intermembrane space. The addition of the prosthetic group triggers the folding of the polypeptide chain in such a way, the molecule is completely translocated across the outer membrane and bound to its functional site on the inner membrane.

ADP/ATP CARRIER

The synthesis of the ADP/ATP carrier protein can be observed in homologous and heterologous cell free systems. The translation product (apparent molecular weight 32 000) in all cases has the same electrophoretic mobility on SDS polyacrylamide gels as the authentic protein in the inner membrane. The *in vitro* translation product was found in the postribosomal supernatant. Analysis by sucrose density gradient centrifugation and gel filtration showed that the *in vitro* product occurs in the form of a higher molecular weight complex and that it interacts with detergents.

Reconstitution experiments were carried out to demonstrate the transmembranous transfer *in vitro*. Reticulocyte lysates were programmed with *Neurospora* mRNS and the postribosomal supernatant was separated after translation. Mitochondria were isolated from *Neurospora* spheroplasts. Supernatant and mitochondria were incubated for various time periods, then they were separated again by centrifugation. The mitochondrial samples were divided into two equal portions. One part remained untreated and the other half was treated with proteinase K at 0°C. The latter treatment leads to digestion of extramitochondrial ADP/ATP carrier but not, or only to a limited degree, of the carrier in the intact mitochondria. ADP/ATP carrier was immunoprecipitated from the supernatants, and from proteinase treated and control mitochondria. The immunoprecipitates were analysed by SDS gel electrophoresis, and yielded one single band (32 K) by autoradiography. The X-ray films were subjected to densitometry and the extinction of the 32 K band was plotted vs. the time of incubation of mitochondria with supernatant. The protein is rapidly bound to the mitochondria (Fig. 5). In studies, in which the radioactivity was determined in sliced gels, 70 - 90% of the *in vitro* synthesized carrier was found to be bound to the mitochondrial fraction after 60 min incubation. The appearance of protease resistant carrier is also shown in Fig. 5. It makes up ca. 20 - 30% of the

total carrier synthesized in vitro.

These in vitro transfer experiments were also made in the presence of carbonylcyanide-*m*-chlorophenyl-hydrazone (CCCP). As can be seen from Fig. 5, CCCP does not inhibit the binding but it does inhibit transfer of the carrier into a protease resistant position. A similar effect was observed, when the temperature was lowered to 0 - 4°C. Inhibition of transfer of ADP/ATP carrier by CCCP in intact cells has already been reported⁷. Furthermore, with yeast cells it was found that CCCP does not inhibit the synthesis but the proteolytic processing of a number of mitochondrial proteins which are synthesized as larger precursors¹².

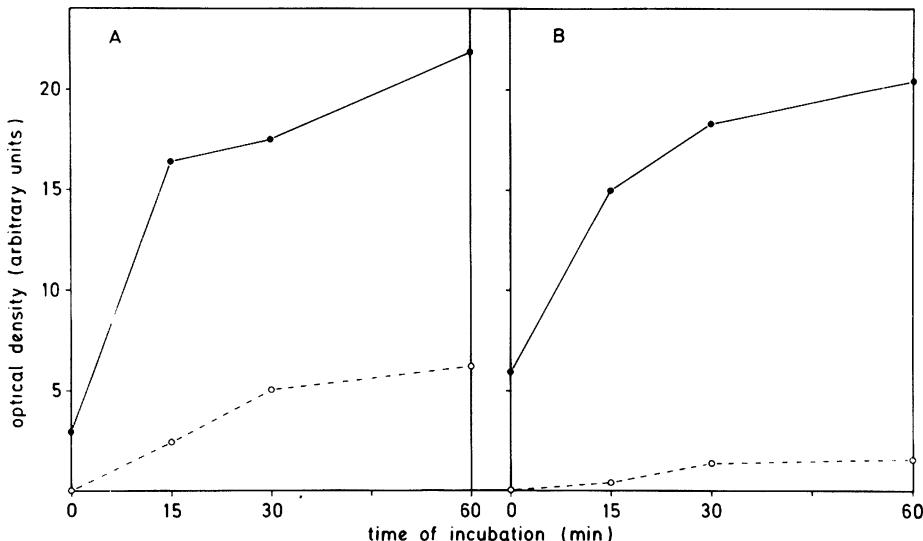


Fig. 5. Transfer of ADP/ATP carrier into mitochondria in vitro.

A reticulocyte lysate was programmed with *Neurospora* poly(A)RNA and, after incubation of ^{35}S methionine, the postribosomal supernatant was prepared. Mitochondria isolated from *Neurospora* spheroplasts were resuspended in the supernatant. After incubation for 60 min at 25°C, supernatant and mitochondria were separated again by centrifugation. One half of the mitochondria were treated with proteinase K at 0°C for 60 min. In a parallel experiment, incubation of supernatant with mitochondria was carried out after addition of 12.5 μM CCCP. Then Triton was added, the ADP/ATP carrier was immunoprecipitated from all fractions, the immunoprecipitates were electrophoresed, the gels autoradiographed and the X-ray films subjected to densitometry.

A: without CCCP; B: with CCCP. \bullet — \bullet : total ADP/ATP carrier bound to mitochondria; $-o--o-$: proteinase K resistant ADP/ATP carrier in mitochondria.

Isolation of the ADP/ATP carrier from the membrane includes as the most important step passage of Triton-solubilized mitochondria over hydroxyapatite¹⁰. Only a few mitochondrial proteins pass through this bed, the major component is the ADP/ATP carrier. When the postribosomal supernatant of a reticulocyte lysate containing newly synthesized carrier and supplemented with Triton was passed over hydroxyapatite, the ADP/ATP carrier protein was found to be completely retained (Fig. 6). However, when mitochondria after transfer *in vitro* were lysed and subjected to the same procedure, part of the carrier was detected in the eluate. This finding supports the view that part of the carrier after its interaction with the mitochondria, is actually integrated into the membrane.

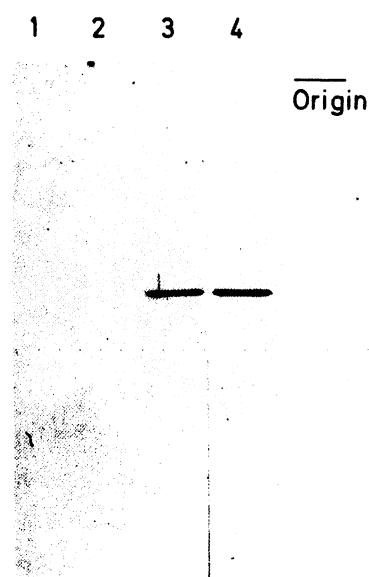


Fig. 6. Chromatography of ADP/ATP carrier on hydroxyapatite before and after transfer *in vitro*.

After translation of poly(A)RNA in a reticulocyte lysate in the presence of ³⁵S methionine, one aliquot of the postribosomal supernatant was made 1% Triton and passed over hydroxyapatite (lane 2). To a second aliquot, Carboxyatractyloside (CAT) was added and the sample was treated in the same way as in 2 (lane 1). A third aliquot was incubated with isolated mitochondria, mitochondria were reisolated and incubated with CAT; then one half was treated with proteinase K (lane 3) one half remained untreated (lane 4). Mitochondria were solubilized and passed over hydroxyapatite. The eluates were subjected to immunoprecipitation; the immunoprecipitates were analysed by electrophoresis and autoradiography.

These results show that there is an extramitochondrial (cytosolic) precursor form of the ADP/ATP carrier which is transferred into mitochondria *in vitro*. They clearly confirm earlier conclusions derived from *in vivo* experiments, that the intracellular translocation of this protein occurs via a posttranslational mechanism^{1,7}. They further show that integral membrane proteins can have precursors existing in the cytosol compartment without a "signal extension" in their amino acid sequence which is cleaved upon membrane insertion. The complicated assembly pathway can be divided into at least two steps: binding to the outer membrane and integration into the inner membrane. We envision that the first

step entails a "receptor" type molecule at the surface of the outer membrane. The second, rather complex, step is apparently dependent on energy, e.g. in the form of a membrane potential.

"SUBUNIT 9" OF ATPASE COMPLEX

This integral membrane protein is synthesized as a precursor possessing a larger apparent molecular weight, as first shown by translation of *Neurospora* poly(A)RNA in a wheat germ extract¹³. Fig. 7 shows a comparison of the protein isolated from the membrane with that obtained by translation in a reticulocyte lysate. The apparent molecular weight difference is ca. 6 000. "Subunit 9" shares the property of being synthesized as a larger precursor with a number of other mitochondrial proteins^{9, 14-18}.



Fig. 7. Synthesis in vitro of "subunit 9" of ATPase complex and transfer in vitro to mitochondria.

A reticulocyte lysate was incubated with poly(A)RNA and ³⁵S methionine for 60 min and the postribosomal supernatant was prepared. From an aliquot "subunit 9" was immunoprecipitated (lane 2). A second aliquot was incubated with isolated mitochondria, then "subunit 9" immunoprecipitated from the reisolated mitochondria (lane 3). "Subunit 9" was also immunoprecipitated from mitochondria isolated from cells grown on ³⁵S sulfate (lane 1). Immunoprecipitates were analysed by SDS gel electrophoresis and autoradiography.

We have also evaluated for this precursor, where and how it is present in the reticulocyte lysate. Similar to the ADP/ATP carrier it was found in the postribosomal supernatant, not as a monomer but with an apparent size larger or comparable to haemoglobin. In vitro transfer was also accomplished by incubating the reticulocyte supernatant with isolated mitochondria. Not only was "subunit 9" then found associated with mitochondria, it was also processed to the size of the authentic membrane protein (Fig. 7).

CONCLUSIONS

The three proteins investigated appear to have some common characteristics with regard to their assembly pathway, but they also show significant differences. A first common feature is that they are translocated by a posttranslational mechanism; a second one is that the extramitochondrial precursors differ in their properties, in particular in their conformation, from the functional products; a third one is that the extramitochondrial precursors pass through the cytosolic compartment.

The type of conformational change which leads to the final product apparently differs among the three proteins. In case of cytochrome *c* it is the covalent linkage of the haem group which leads to a drastic refolding of the molecule; in case of the ADP/ATP carrier refolding may occur in the step in which the precursor, bound to the outer membrane, is inserted into the inner membrane; and "subunit 9" probably experiences a conformational change when the additional sequence is cleaved. An additional sequence is not necessary for the construction of a precursor molecule; and it can be concluded that the signalling device which directs a precursor protein to its organelle may lie in the tertiary structure ("signal structure"). Those proteins which do have a larger precursor would then contain a "signal structure" which may or may not reside in the additional sequence.

Thus, in several respects posttranslational transfer into mitochondria (and chloroplasts¹⁹) appears to differ from cotranslational membrane transfer of secretory proteins according to the "signal hypothesis"²⁰.

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