Genetics, Biogenesis and Bioenergetics of Mitochondria

Proceedings of a Symposium held at the Genetisches Institut der Universität München Munich, September 11-13, 1975

Editors
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Walter de Gruyter · Berlin · New York 1976

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Session 3. Components and Mechanisms of Electron Transfer and Oxidative Phosphorylation
Chairmen: M. Klingenberg and D. F. Wilson

Translation Products in Isolated Mitochondria from Neurospora Crassa: Partial Assembly of Cytochrome Oxidase Components

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Introduction

The formation of a mitochondrial membrane which is fully competent in electron transfer and oxidative phosphorylation requires the cooperation of cytoplasmic and mitochondrial protein synthesis. Particularly, this holds for the membrane constituent cytochrome oxidase. This enzyme is composed of subunits which can be divided into two groups: one group is synthesized on mitochondrial ribosomes (subunits 1, 2 and 3) and the second group on cytoplasmic ribosomes (subunits 4, 5, 6 and 7)(1, 2). This finding implies that many complex processes must be involved in the formation of the enzyme. Accordingly, we are faced with a large number of open questions, concerning e. g. the regulation of the synthesis of the two groups of subunits, the transfer of the subunit precursors to the sites of assembly, the sequence of the assembly reaction, etc.

Two types of approaches to answer these questions appear to be promising: a) *in vivo* studies, employing radioactive pulse-chase labeling techniques together with specific inhibitors of mitochondrial and cytoplasmic translation (3); b) *in vitro* studies with cell fractions and reconstituted systems.

We have described a system in which isolated mitochondria are able to form subunits 1, 2 and 3 of cytochrome oxidase

(4). Here we present experiments which show that the cytoplasmic fraction of Neurospora contains components which have a stimulatory effect on polypeptide synthesis in isolated mitochondria and on the formation of subunits of cytochrome oxidase. Furthermore, the cytoplasmic fraction inhibits proteolysis of in vitro formed polypeptides. Finally, it is shown that partial assembly of subunit precursors of cytochrome oxidase occurs in such a reconstituted system.

Results

1. Amino acid incorporation into the protein of isolated mitochondria

Fig. 1 shows the rate of incorporation of leucine into the protein of isolated mitochondria. Incorporation in an incubation medium (see ref. 4) containing 3-H-leucine, proceeds for about 30 min. When a chase of unlabeled leucine is given after 30 min it can be observed that leucine which was already incorporated is lost from TCA-precipitable protein. This indicates a breakdown of newly formed chains. Thus, in isolated mitochondria formation and breakdown of polypeptide chains take place simultaneously.

When isolated mitochondria are resuspended in an incubation medium which contains post-ribosomal cytoplasmic supernatant, incorporation of leucine is strongly stimulated. After a chase of leucine, the amount of incorporated leucine does not decrease. Thus, the cytoplasmic supernatant has two effects: it stimulates protein synthesis and inhibits breakdown. These two effects may be based on different mechanisms since it was found that a proteinase inhibitor fraction isolated from Neurospora blocks breakdown but does not stimulate the initial rate of leucine incorporation (unpublished results).

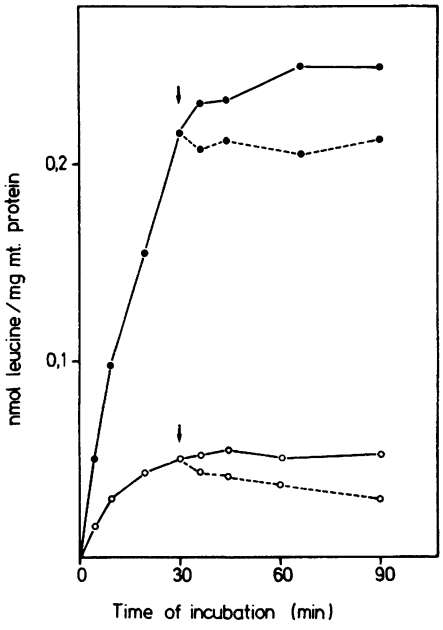

Fig. 1. Incorporation of leucine into the protein of isolated mitochondria from Neurospora crassa

Isolated mitochondria were incubated with 3-H-leucine in a medium optimized for amino acid incorporation (4) without and with cytoplasmic supernatant.

-o——o- mitochondria in incubation medium
-●——●- mitochondria in medium containing cytoplasmic supernatant

Cytoplasmic supernatant was prepared by grinding cells with sand in the presence of incubation medium (3 ml/g hyphae wet weight). The resulting suspension was centrifuged to remove cells walls, mitochondria and cytoplasmic ribosomes.

At the time indicated by arrows, a chase of unlabeled leucine (5 mM) was given to stop incorporation of 3-H-leucine

2. Products of translation in isolated mitochondria

Mitochondria were isolated from cells which were preincubated with 14-C-leucine to obtain homogeneous labeling of total mitochondrial protein. Samles of mitochondria were incubated for 30 min with 3-H-leucine in the absence and presence of cytoplasmic supernatant. Then a chase of unlabeled leucine was given for 60 min. The polypeptides synthesized under these conditions were analysed by SDS-gel electrophoresis. Fig. 2 shows the distribution of 3-H- and 14-C-label on the gels. The homogeneous 14-C-label displays the same pattern in the two samples, without (A) and with (B) added cyto-

plasmic supernatant. This pattern is very similar to the absorbance pattern after staining gels with Coomassie Brilliant Blue obtained when isolated mitochondria were subjected to gel electrophoresis. It is concluded that during the incubation no significant breakdown of mitochondrial proteins has taken place.

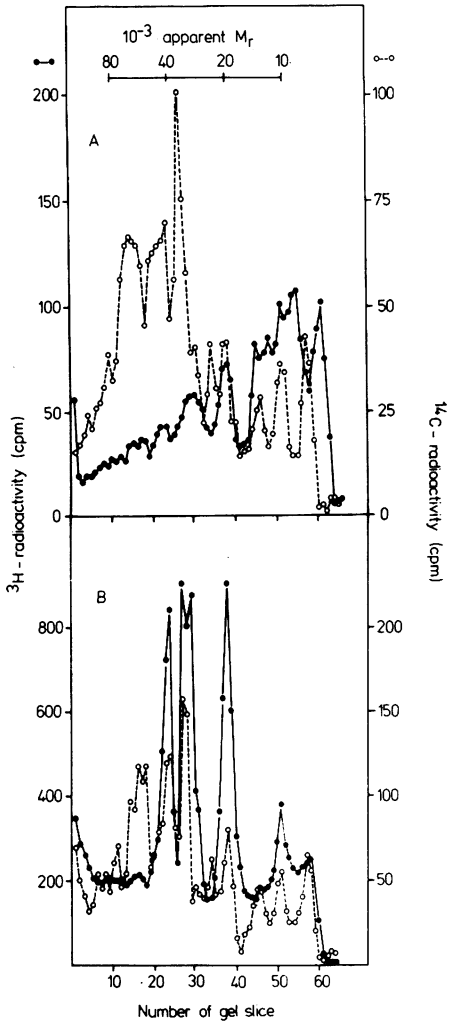

Fig. 2. Gel electrophoretic analysis of polypeptides synthesized in isolated mitochondria

Mitochondria were isolated from cells labeled with 14-C-leucine for 2 hrs. After 30 min incubation with 3-H-leucine and a 60 min chase of unlabeled leucine, mitochondria were dissolved in SDS containing buffer, dialysed, and subjected to gel electrophoresis (4).

A: Mitochondria incubated without cytoplasmic supernatant

B: Mitochondria incubated with cytoplasmic supernatant

When mitochondria are incubated without adding cytoplasmic supernatant, the bulk of mitochondrial translation products displays apparent molecular weights of less than 20 000

(Fig. 2A, 3-H-label). In contrast, the polypeptides formed in isolated mitochondria in the presence of cytoplasmic supernatant have higher apparent molecular weights and migrate as well defined bands. These polypeptides, as judged by their electrophoretic mobility, are very similar to those formed in vivo, when cells are labeled with radioactive leucine in the presence of cycloheximide (5).
The data from Figs. 1 and 2 suggest that the majority of the polypeptides formed in the absence of cytoplasmic supernatant is broken down by a specific proteinase, which attacks newly formed mitochondrial translation products but not mitochondrial proteins in general. This breakdown is blocked by cytoplasmic supernatant. After addition of the above mentioned proteinase inhibitor fraction to the incubation medium a labeling pattern very similar to that in Fig. 2A is observed.

3. Formation of subunits of cytochrome oxidase

Cytochrome oxidase was immunoprecipitated from mitochondria incubated as described in Fig. 2 with a specific antibody against the whole enzyme (3). The immunoprecipitate was analysed by SDS gel electrophoresis (Fig. 3). The 14-C-radioactivity peaks in Figs. 3A and B represent the seven subunits of cytochrome oxidase. When isolated mitochondria are incubated in the absence of cytoplasmic supernatant, only subunit 3 is found to be labeled with 3-H-leucine. The same is observed when the proteinase inhibitor fraction (see above) is added to the incubation medium. However, when the incubation medium contains cytoplasmic supernatant, 3-H-label is associated with subunits 1, 2 and 3.

These findings suggest that the cytoplasmic supernatant contains a factor which is necessary for the integration of subunits 1 and 2, and of higher amounts of subunit 3 into a complex which can be immunoprecipitated with antibody against the whole cytochrome oxidase.

In order to analyse the nature of this factor, isolated mitochondria were incubated with cytoplasmic supernatant which was isolated from cells kept for 2 hrs in the presence of cycloheximide. This cytoplasmic supernatant stimulates protein synthesis to almost the same extent as does the supernatant from control cells. However, subunits 1 and 2 were not labeled in the immunoprecipitate. These observations suggest that the factor in the cytoplasmic supernatant consists of one or more proteins, the pools of which are exhausted during cycloheximide treatment of the cells.

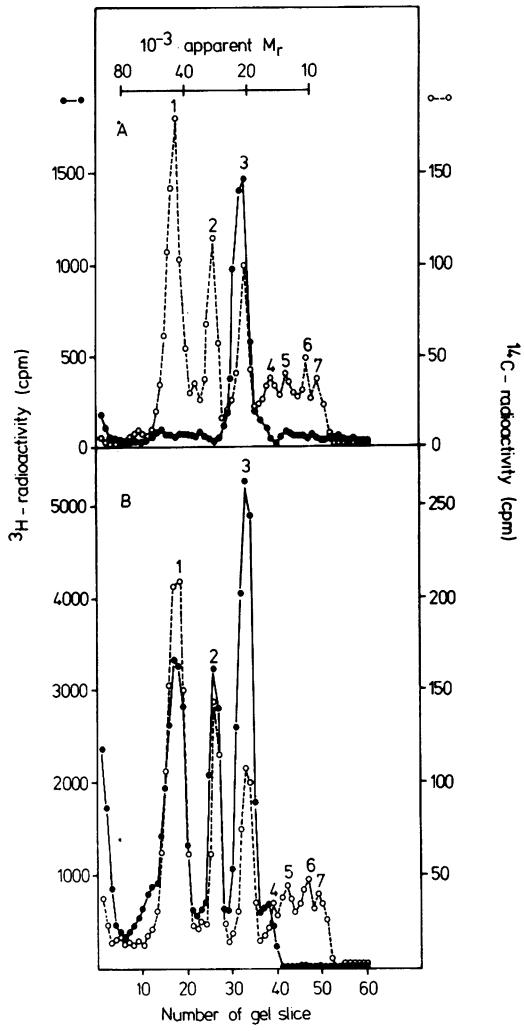

Fig. 3. Gel electrophoretic analysis of immunoprecipitated components of cytochrome oxidase from isolated mitochondria.

Mitochondria labeled as described in Fig. 2 were dissolved with Triton X-100 and treated with an antibody against whole cytochrome oxidase. The precipitate was dissolved in SDS containing buffer and analysed by SDS gel electrophoresis.

A: Immunoprecipitate from mitochondria labeled as in Fig. 2A

B: Immunoprecipitate from mitochondria labeled as in Fig. 2B

There are mainly two possibilities to explain the nature of such a factor: a) it may have some unknown catalytic function in the assembly of cytochrome oxidase; b) it may represent (a) component(s) of cytochrome oxidase, i. e. cytoplasmically made subunits of the enzyme

The second possibility is in accordance with the finding of Schwab et al. (6) that cytoplasmically made subunits are present in precursor pools of different sizes in Neurospora. Furthermore, it is supported by the following experiment.

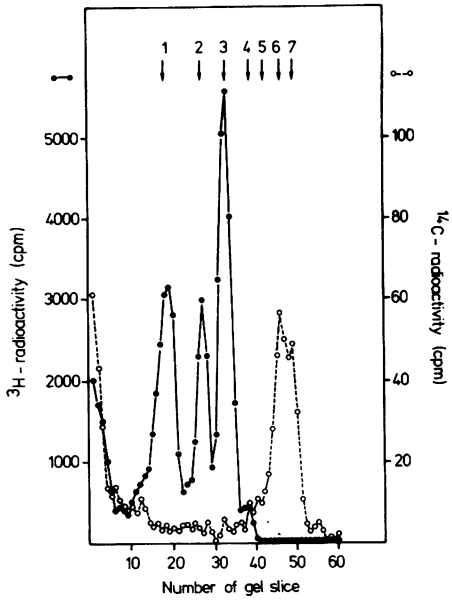

Fig. 4. Gel electrophoretic analysis of cytochrome oxidase components labeled in vitro in the presence of cytoplasmic supernatant containing 14-C-labeled proteins

Isolated mitochondria were incubated with 3-H-leucine in the presence of cytoplasmic supernatant for 30 min. The cytoplasmic supernatant was isolated from cells labeled with 14-C-leucine for 10 min. Positions of subunits 1 - 7 were determined by separating homogeneously labeled cytochrome oxidase on the same gel slab.

Whole cells were pulse labeled with 14-C-leucine and the post-ribosomal cytoplasmic supernatant was isolated. Mitochondria from unlabeled cells were incubated after addition of the 14-C-labeled supernatant in the presence of 3-H-leu-

cine. After incubation immunoprecipitation was carried out. The gel electrophoretic separation of the immunoprecipitate is shown in Fig. 4. The 3-H-label displays the integration of subunits 1, 2 and 3 into an immunoprecipitable complex, as expected from the data in Fig. 3. The 14-C-radioactivity is found in a double band. This double band coincides with the positions of subunits 6 and 7. In a control experiment it was found that no 14-C-label was precipitated when isolated mitochondria were mixed but not incubated with 14-C-labeled cytoplasmic supernatant.

These observations may be interpreted in the following way : Subunits 6 and 7 are present in the cytoplasmic supernatant as precursors and their integration into an assembly complex is necessary for the further integration of subunits 1 and 2 in an immunoprecipitable complex.

Conclusions.

Cell sap components stimulate the formation of mitochondrially synthesized polypeptides in isolated mitochondria. Cytoplasmically synthesized proteins appear to be necessary for the integration of mitochondrially made subunits 1 and 2 of cytochrome oxidase into a complex which can be immunoprecipitated with antibody against whole cytochrome oxidase. This complex may be the result of a partial assembly reaction. Mitochondria contain a specific proteinase which affects newly formed in vitro translation products but not properly integrated proteins of the inner mitochondrial membrane. It is supposed that this proteinase becomes active when mitochondrial translation products accumulate which cannot be properly integrated into mitochondrial membrane complexes. Thus, experiments with isolated mitochondria are considered to be a useful approach to the understanding of the mechanism and the regulation of the assembly of mitochondrial membrane complexes.

Acknowledgements. This work was supported by the Deutsche Forschungsgemeinschaft, We 107/1 and Ne 101/11 (Schwerpunktprogramm "Biochemie der Morphogenese").

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