GENETICS AND BIOGENESIS OF CHLOROPLASTS AND MITOCHONDRIA

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COORDINATION OF MITOCHONDRIAL AND CYTOPLASMIC PROTEIN SYNTHESIS IN NEUROSPORA CRASSA

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INTRODUCTION

The formation of some multi-component mitochondrial enzyme complexes such as cytochrome oxidase requires the cooperation of cytoplasmic and mitochondrial translation. Products from both systems are integrated to form functional enzyme complexes with definite subunit stoichiometry. Control of the coordination of these two distinct systems may be exerted at many levels, so that a general study of the coordination presents many difficulties. Recently, a detailed review on these problems was presented by SCHATZ and MASON (1).

In the present investigation we have limited ourselves to one facet of the interaction between cytoplasmic and mitochondrial translation, viz. the synthesis and membrane integration of mitochondrial translation products under conditions when cytoplasmic translation does not operate. We have linked the general aspects of translation to the synthesis and assembly of specific mitochondrial products, namely the subunits of cytochrome oxidase.

RESULTS

1. Amino acid incorporation into mitochondrial membrane proteins of cells with blocked cytoplasmic translation

Cells were prelabelled with ^{14}C-leucine to obtain homogeneous labelling of total cellular protein. At 16 hrs growth (log phase) cycloheximide (CHI) was added. After the times indicated in Fig. 1, aliquots were withdrawn, mitochondria prepared and the ^{3}H/^{14}C-ratios of the mitochondrial membrane protein determined. Labeling with ^{3}H-leucine was performed at a very low added leucine concn. (0.3 nmol/mg cell protein), a procedure usually employed to effectively label mitochondrial translation products.

When leucine was added 2.5 min after addition of CHI, incorporation proceeds for about 10-15 min and then stops. When the cells were preincubated for 62.5 min, incorporation of ^{3}H-labelled leucine still takes place and furthermore continues for a longer time span. The level of incorporation is lower, however, than in the cells exposed to CHI for a shorter time.

This observation leads to suggest that the incorporation of ^{3}H-leucine in the presence of CHI is not a true measure of the rate of mitochondrial protein

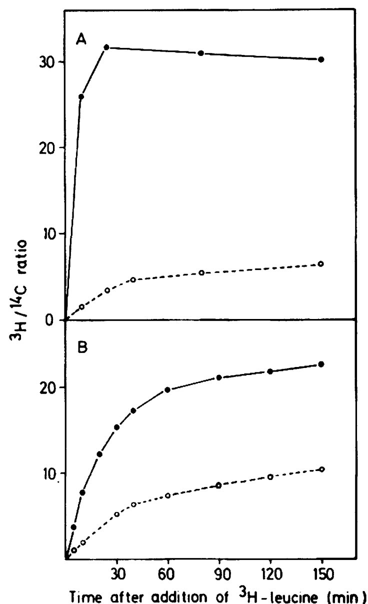

Fig. 1. Time course of 3H-leucine incorporation into mitochondria in whole cells as a function of time of incubation in cycloheximide (0.1 mg/ml).
Cells were prelabelled with 14C-leucine. Incorporation was carried out with (A) low and (B) high concn. of added leucine (see text)
-•——•- 2.5 min CHI preincub.;
-o----o- 62.5 min CHI preincub.

synthesis (see also ref. (2)). This lack of validity may be related to changes in pool size and to metabolism of intracellular leucine.

To obviate these effects, leucine was added at concns. sufficiently high to offset these changes. At the concn. added (500 nmol/mg cell protein), leucine uptake still takes place and within 10 min about 75% of the added radioactivity was accumulated in the cells. At this high added leucine concn. incorporation proceeds in both the short and long time CHI preincubation. Incorporation even continues at 150 min after addition of ^{3}H-leucine. When the initial rate of the 62.5 min preincubation sample is compared with the rate observed at 60 min with the 2.5 min preincubated sample, it can be seen that the rates are not equal. The longer preincubated sample shows a rate faster by a factor of six than the 2.5 min sample. Therefore, even at this high added leucine concn. the incorporation does not truely reflect the real rate of protein synthesis. This discrepancy between real and apparent rates can be explained by the following observations:
(a) The intracellular concns. of amino acids were found to strongly increase after exposing cells to CHI. Leucine concn. increases by a factor of about 10 during the first 90 min. (b) It was found that leucine is rapidly metabolized, a large part of the ^{3}H-radioactivity appearing in a-ketoisocaproic acid.

The effect of preincubation of cells with CHI on the protein synthesis of isolated mitochondria was measured and the results are presented in Fig. 2. The mitochondria from cells kept for 1 hr in the presence of CHI displayed a rate of incorporation which was very similar to that of mitochondria from cells treated for 2.5 min with CHI (or to that of mitochondria from untreated cells). Even after treatment of cells for 4 hrs the rate of incorporation was not reduced by more than about 30%. Similar observations were made by Sebald et al. (3).

It appears therefore that the mitochondrial protein synthesizing system is not essentially linked to the cytoplasmic system as suggested by some investigators (see ref. (1)).

2. Translational activity of mitochondrial ribosomes in cycloheximide blocked cells

In view of the unexpected lack of tight coupling of cytoplasmic and

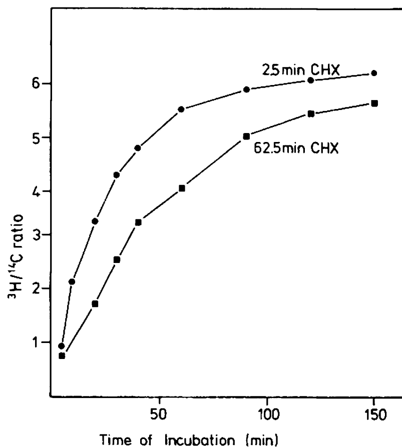

Fig. 2. Time course of 3H-leucine incorporation into isolated mitochondria from 14C-prelabelled cells and the effect of preincubation of cells in cycloheximide on this incorporation.

For experimental details see ref.(8).

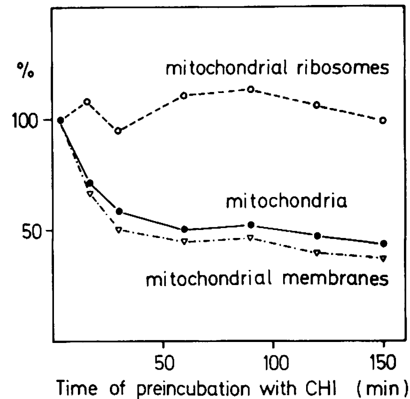

Fig. 3. The effect of cycloheximide preincubation on the in vivo incorporation of leucine into different mitochondrial fractions.
Whole cells were pulse labelled with 3H-leucine for 5 min at the indicated times. All incorporation measurements were corrected for the intracellular leucine pool. The 100% value represents incorporation after 2.5 min preincub.

mitochondrial protein synthesis, the rate of translation on mitochondrial ribosomes was measured by a different approach. Cells were preincubated for different time periods in the presence of CHI and then pulsed with ^{3}H-leucine for 5 min. To aliquots of these samples a chase of unlabelled leucine was given for 10 min. Mitochondrial ribosomes were then isolated from the pulsed cells. Mitochondria and mitochondrial membranes were isolated from the chased cells. ^{3}H-radioactivity was measured in these fractions and the actual rates of leucine incorporation were determined by correcting for the intracellular leucine concns. at the different times. The result is shown in Fig. 3. The amount of leucine in nascent polypeptide chains is found to be constant at all times of CHI preincubation. The amount of leucine incorporated into mitochondrial membrane protein however decreases during the first 30 min to about 50% compared to the control. (Preincubation for 2.5 min is taken as a control because the addition of CHI is necessary to assess specifically radioactivity in mitochondrial translation products). After 30 min preincubation incorporation stays constant at a level of about 40%.

To measure the velocity of replacement of labelled nascent chains on mitochondrial ribosomes by unlabelled ones, i.e. to determine the rate of elongation, cells were pulse labelled in the presence of CHI and then exposed to a leucine chase of increasing times. The rate of elongation was found to be the same, irrespective of

whether the cells were preincubated with CHI for 2.5 min or for 62.5 min before the pulse. Furthermore, when ribosomes from corresponding mitochondria were analysed by gradient centrifugation, the same distribution of radioactive nascent chains was seen among mono- and polymeric ribosomes, more than 80% of the ^{3}H-leucine being associated with polymers.

These observations firmly support the conclusion that mitochondrial translation continues in the absence of cytoplasmic translation. They also suggest that at least part of the polypeptides formed, are integrated into the mitochondrial membrane. They further suggest that part of the translation products are degraded since completed mitochondrial translation products were neither detected in the mitochondrial matrix nor in the postmitochondrial supernatant.

3. Translation products formed in mitochondria in cycloheximide blocked cells

Cells homogeneously labelled with ^{14}C-leucine were exposed to CHI for 2.5 min, then a pulse of ^{3}H-leucine was given for 2.5 min, followed by a 60 min chase. This experiment was made in an attempt to catch the two systems as close to equilibrium as possible. Mitochondria were isolated and subjected to SDS gel electrophoresis (Fig. 4). ^{14}C-labelled total mitochondrial proteins are mainly found at apparent MWs between 60,000 and 10,000. Welldefined mitochondrial translation products are displayed by the ^{3}H-label, which can be attributed to cytochrome oxidase, cytochrome b and OS-ATPase. It should be especially noted that the proportion of mitochondrial translation products with low apparent MWs (10,000 and less) is extremely low.

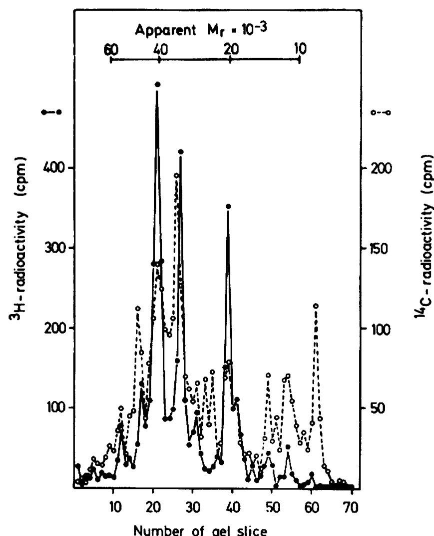

Fig. 4. SDS gel electrophoretic analysis of mitochondrial translation products synthesized in 14C-prelabelled cells during a short pulse.
Cells were pretreated with cycloheximide for 2.5 min, 3H-leucine was then applied for 2.5 min and chased for 60 min with unlabelled leucine.
For experimental details see (8).

In a further experiment, cells were preincubated with CHI for 2.5 min, 62.5 min and 122.5 min, respectively, then ^{3}H-leucine was incorporated for 45 min and a chase of unlabelled leucine was given for further 10 min. The SDS gel electrophoretic analysis of the isolated mitochondria is presented in Fig. 5. Clearly, the same products are formed in cells, in which cytoplasmic translation was blocked for 120 min compared to those blocked only for 2.5 min. However, the translation products with apparent MWs of more than 20,000 are present in lower proportion in cells treated with

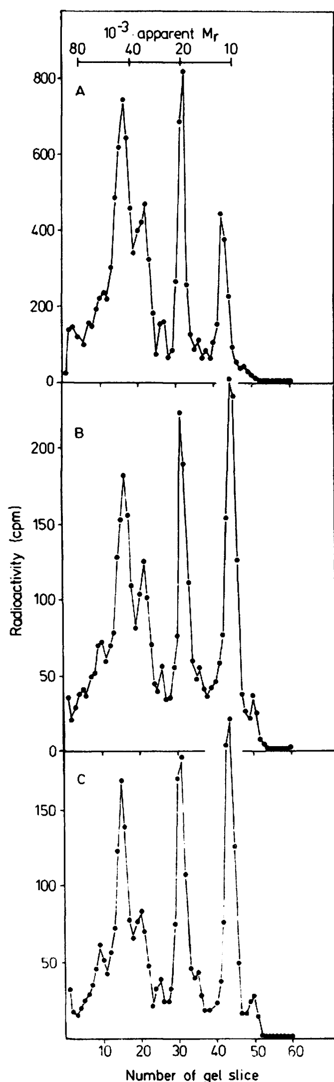

Fig. 5. SDS gel electrophoretic analysis of mitochondrial translation products synthesized after different times of pretreatment of cells with cycloheximide.
Cells were incubated with cycloheximide for (A) 2.5 min, (B) 62.5 min and (C) 122.5 min.3H-leucine was added to the cells and after further 45 min incubation a chase of unlabelled leucine was given for 10 min.

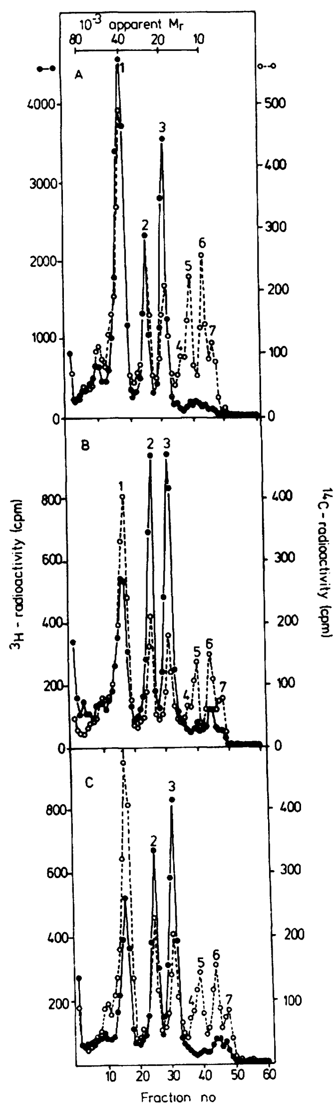

Fig. 6. SDS gel electrophoretic analysis of cytochrome oxidase immunoprecipitated from the total homogenate of cells which were prelabelled with 14C-leucine. Incubation of cells with cycloheximide and labelling with 3H-leucine was carried out as described in legend to Fig. 5. Samples A, B and C correspond to samples A, B and C in Fig. 5.

cycloheximide for longer time. Furthermore, translation products are found in increasing amounts in the low apparent MW region (11,000 and less) after longer preincubation of cells with CHI (cf. Fig. 4).

These observations suggest that original translation products are degraded to low MW components and that part of the degradation products are retained in the membrane. When mitochondrial translation products are formed only during a short pulse period immediately after blocking cytoplasmic translation, this degradation appears to be negligible. It is tempting to suggest that under these conditions cytoplasmically synthesized partner proteins are available in such amounts that the newly formed mitochondrial partners can be assembled to functional complexes and thereby preserved in the mitochondrial membrane. After longer exposure of the cells to CHI, the pools of cytoplasmic partner proteins are exhausted, the mitochondrially translated components are continued to be synthesized but only part of them can be integrated into the membrane. Another part may be degraded probably before being integrated.

4. Synthesis and assembly of cytochrome oxidase components in cells with blocked cytoplasmic translation

We next studied the influence of the availability of cytoplasmically synthesized subunits of cytochrome oxidase on the production and insertion of the mitochondrially synthesized subunits. ^{14}C-homogeneously labelled cells were exposed to ^{3}H-leucine in the presence of CHI as described in Fig. 5. After incubation, the cells were homogenized and extracted with a buffer containing Triton X-100. Cytochrome oxidase was precipitated from these extracts with an antibody against the whole enzyme. SDS gel electrophoretic analysis of the immunoprecipitates is shown in Fig. 6.

The ^{14}C-radioactivity on the gels displays the seven subunits of the enzyme. After short CHI treatment, the three mitochondrially synthesized subunits (1-3) are found to be labelled with ^{3}H in about the same ratio, at which they are present in the ^{14}C-profile, subunit 3 being somewhat higher. After 62.5 min and 122.5 min preincubation of cells still considerable synthesis of subunits 1-3 occurs. The proportions of the three subunits however change. Subunits 1 and 2 decrease with respect to subunit 3. This is in close agreement with the findings with whole mitochondrial membranes (cf. Fig. 5). In the samples preincubated with CHI for longer times, lower MW material is precipitated which may represent breakdown products of subunits 1-3 which are recognized by the antibody.

These data show that immunoprecipitation of cytochrome oxidase from total cell homogenate appears to capture not only fully assembled enzyme but also precursor polypeptides. However, if immunoprecipitation was carried out with isolated mitochondria, a quite different picture was observed. The labelling pattern in this case resembles that of the chemically isolated complex (4), showing that after short CHI exposure only small amounts of ^{3}H-labelled subunits 1 and 2 are found

(see also (5)), whereas after 62.5 min and 122.5 min CHI none of these subunits were detected. This is in agreement with the data of Schwab et al. (4), that the pool of cytoplasmically formed subunits allows assembly of total enzyme only for a few min. Subunit 3 may get into the immunoprecipitate by an exchange process or by specific attachment to a complete enzyme complex.

DISCUSSION

The data presented show that mitochondrial translation in Neurospora continues even when cytoplasmic translation is shut off for periods as long as 2 hrs, i.e. one half of a doubling period of the cells. We find no evidence for a mechanism which regulates mitochondrial translation in such a way that synthesis of mitochondrial translation products is stopped, when these products cannot any longer become assembled to functional membrane complexes. With respect to the question, what then happens to these products, the experimental data presented would suggest that (a) part of these products are taken up into the membrane and remain there, and (b) another part of the translation products is degraded to lower MW components which partly remain in the mitochondrial membrane. Such a mechanism would require the existence of specific mitochondrial proteinases, since it was observed that preexisting mitochondrial proteins are not degraded when the cells are kept in the presence of CHI. Based on experiments which were carried out on isolated mitochondria to investigate the formation of cytochrome oxidase components in a reconstituted system, we have arrived at a similar conclusion, viz. that mitochondrial translation products are formed in the absence of cytoplasmic partner proteins but are subject to proteolytic breakdown, probably since they cannot be assembled to functional complexes (6).

Cycloheximide treatment of cells may result in a situation analogous to that in some nuclear mutants. In such mutants defective assembly of cytochrome oxidase or other complexes may result from the inabilty of cytoplasmic protein synthesis to provide certain partner proteins (for review see (1)). Specific degradation of mitochondrially synthesized partner proteins may occur in these mutants in the same way as in CHI treated cells.

Further studies of the regulatory processes on the level of transcription and translation seem desireable (e.g. (7)), however our limited knowledge of these reactions imposes severe experimental limitations.

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