SYNTHESIS OF CYTOCHROME OXIDASE COMPONENTS IN ISOLATED MITOCHONDRIA OF *NEUROSPORA CRASSA*

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1. Introduction

The formation of polypeptides within the mitochondrion has been documented by a large body of evidence. The ability of isolated mitochondria to incorporate amino acids into membrane protein is an expression of this cellular capacity (for reviews see [1–6]). However, until now it has not been demonstrated that isolated mitochondria are able to synthesize definite polypeptides which are components of mitochondrial membrane enzymes.

In this communication we report that isolated mitochondria of *Neurospora crassa* under appropriate conditions do synthesize definite polypeptides which can be shown to be components of cytochrome oxidase. As described by several laboratories, this enzyme consists of seven subunits, three of which are made by mitochondrial ribosomes, and four of which are made by cytoplasmic ribosomes [7–9]. The components of cytochrome oxidase produced in isolated mitochondria were compared to the components produced in whole cells. The enzyme components were isolated by means of immunoprecipitation. It was found that the same components are made in vitro and in vivo in the presence of cycloheximide. A partial assembly of newly made cytochrome oxidase components in isolated mitochondria is discussed.

2. Materials and methods

2.1. *Protein synthesis in vivo*

Growth of *Neurospora crassa* wild type 74A was performed as described earlier [10]. Cells were labeled homogeneously with L-[^{3}H]leucine (specific radioactivity 54 Ci/mmol) (Radiochemical Centre, Amersham, England) by growing a 16 hr culture for 1 hr in the presence of the amino acid prior to isolation of mitochondria [11]. In vivo labeling in the presence of cycloheximide occurred by adding the drug (0.1 mg/ml) 2.5 min prior to the application of radioactive leucine (2 μCi L-[^{3}H]leucine/ml). After incubation of the cells for 45 min, a chase of unlabeled leucine (0.01 M) was given for 10 min. Then mitochondria were prepared [11].

2.2. *Protein synthesis in a cell free system*

For experiments with isolated mitochondria, cells were ground with fine quartz sand (Riedel–De-Haen AG, Seelze-Hannover, West Germany) and buffer A (2 g cells, 3 g sand, 1 ml buffer A). Buffer A consisted of KCl (0.03 M), sucrose (0.15 M), EDTA (0.002 M), $MgCl_2$ (0.01 M), KH_2PO_4 (0.02 M), ATP (0.004 M), triethanolamine-HCl (0.09 M), plus a mixture of all amino acids (0.00025 M), except leucine; pH was adjusted to 7.9 with KOH. After disruption of cells further 5 ml of buffer A were added. The suspension was centrifuged for 5 min at 3000 *g*. From the supernatant mitochondria were precipitated by centrifugation for 6 min at 48 000 *g*. They were washed by resuspension in 0.44 M sucrose, 0.002 M EDTA and 0.03 M Tris–HCl, pH 7.6 and centrifugation for 6 min at 48 000 *g*. The resulting mitochondrial preparation was free of any intact cells. This was verified by microscopic examination. Contribution of contaminating intact cells to leucine incorporation is also excluded by the data in fig. 2.

For preparing the incubation medium for isolated

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mitochondria, cells were disrupted as described above. After the first centrifugation step the supernatant was centrifuged for 2 hr at 144 000 *g*. The isolated mitochondria were resuspended in the resulting supernatant at a concentration of 1–2 mg mitochondrial protein per ml. Streptomycin–sulfate was added (0.06 mg/ml). 0.2 mCi of L-[^{3}H]leucine were applied per ml. Incorporation was started by adjusting the temperature to 25°C. Samples were withdrawn after different time periods up to 120 min. They were put on glass fibre papers soaked with trichloroacetic acid, and radioactivity was determined as described [12].

The concentration of leucine in the incubation medium was determined by adding definite amounts of unlabeled leucine to the medium. The leucine concentration necessary to diminish the incorporation of radioactivity by 50% represents the actual leucine concentration in the assay. In all experiments it was about ten times the K_m of the leucine incorporation. ($K_m = 5 \times 10^{-6}$ M).

2.3. *Analysis of membrane proteins*

To analyse the labeled proteins, samples of mitochondria (0.2–0.5 mg) were centrifuged for 5 min at 48 000 *g*. The pellet was dissolved in buffer B (1% sodium dodecyl–sulfate (SDS), 0.1 M Tris–HCl pH 8.0) and dialysed against buffer B until no more radioactivity appeared in the dialysis buffer. The solubilized mitochondria were then subjected to SDS-gel electrophoresis. Immunoprecipitation with antiserum against cytochrome oxidase was performed as described [13]. Conditions of SDS-gel electrophoresis and radioactivity measurement were as reported previously [14].

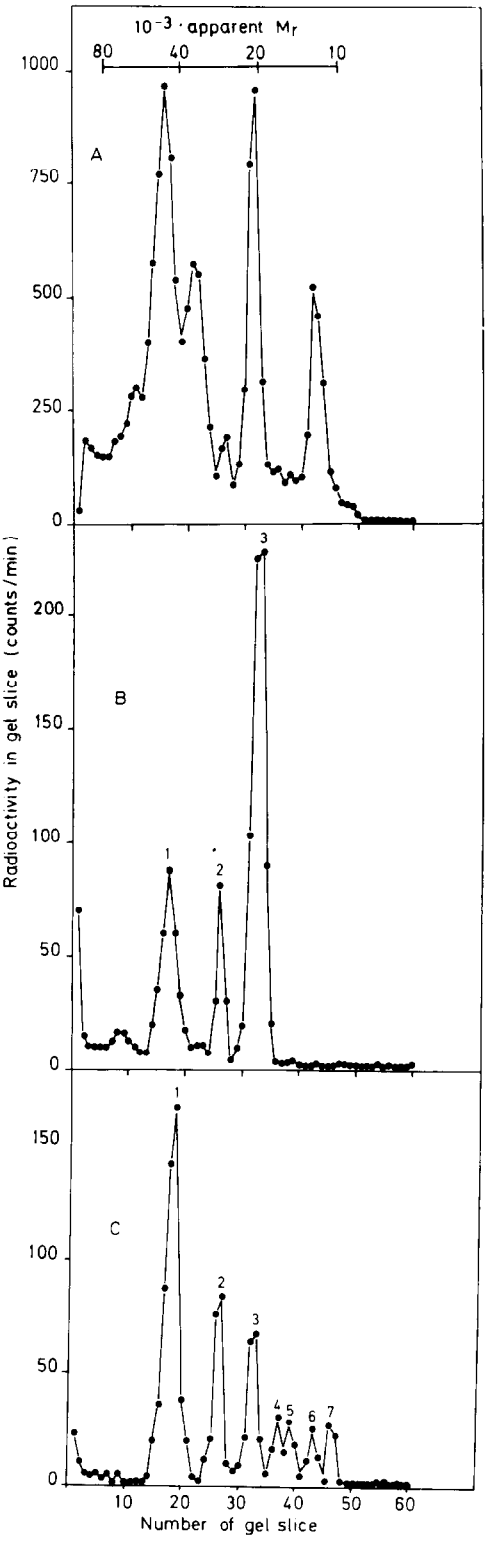

Fig. 1. Gel electrophoretic analysis of membrane proteins and of cytochrome oxidase components synthesized in mitochondria of whole cells. Cells were labeled in the presence and absence of cycloheximide. Mitochondria were prepared and either dissolved in SDS containing buffer, or solubilized with Triton X-100 and treated with antibodies against cytochrome oxidase. A) membrane proteins labeled in the presence of cycloheximide; B) immunoprecipitated components of cytochrome oxidase labeled in the presence of cycloheximide; C) immunoprecipitated components of cytochrome oxidase labeled in the absence of cycloheximide.

3. Results

3.1. *Formation of membrane proteins and of cytochrome oxidase components in mitochondria of whole cells*

Cytoplasmic protein synthesis was inhibited by addition of cycloheximide. Then cells were incubated with radioactive leucine to obtain selective labeling of mitochondrial translation products. SDS-gel electrophoresis of mitochondria resolves at least six distinct bands with apparent mol. wt. between 50 000 and 10 000 (fig. 1A). Mitochondria were solubilized with Triton X-100 and treated with antibodies against cytochrome oxidase. The resultant precipitate was analysed by SDS-gel electrophoresis. Fig. 1B shows three discrete labeled bands.

For comparison cells were incubated in the absence of cycloheximide with radioactive leucine to obtain homogeneous labeling of cytochrome oxidase. Mitochondria were prepared and cytochrome oxidase was immunoprecipitated as above. In this case gel electrophoresis resolves seven labeled bands (fig. 1C). They represent the subunits of cytochrome oxidase. The subunits 1, 2, and 3 have been reported to be synthesized by mitochondrial ribosomes, whereas subunits 4–7 are formed by cytoplasmic ribosomes [7,13]. The three components which are labeled in the presence of cycloheximide correspond to subunits 1–3 of cytochrome oxidase (fig. 1B and 1C).

3.2. *Formation of membrane proteins and of cytochrome oxidase components in isolated mitochondria*

The time course of leucine incorporation into the protein of mitochondria in a cell free system (see Materials and methods) is shown in fig. 2. Incorporation proceeds for about 30–40 min at a constant rate (approx. 6 pmol per mg mitochondrial protein and min), and stops after 70–100 min. Chloramphenicol is strongly inhibitory. Cycloheximide does not influence the rate of incorporation (fig. 2).

The gel electrophoretic labeling pattern of mitochondria incubated with radioactive leucine for 70 min is seen in fig. 3A. It is very similar to the pattern of mitochondria labeled in intact cells in the presence of cycloheximide (compare fig. 1A). In in vitro systems, which were less efficient with regard to amino acid incorporation, a much lower labeling of the proteins

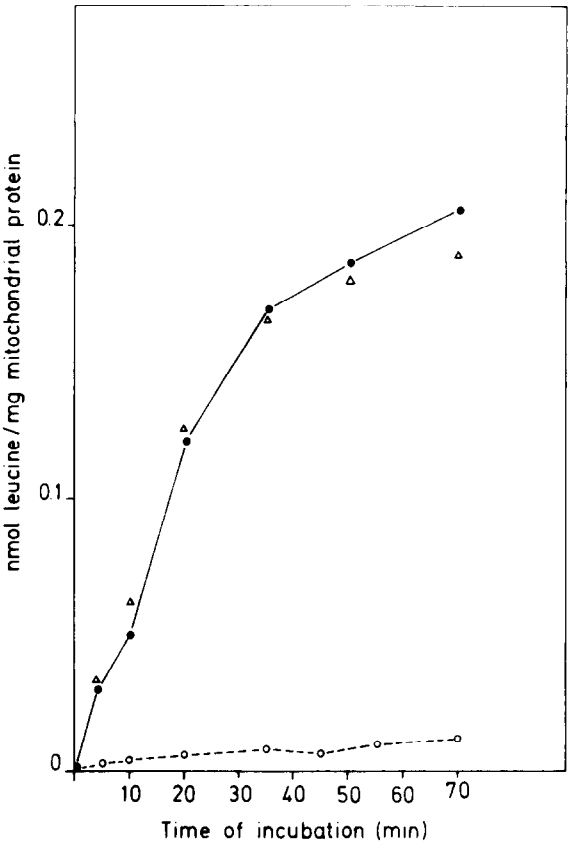

Fig. 2. Time course of leucine incorporation into the protein of isolated mitochondria in the absence and presence of inhibitors. Mitochondria were isolated and resuspended in [^{3}H]leucine containing incubation medium. One portion was incubated without any further addition (●–●). To the second portion cycloheximide (0.1 mg/ml) (△–△), and to the third one chloramphenicol (0.5 mg/ml) (○–○) was added before starting incorporation.

with apparent mol. wt. between 25 000 and 50 000 was observed [14].

Mitochondria labeled in vitro were treated with antibodies against cytochrome oxidase. The antibody precipitated approx. 4–6% of the total radioactivity incorporated. The gel electrophoretic analysis of the immunoprecipitate is shown in fig. 3B. Three radioactive bands are found. They correspond to components 1–3 of cytochrome oxidase. The relative amounts of label in the individual components are very similar to those observed after labeling of

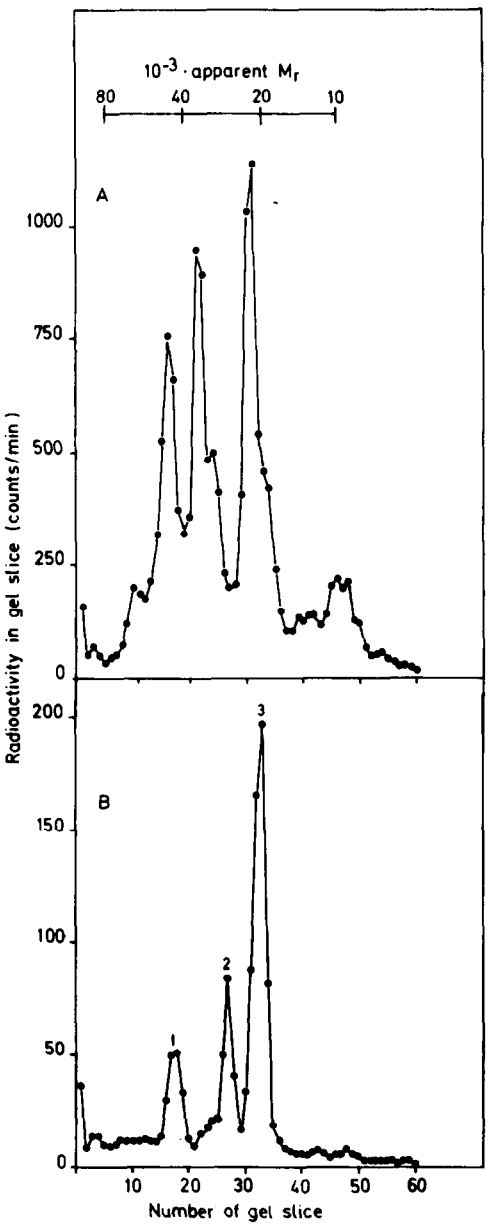

Fig. 3. Gel electrophoretic analysis of membrane proteins and of cytochrome oxidase components synthesized in isolated mitochondria. Isolated mitochondria were incubated at 25°C for 70 min in the presence of [^{3}H]leucine. Mitochondria were then analysed as described in fig. 1. A) membrane proteins; B) immunoprecipitated components of cytochrome oxidase.

whole cells in the presence of cycloheximide. Bands 1 and 2 are low, band 3 is high.

4. Discussion

The pattern of membrane proteins synthesized in isolated mitochondria under the conditions described in this report appears to be very similar to the pattern of proteins synthesized in whole cells, in which cytoplasmic protein synthesis is blocked. The components 1–3 of cytochrome oxidase constitute part of the membrane proteins synthesized in isolated mitochondria.

The question arises whether these components are subunits of completely assembled cytochrome oxidase, or whether they are present in some other state. Information for answering this question comes from experiments with whole cells.

The relative amounts of radioactive components 1–3 immunoprecipitated from mitochondria labeled in vivo under cycloheximide are different from the relative amounts observed with cells after labeling in the absence of cycloheximide. This difference is most likely explained by an incomplete assembly of cytochrome oxidase in the presence of this drug [15]. On the other hand, in vivo experiments with cycloheximide and in vitro experiments are much alike with regard to the proportions of components 1–3. Therefore, most of the material precipitated from isolated mitochondria appears to represent partially assembled cytochrome oxidase.

The precipitated components may not account for all of the precursors made in the mitochondria, but only for those present in the form of precursor complexes. This has been suggested from experiments with cycloheximide treated cells in which antibodies against isolated component 3 were employed. The antibody against the complete enzyme did not precipitate the free component 3 [13].

It is not excluded that a minor part of the labeled components represents subunits of a completely assembled cytochrome oxidase, both in isolated mitochondria and in vivo after blocking cytoplasmic protein synthesis.

These studies are regarded as an initial step to investigate the formation of cytochrome oxidase in a reconstituted system.

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