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BIOGENESIS OF THE CYTOCHROME bc_1 COMPLEX IN ISOLATED RAT HEPATOCYTES

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SUMMARY

Isolated rat hepatocytes were labeled with [^{35}S]methionine in the absence or presence of cycloheximide or chloramphenicol. The cytochrome bc_1 complex was isolated from labeled cells by a micromethod and analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. All subunits except the two smallest, subunits VII and VIII, were labeled in the absence of translational inhibitors. In the presence of cycloheximide only subunit III (molecular weight, 30 000) was labeled. This polypeptide, identified as an apo-cytochrome b, was weakly labeled with [^{35}S]methionine in the presence of cycloheximide, indicating a strict dependence of cytoplasmically synthesized products for its assembly. In the presence of chloramphenicol, labeling was inhibited only in subunit III.

INTRODUCTION

Biosynthesis of the cytochrome bc_1 complex has been studied in yeast (1-4) and in Neurospora crassa (5). No studies have been forthcoming on the biosynthesis and assembly of the mammalian cytochrome bc_1 complex. Such studies, however are increasingly important now that it is becomming clear that the structure and regulation of mitochondrial genes are very different in lower eucaryotic cells and in mammalian cells (5,6).

We have previously reported the development of a system allowing us to study mitochondrial (7) and cytochrome oxidase (8) biosynthesis in isolated rat hepatocytes. The study of rat liver cytochrome bc_1 complex was, however, made possible only recently by the development of an isolation procedure for this enzyme (9). This purification method has now been adapted for the small amount of material available using isolated hepatocytes. The present paper reports our preliminary findings on the synthesis and assembly of the cytochrome bc_1 complex in isolated rat hepatocytes.

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METHODS

Rat hepatocytes were isolated from 180 g male Sprague-Dawley rats as in (10). Cells (100-200 mg of protein) were labeled by [^{35}S]methionine as described previously (7,8). Other conditions of the incubation are indicated in the legends to the figures and tables. Labeled cells were washed twice in 0.25 M sucrose containing 10 mM unlabeled methionine and 0.5 mM phenylmethylsulfonyl fluorid (PMSF). Cells were disrupted by sonication and mitochondria isolated as in (11). The cytochrome bc_1 complex was isolated from labeled mitochondria by a small scale procedure adapted from Gellerfors et al. (9). The following modifications were made. The dimension of the column used in the hydroxylapatit chromatographic step (9,12) was decreased to 6.0 x 0.7 cm. All elutions were made at a flow rate of 0.2 ml/min. The buffer volumes used in step 1-3 (9,12) were decreased to 40 ml, 5 ml and 10 ml respectively. The elution was followed simultaneously at 279 nm and 418 nm using a LKB 2089 Uvicord III. The enzyme was eluted from the column at a protein concentration of approximately 0.05 mg/ml. These adaptions allow isolation of the cytochrome bc_1 complex from as little as 20 mg of mitochondrial protein. The isolated complex was concentrated for SDS-polyacrylamide gel electrophoresis, by dialysis against 1 l of distilled H_2O at 0-4^{o}C over night followed by freeze drying. Electrophoresis was done on 11 percent polyacrylamide gels using the buffer system of Laemmli (13). In order to avoid aggregation of cytochrome b, warming of the SDS-solubilized complex was avoided. Successful dissociation of the peptides was achieved by adding sample buffer (13) directly to the sample at 20^{o}C and electrophoresing as rapidly as possible, usually within 15 minutes. Fluorography was done according to (14) using kodak X-Omat films.

RESULTS

Isolated rat hepatocytes were labeled with [^{35}S]methionine in the absence or presence of various inhibitors of protein synthesis. Labeled mitochondria and cytochrome bc_1 complex were isolated (see methods). The yield of the cytochrome bc_1 complex was approximately 200 µg protein starting from 200 mg of cell protein. These preparations were judged to be free of major contaminating peptides by SDS-polyacrylamide gel electrophoresis (Fig. 1, track A).

The effects of different inhibitors on the specific radioactivity of isolated cytochrome bc_1 complex is shown in Table I. Labeling is inhibited to 85 percent by cycloheximide. The cycloheximide-insensitive labeling is not completely inhibited by chloramphenicol, as previously described (7). This is due, in part, to the suboptimal concentration of chloramphenicol used to prevent inhibition of protein synthesis on cytoplasmic ribosomes (unpublished results).

Electrophoretic and fluorographic analysis of chemically isolated cytochrome bc_1 complex is shown in Fig. 1. Eight major Coomassie blue staining peptides can be resolved on polyacrylamide gels containing SDS (Fig. 1, track A).

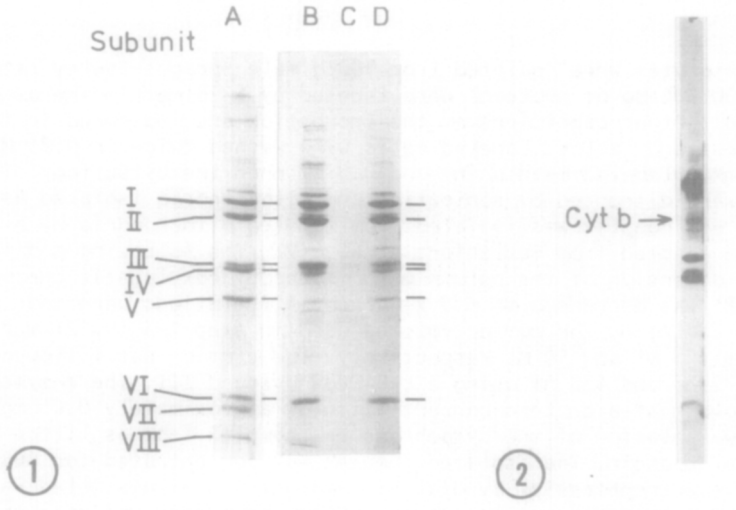

Fig. 1. Electrophoretic and fluorographic analysis of the cytochrome bc_1 complex isolated from in vitro labeled rat hepatocytes.

Hepatocytes were labeled with [^{35}S]methionine as in Table I. Cytochrome bc_1 complex was isolated from cells labeled in the absence of inhibitors (lane B), or in the presence of 0.5 mM cycloheximide (lane C) or 0.05 mM chloramphenicol (lane D). Gels were either stained with Coomassie blue (lane A) or fluorographed (lanes B-D).

Fig. 2. Identification of the cytochrome b polypeptide among the total rat liver mitochondrial translational product.

Hepatocytes were labeled in the presence of 0.5 mM cycloheximide as in Table I, and Brij-58 washed inner mitochondrial membranes were isolated (9). These membranes were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. The arrow indicates the component of the isolated cytochrome bc_1 complex corresponding to cytochrome b (subunit III).

All of the subunits except the two smallest (VII and VIII) are labeled in the absence of inhibitors (Track B). Under these conditions a number of labeled contaminants, which are not revealed by Coomassie blue staining, also appear on the gels.

In the presence of cycloheximide three weakly labeled bands are observed (Track C), only one of which (subunit III) corresponds to a major Coomassie blue staining peptide of the cytochrome bc_1 complex. The others are contaminants from cytochrome oxidase and OS-ATPase (not shown). Band III is identified as cytochrome b based upon its molecular weight (15-17) and its tendency to aggregate

Table I. Radioactivity found in cytochrome bc_1 complex isolated from in vitro labeled rat hepatocytes.

Conditions	Specific activity cpm/mg protein $X10^{-3}$
No inhibitor	348
Cycloheximide (0.5 mM)	48
Chloramphenicol (0.05 mM)	338
Cycloheximide (0.5 mM) + Chloramphenicol (0.05 mM)	17

Hepatocytes were labeled with [$^{S}35$]methionine (30 µCi/ml) for 3 hours under different conditions. Cytochrome bc_1 complex was isolated by a micromethod (see methods) and analyzed for radioactivity.

(18) on SDS-polyacrylamide gel electrophoresis (not shown). Band III is also the only major peptide of the complex whose labeling is inhibited by chloramphenicol (Fig. 1, track D).

The decreased labeling of cytochrome b (subunit III) observed in the presence of cycloheximide (Fig. 1, track C) suggests that cytoplasmically synthesized proteins might be needed for its proper assembly into the holo-cytochrome bc_1 complex. This can not, unfortunately, be tested until specific antibodies are raised against cytochrome b. The lack of labeling of subunit VII and VIII observed in the absence of inhibitors (Fig. 1, track B) can be explained if these subunits lack methionine in their amino acid sequence or if larger cytoplasmic pools of these peptides are present.

Fig. 2 shows the total mitochondrial translational products which are found associated with the inner mitochondrial membrane. The identity of one of these, the cytochrome b, has now been possible to assess.

DISCUSSION

Biogenesis of the cytochrome bc_1 complex has been previously studied in yeast (1-4) and *Neurospora crassa* (5). The present study provides the first report on the biosynthesis and assembly of a mammalian cytochrome bc_1 complex. In

the present experiments the holo-complex was isolated by a small scale chemical procedure. Labeling of subunits, therefore, represents only assembled products.

Of the eight major Coomassie blue staining peptides of the cytochrome bc_1 complex, only the two smallest (VII and VIII) do not appear to be labeled in the absence of translation inhibitors. This could be due to the absence of methionine residues in these peptides. However, the fact that methionine is present in these subunits from beef heart cytochrome bc_1 complex (19) suggests an alternative explanation i.e., that labeling is weak due to dilution of the newly synthesized peptides into larger pool of unlabeled peptides. Different pool sizes have been reported for the subunits of Neurospora crassa cytochrome oxidase (18).

In agreement with results from lower eucaryotic cells (1-4), only one peptide of rat liver cytochrome bc_1 complex is translated on mitochondrial ribosomes. This peptide is assumed to be cytochrome b based upon several criteria, including its sensitivity to chloramphenicol and its molecular weight, which is similar to that of cytochrome b from beef heart (19), Neurospora crassa (5), and yeast (14). Labeling of cytochrome b is also partially inhibited in cells incubated with cycloheximide, suggesting that its assembly into the holoenzyme requires the presence of cytoplasmically synthesized proteins.

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