SITE OF BIOSYNTHESIS OF MAMMALIAN CYTOCHROME *c* OXIDASE SUBUNITS

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1. Introduction

In *Saccharomyces cerevisiae* [1–3] and in *Neurospora crassa* [1,4] it has been shown that the three largest subunits of cytochrome *c* oxidase (EC 1.9.3.1) are synthesized in mitochondria, while the remaining subunits are synthesized on cytosolic ribosomes. In *Locusta migratora,* at least one of the subunits of insect cytochrome *c* oxidase is synthesized in mitochondria [5]. Previous work with mammalian cells has suggested a mitochondrial origin for at least some of the subunits of cytochrome *c* oxidase because formation of the enzyme is inhibited by chloramphenicol or its sulfamoyl analog, Tevenel [1,6,7]. The present work provides immunological evidence that at least two of the subunits of mammalian cytochrome *c* oxidase are synthesized in mitochondria.

2. Materials and methods

The sources of all materials used are described elsewhere [8].

Embryonic bovine trachea cells (CCL-44) were obtained from the American Type Culture Collection (Washington, DC). They were grown in monolayer culture with α-minimal essential medium [9] with a reduced bicarbonate content (0.85 g/l) and 10% fetal calf serum. The products of mitochondrial protein synthesis were labelled as described previously [8]. Cells (5×10^6/ml in 20 ml) were incubated with 4 μCi L-[^{35}S]methionine (300–600 Ci/mmol)/ml for 1 h in the presence of 300 μg cycloheximide/ml. In one experiment Tevenel was added to 100 μg/ml. Mitochondria were isolated by differential centrifugation and purified on a sucrose step gradient as described by Wallace et al. [10].

Labelled mitochondria (2.5 mg protein/ml 0.6 M sucrose/2 mM EDTA/10 mM Tris–HCl, pH 7.4) were solubilized by adding 20% sodium deoxycholate, pH 7.0, to a final concentration of 3 mg/mg protein. The mixture (0.5–1.0 ml) was sonicated for 10 min at 4°C, incubated for 1 h at 22°C and then centrifuged for 15 min at 12 000 × *g*. The resulting supernatant solution contained 50 to 70% of the total counts present in the mitochondrial protein.

Bovine heart cytochrome *c* oxidase was prepared by the method of Hartzell [11] and labelled by reductive methylation with [^{14}C]formaldehyde (10–20 mCi/mmol) [12].

The preparation and properties of the rabbit antiserum against bovine heart cytochrome *c* oxidase will be described later (W. J. Vail, in preparation). The original titre was 1:1020 and the antibody was partially purified by $(NH_4)_2SO_4$ precipitation. It was specific for cytochrome *c* oxidase as judged by the following:

(i) Ouchterlony double diffusion showed only one precipitin line.
(ii) Precipitation of cytochrome *c* oxidase from solution as determined spectrophotometrically.

(iii) Interaction with cytochrome *c* oxidase on both surfaces of lipid vesicles.

Precipitin mixtures contained 350 μg purified bovine heart cytochrome *c* oxidase in 0.3 ml of 10 mM Tris–HCl, pH 7.4, 50 μl of purified antibody in 20% $(NH_4)_2SO_4$/10 mM Tris–HCl, pH 7.4 and in some cases about 300 μl solubilized mitochondrial protein. The mixtures were incubated for 15 h at 4°C or for 5 h at 22°C. The resulting precipitate was recovered at 10 000 × *g* for 10 min and washed three times by resuspension in 10 mM Tris–HCL, pH 7.4.

Electrophoresis was performed in 12.5% gels with an acrylamide to bis-acrylamide ratio of 40:1 and the buffer system of Laemmli [13]. The preparation of markers for molecular weight determination, the preparation of gels for fluorography and the scanning of the fluorograms by a Joyce-Loebl densitometer is described elsewhere [8].

Table 1
Apparent molecular weights of bovine cytochrome *c* oxidase subunits and bovine cell mitochondrially-synthesized proteins

Bovine cytochrome *c* oxidase		Bovine cell mitochondrially-synthesized proteins	
I	45 000	1	49 500
II	29 000	2	45 000
III	20 000	3	41 500
IV	17 000	4	37 500
V	13 000	5	36 000
VI	10 500	6	29 000
		7	27 500
		8	21 500
		9	18 500
		10	12 500
		11	10 000
		12	6500

Apparent molecular weights were calculated as described elsewhere [8]

3. Results and discussion

A densitometric scan of the fluorographic pattern of proteins synthesized by embryonic bovine trachea cells in the presence of cycloheximide is shown in fig.1 (dotted line). Twelve bands were observed. These are the protein components synthesized in mitochondria as confirmed by the inhibition of their synthesis by Tevenel, a chloramphenicol analog (dashed line, fig.1) [8,10]. The apparent molecular weights of these components are given in table 1.

Figure 1 also shows that an antibody specific to cytochrome *c* oxidase, precipitated an enzyme containing 6 subunits (solid line). The same components were also observed with Coomassie Blue staining. The apparent molecular weights are given in table 1. This antibody also precipitated 10–20% of the counts of deoxycholate-solubilized mitochondrial protein while virtually no radioactivity was recovered if non-immune rabbit serum was used. As shown in fig.1, of the twelve protein components synthesized in mitochondria, only two (numbers 2 and 6) were precipitated by the antibody (dashed and dotted line) and these two have the same electrophoretic mobility as the two largest subunits of cytochrome *c* oxidase (numbers I and II, respectively). Band number 2 was broad, usually had

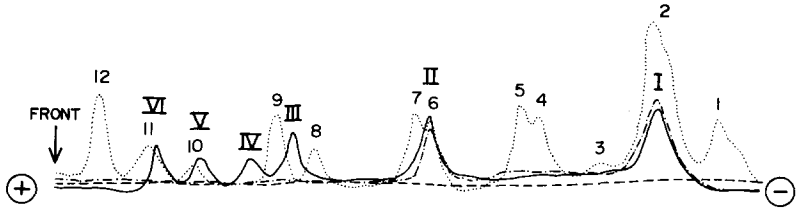

Fig.1. Densitometric scans of fluorograms of mitochondrially-synthesized proteins and of cytochrome *c* oxidase. Mitochondrially-synthesized proteins were labelled with [^{35}S]methionine in the presence of cycloheximide (· · · · · · · ·), cycloheximide and Tevenel (- - - - - - - -), or cycloheximide followed by precipitation with antibody against cytochrome *c* oxidase (- · - · - · -). Cytochrome *c* oxidase was labelled with [^{14}C]formaldehyde by reductive methylation and precipitated with the antibody (————). Proteins were separated by sodium dodecyl sulfate–polyacrylamide slab-gel electrophoresis with the anode to the left. After fluorography, the fluorograms were scanned by a Joyce-Loebl densitometer. Details are found in Materials and methods or are described previously [8].

a shoulder of higher molecular weight and was more intensely labelled than the other components. It has been suggested that in other cell lines a band having a similar electrophoretic mobility consists of two proteins [8,14]. In various electrophoretic runs, the position of subunit I of cytochrome *c* oxidase and of the antibody-precipitate of proteins synthesisized in mitochondria varied with respect to band 2 and its shoulder. Further, while the ratio of labelling of proteins 2–6 is 1.85 ± 0.18 (SE for 5 runs), normalized for molecular weight [8], the ratio is 0.74 ± 0.01 for the precipitate of mitochondrially-synthesized proteins and 0.76 ± 0.02 for subunits I and II of cytochrome *c* oxidase. This supports the suggestion that there are at least two components in band 2. Alternatively, there is one component and only part was incorporated into cytochrome *c* oxidase and precipitated by the antibody.

The results demonstrate that mammalian mitochondria synthesize the two largest components of cytochrome *c* oxidase. This is similar to yeast [1–3] and *Neurospora* [1,4] where the three largest components are synthesized in mitochondria. At present the relationship between the subunits of the mammalian and fungal enzyme is unclear. This is in part because there is no agreement on the number of subunits in the mammalian enzyme [15–20]. We have separated the subunits of the antibody-precipitated bovine enzyme by electrophoresis in the three buffer systems used by Capaldi [20]. Although Capaldi et al. [20] reported seven subunits in each we observed only six. It is possible that one subunit is not precipitated by the antibody, but the mammalian enzyme might lack one mitochondrially-synthesized subunit present in yeast and *Neurospora*.

It was not possible to demonstrate whether the other four subunits of cytochrome *c* oxidase were synthesized on cytosolic ribosomes by labelling in the presence of Tevenel.

Subsequent to this work, Jeffreys and Craig [21] reported that one subunit of the four in their preparation of mouse and human cytochrome *c* oxidase was synthesized in mitochondria. This subunit had a similar electrophoretic mobility to subunit II seen here. The enzyme isolated by them does not have subunit I. The discrepancies between the results will only be resolved when the subunit structure of cytochrome *c* oxidase is known.

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