BIOSYNTHESIS OF CYTOCHROME *c* OXIDASE IN ISOLATED RAT HEPATOCYTES

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1. Introduction

Cytochrome *c* oxidase from yeast contains 7 polypeptide chains [1] and the enzyme from *Neurospora crassa* 8 [2]. Recently 12 different polypeptide chains were identified in cytochrome *c* oxidases from various mammalian species [3–5]. Studies on the biosynthesis of cytochrome *c* oxidase in yeast [6] and *N. crassa* [7] have established the mitochondrial site of synthesis of the 3 large subunits and the nucleocytoplasmic origin of the smaller ones. This result was confirmed in studies with embryonic bovine trachea cell cultures [8], and with oocytes of *Xenopus laevis* [9]. To understand the biosynthesis and assembly of the 12 polypeptide chains of cytochrome *c* oxidase in nongrowing mammalian cells, an isolated cell system is required, which allows the incorporation of radioactive amino acids for several hours. Isolated hepatocytes represent a well-investigated system, which, in contrast to continuously growing cell cultures, is not dedifferentiated [10].

We have described the biosynthesis of cytochrome *c* oxidase in hepatocytes, using antibodies against the enzyme [11]. The limited separation power of the applied gel system, and the low incorporation of radioactivity did not allow a clear identification of synthesized subunits. Here, the biosynthesis of cytochrome *c* oxidase is described in hepatocytes with a high rate of protein synthesis. The results suggest differences between continuously growing and non-growing cells.

2. Materials and methods

L-[^{35}S]methionine (1300 Ci/mmol) was purchased from Amersham Buchler (Braunschweig), PMSF from Serva (Heidelberg) and emetine from Boehringer (Mannheim).

Antibodies against rat liver cytochrome *c* oxidase were raised in rabbits ([11], scheme B). Hepatocytes were isolated and incubated as in [11] with some modifications [12] improving total [^{35}S]methionine incorporation into protein up to 40–60% in <5 h incubation. Rat liver mitochondria were isolated and incubated as in [13]. Mitochondria from labelled hepatocytes were isolated according to [14]. Immunoprecipitations were performed with labelled cells or mitochondria, dissolved in 150 mM NaCl, 20 mM sodium phosphate (pH 7.2), 4% Triton X-100, 1 mM PMSF, or dissolved in a dissociating buffer containing SDS, as in [15]. SDS slab gel electrophoresis was done as in [3] but without glycerol or urea in the separation gel (15% acrylamide). Photographs were taken from the Coomassie blue G-250 stained gels and fluorography was performed according to [16]. Radioactive bands were cut out from dried gels, dissolved at 45°C in 0.5 ml 30% H_2O_2 containing 1% of 25% NH_3, mixed with scintillation liquid and counted.

3. Results and discussion

The kinetics of [^{35}S]methionine incorporation by isolated mitochondria into protein is presented in fig.1. After a chase of unlabelled methionine at 15 min the specific radioactivity remains constant until 75 min incorporation. At optimal antibody/antigen ratio, maximally 8% of total radioactivity, incorporated within 75 min, could be immunoprecipitated with an antibody against cytochrome *c* oxidase as shown by a titration curve (fig.2).

Whereas the immunoprecipitated radioactivity shows a maximum at 0.5 ml serum the precipitated protein increases further up to 1.0 ml serum. The

Abbreviations: SDS, sodium dodecylsulfate; PMSF, phenylmethane sulfonylfluoride

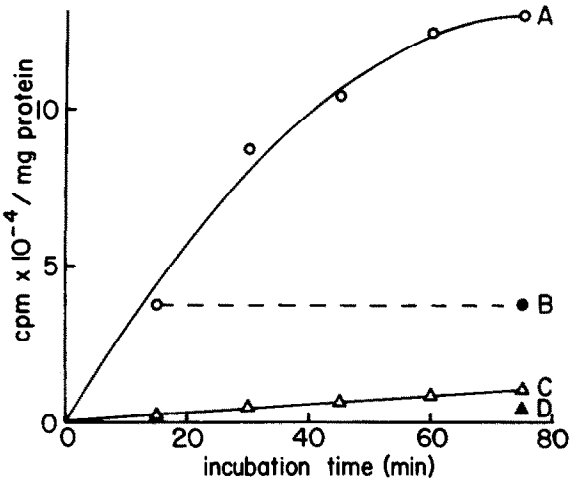


Fig.1. Incorporation of [^{35}S]methionine into total and immunoprecipitated protein of rat liver mitochondria. Mitochondrial protein (2.7 mg) was incubated with 30 μCi [^{35}S]methionine (total vol. 2 ml). Chase: 0.2 ml 100 mM methionine. Immunoprecipitations were performed at optimal antibody/protein ratio. (A,B) Radioactivity in total protein; (B) after a chase at 15 min; (C) radioactivity in immunoprecipitate; (D) after a chase at 15 min.

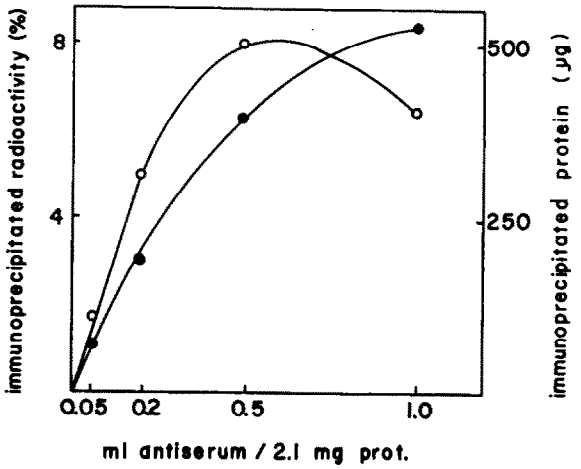


Fig.2. Titration of in vitro labelled rat liver mitochondria with an antiserum against cytochrome *c* oxidase. Mitochondria were labelled for 75 min (see legend to fig.1). After solubilization in Triton X-100 buffer (see section 2) immunoprecipitations were done with the indicated amounts of antiserum. Aliquots of the immunoprecipitates were used for measurement of radioactivity (o—o) and protein (•–•).

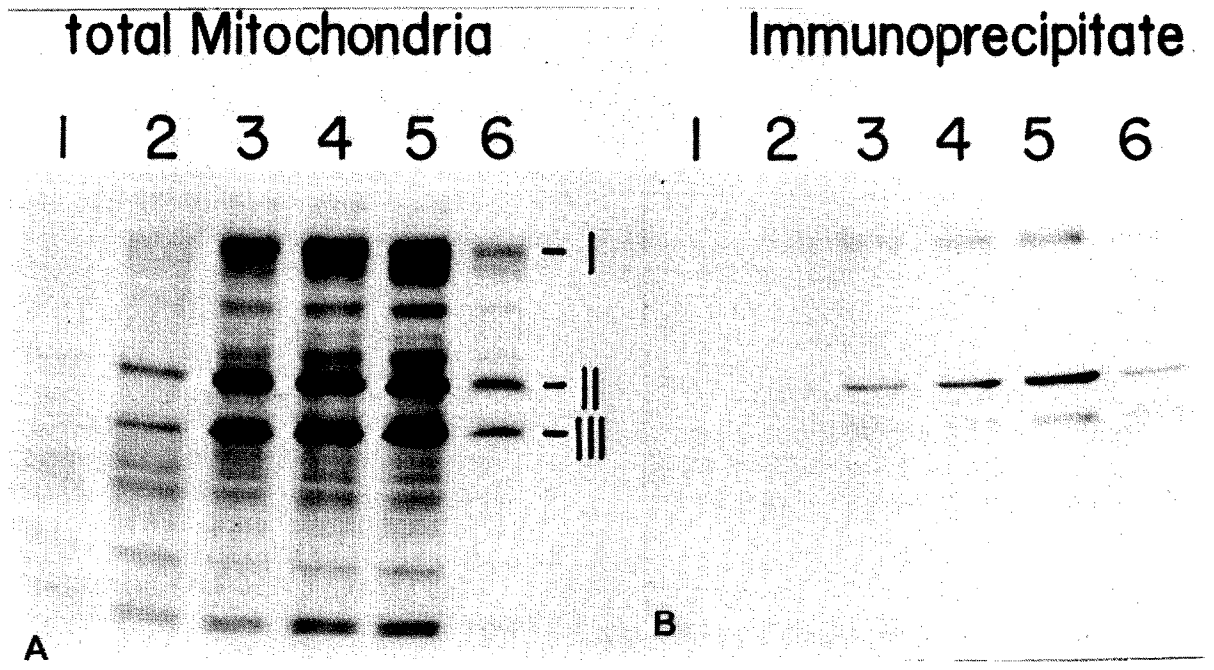


Fig.3. Fluorography of total and immunoprecipitated labelled mitochondrial proteins, separated by SDS gel electrophoresis. Aliquots from the fractions of fig.1 were separated and the gels further treated as in section 2. Labelling conditions: (1) 15 min; (2) 30 min; (3) 45 min; (4) 60 min; (5) 75 min; (6) 15 min [^{35}S]methionine pulse, 15–75 min methionine chase.

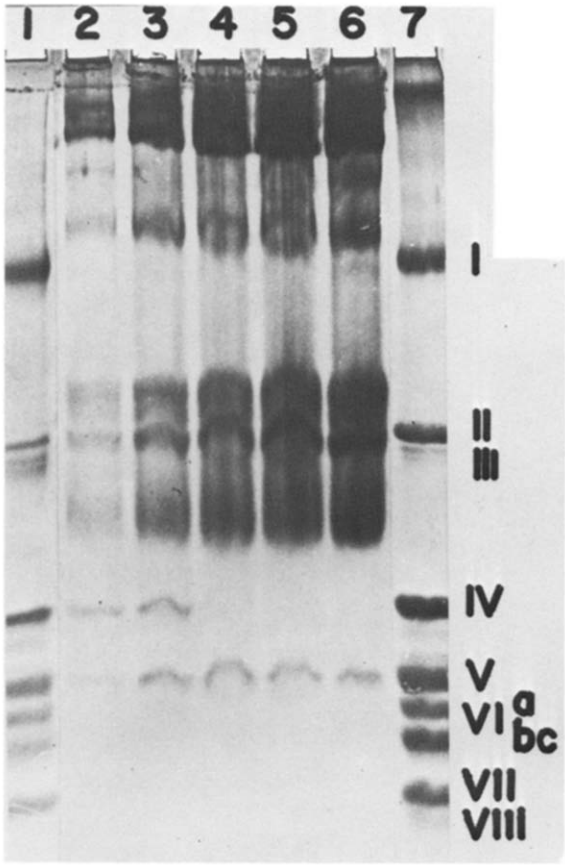


Fig.4. Immunotitration of SDS-dissociated rat liver mitochondria with an anti-serum against cytochrome *c* oxidase. Mitochondria were dissolved in 4% SDS, 1 mM EDTA, 1 mM EGTA, pH 7.4 [14]. They were diluted 10-fold with a buffer containing 3% Triton X-100, 150 mM NaCl, 1 mM EDTA, 100 mM Na_2HPO_4 (pH 7.4) and 4 ml of each were mixed with 0.1 ml (lane 2), 0.3 ml (lane 3), 0.6 ml (lane 4), 1.0 ml (lane 5) or 1.4 ml serum (lane 6). Lanes 1,7: isolated cytochrome *c* oxidase.

maximum of radioactivity corresponds to a maximum of immunoprecipitated cytochrome *c* oxidase, indicated by a decrease of Coomassie blue staining intensity of the subunits at 1.0 ml serum (not shown). The increase of total protein at 1.0 ml serum is due to an increase of precipitated immunoglobulin, which exceeds the decrease of cytochrome *c* oxidase protein.

The SDS gel electrophoretic pattern of total radioactivity shows 3 main labelled bands, representing subunits I–III of cytochrome *c* oxidase (fig.3A). The radioactivity within these 3 bands amounts to 35% of total radioactivity, after 75 min incubation, as measured by scintillation counting (see section 2). During the chase period from 15–75 min, some radioactivity in the lower region of the gel decreases, whereas the label within subunits I–III increases, suggesting a very slow rate of protein synthesis in isolated liver mitochondria. In fig.3B the labelled pattern of immunoprecipitated proteins is presented. The antibody precipitates only subunits I–III of cytochrome *c* oxidase. But only a small portion of the radioactivity, within subunits I–III (23%) can be immunoprecipitated. This may be explained by the inability of the antibody to precipitate unassembled subunits of cytochrome *c* oxidase. In fig.4 the pattern of immunoprecipitated cytochrome *c* oxidase, which has been dissociated prior to addition of the antibody is presented. Subunits II, IV and V are precipitated, but not subunit I. Therefore we suggest that the antibody cannot precipitate unassembled subunit I.

The biosynthesis of cytochrome *c* oxidase was studied by incorporation of [^{35}S]methionine into proteins of isolated hepatocytes. The SDS gel electrophoretic pattern of the subunits, immunoprecipitated from Triton X-100 extracts of total cells or of mitochondria isolated from the labelled hepatocytes, equals the pattern of the isolated enzyme in the molecular weight range of subunits IV–VIII (fig.5A). The radioactive pattern of the gel (fig.5B) is almost identical with the Coomassie blue staining pattern in the range of subunits IV–VIII. The intensity of labelling increases up to 5 h of incubation, indicating a continuous synthesis and assembling of cytochrome *c* oxidase in hepatocytes for 5 h.

Emetine completely suppressed labelling of all proteins except subunit I–III. In the presence of emetine (lane 7) subunits I, II and III contained 31, 69 and 51%, respectively, of the radioactivity found in the corresponding subunits of the control (lane 4). Since the antibody cannot precipitate unassembled subunit I, it is suggested that in isolated hepatocytes, cytochrome *c* oxidase is assembled in the absence of cytoplasmic protein synthesis for 5 h at ~50% the rate than in its presence. This suggests the occurrence of a large pool of unassembled cytoplasmic precursor proteins for cytochrome *c* oxidase in isolated hepatocytes.

The labelling kinetics of the individual subunits (fig.6) further supports this conclusion. All subunits of cytochrome *c* oxidase show very similar labelling kinetics, characterized by a lag-phage of ~1 h, which is not observed for total hepatocyte protein. This contrasts the results obtained with *N. crassa* cells,

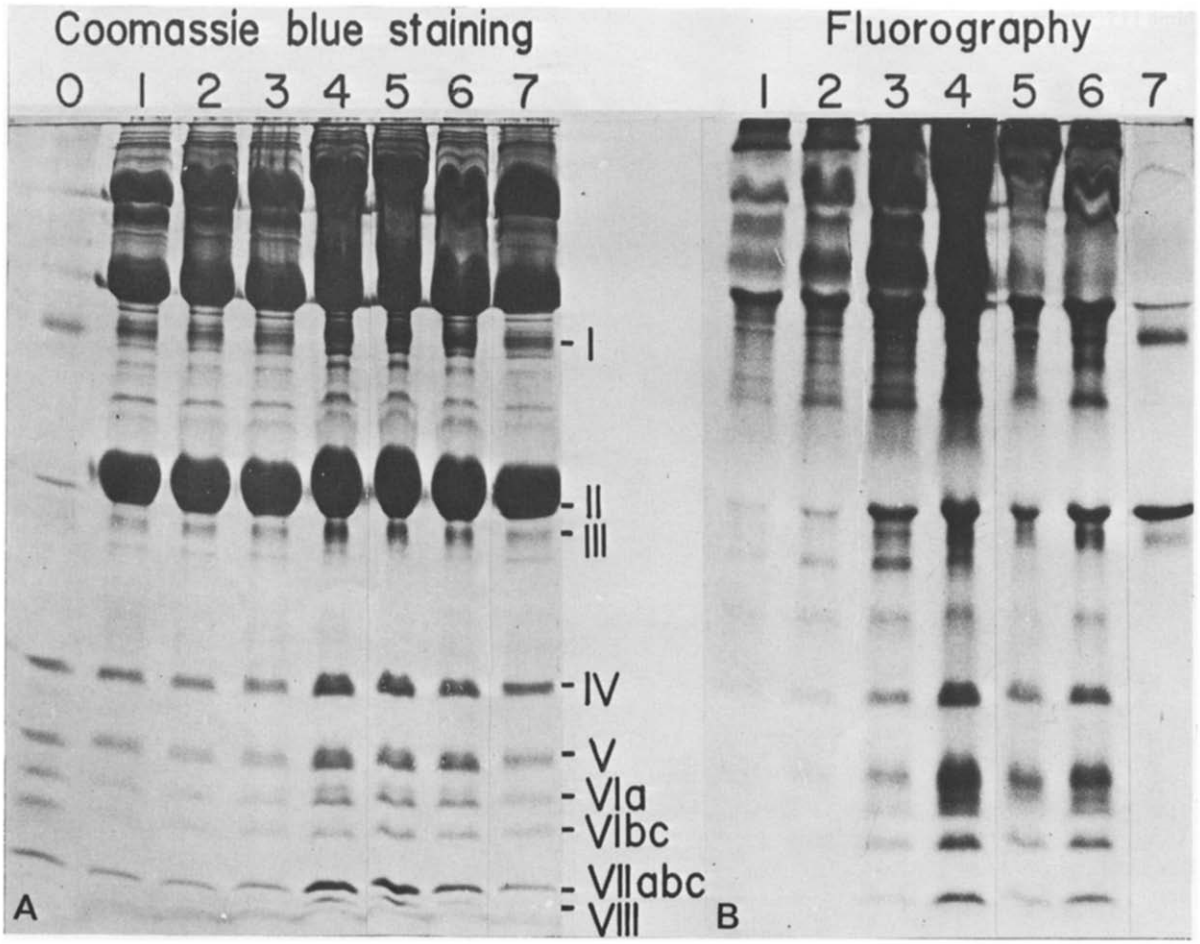
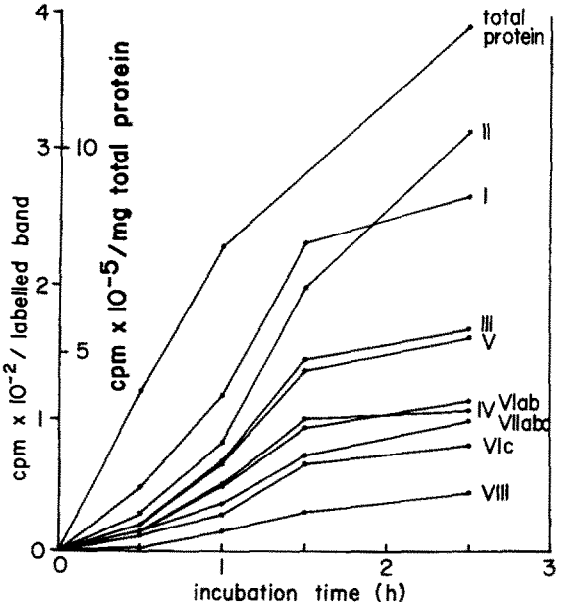


Fig.5



where different labelling kinetics were found for individual subunits [17], and with yeast cells, where very rapid labelling kinetics were described, and the calculated half-lives of unassembled subunits ranged from 1.5–9 min [18]. Our data characterize the biosynthesis of cytochrome *c* oxidase in isolated hepatocytes as a slow process, involving large pools of cytoplasmic precursor proteins.

Fig.6. Kinetics of [^{35}S]methionine incorporation into subunits of cytochrome *c* oxidase. Isolated hepatocytes (10.6 mg protein/1.4 ml) were incubated with 75 μCi [^{35}S]methionine for the indicated times. Immunoprecipitates from total cells were separated by slab gel electrophoresis and stained with Coomassie blue G-250. The labelled subunits of cytochrome *c* oxidase were identified by fluorography, cut out and the radioactivity measured by scintillation counting. Control incubations (4 mg protein/1.0 ml; 1 μCi [^{35}S]methionine) were performed to measure the incorporation into total protein.

Fig.5. SDS gel electrophoresis of immunoprecipitates from [^{35}S]methionine-labelled hepatocytes and their mitochondria. Isolated atocytes (34.5 mg protein/1.7 ml) were incubated with 75 μCi [^{35}S]methionine for various times. From some samples mitochondria were isolated (lanes 5,6). Immunoprecipitates from mitochondria and total cells were separated by slab gel electrophoresis, stained with Coomassie blue G-250, dried and the radioactivity visualized by fluorography. Lane (0), isolated rat liver cytochrome *c* oxidase; lanes (1–7), immunoprecipitates from total cells (1–4,7) or mitochrondria (5,6). Lane (1), 0.5 h incubation time; lanes 2,5, 1 h; lanes 3,6, 2 h; lane 4, 5 h; lane 7, 5 h incubation in presence of 0.12 mg emetine/ml.

Acknowledgements

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