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BIOSYNTHESIS AND INTRACELLULAR TRANSLOCATION OF
MITOCHONDRIAL PROTEINS: CYTOCHROME C AND THE
CARBOXYATRACTYLOSIDE BINDING PROTEIN

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I. INTRODUCTION

The vast majority of mitochondrial proteins is translated on cytoplasmic ribosomes and must eventually be transported to their site of function in the mitochondrion. In previous studies we have presented evidence for a transfer mechanism which involves extramitochondrial precursors (Haller-mayer et al., 1977; Harmey et al., 1977; Zimmermann et al., 1977). These studies strongly argued against the proposal that as a general rule proteins are transferred to mitochondria by direct insertion of nascent polypeptide chains, carried out by a special class of cytoplasmic ribosomes bound to the outer mitochondrial membrane (Kellems and Butow, 1972; Kellems et al., 1975).

Attempts to further elucidate the mechanisms of transfer have to take into consideration that mitochondrial proteins differ widely with respect to their structure, physico-chemical properties and submitochondrial location. Proteins of the outer membrane, the intermembrane space, the inner membrane (peripheral and integral) and of the matrix space have to be distinguished. It may be expected that the mechanisms of transfer are different for these various groups of proteins. Accordingly, the transfer processes have to be studied for individual proteins of each group. The primary translation products have to be analysed, extramitochondrial precursors must be isolated, their subcellular location determined and their structural relationship to the functional proteins

investigated. Finally, the mechanism of transfer of extra-mitochondrial precursors must be studied in reconstituted systems, involving the precursor proteins and isolated mitochondria.

Here we report on the synthesis and transfer of two mitochondrial membrane proteins, cytochrome c and the carboxyatractyloside binding protein, the ATP/ADP carrier of the inner mitochondrial membrane.

II. RESULTS AND DISCUSSION

A. Cytochrome c

Cell free homogenates from Neurospora crassa were employed to study biosynthesis and intracellular translocation of cytochrome c. Antibodies against apocytochrome c and holocytochrome c were used to isolate these proteins by simple immunoprecipitation. Preexistent proteins were discriminated from in vitro synthesized proteins by dual labelling. For this purpose, Neurospora cells were grown in 35-S-sulfate containing medium and labelling in the cell free homogenate was performed with 3-H-leucine.

Apocytochrome c as well as holocytochrome c were found to be synthesized in the cell free homogenate after incubation for 10 min under appropriate conditions. When protein synthesis was then blocked with cycloheximide, the amount of in vitro synthesized apocytochrome c decreased during a further 30 min incubation period. Simultaneously, the amount of newly synthesized holocytochrome c increased (Table I). The identities of the immunoprecipitated apo- and holocytochrome c were verified by analysis of the cyanogen bromide fragments.

A cell free homogenate labelled as described in Table I was separated by differential centrifugation into the mitochondrial fraction, the microsomal fraction and the post-ribosomal supernatant. Immunoprecipitation with antibodies against apocytochrome c and holocytochrome c was carried out with all fractions.

Fig. 1A and B show that in vitro synthesized apocytochrome c is present in the postribosomal supernatant and that its amount decreases after blocking protein synthesis. No pre-existent 35-S-labelled holocytochrome c is precipitated by the antibody against apocytochrome c. From the mitochondrial fraction anti-apocytochrome c precipitated neither 3-H-labelled apocytochrome c nor 3-H- or 35-S-labelled holocytochrome c.

Antibodies against holocytochrome c precipitated from the postribosomal fraction 35-S-labelled holocytochrome c, which represents preexistent cytochrome c leaked out from the mitochondria during preparation of the cell free homogenate. Also

TABLE I. Immunoprecipitation of Apocytochrome c and of Holocytochrome c from a Cell Free Homogenate

A cell free homogenate was prepared from cells grown in the presence of 35-S-sulfate and incubated at 25°C for 10 min in the presence of 3-H-leucine. Then cycloheximide (CHI) (100 µg/ml) was added. One half of the homogenate was withdrawn and kept at 0°C. The other half was further incubated for 30 min at 25°C. Then from both portions apocytochrome c and holocytochrome c were immunoprecipitated. The immunoprecipitates were analysed by SDS polyacrylamide gel electrophoresis. 3-H- and 35-S-radioactivities in the peak fraction were determined.

Antibody		10 min	10 min + 30 min CHI
		Radioactivity (counts x min ⁻¹)	
Anti-apocytochrome c	3-H	1826	836
	35-S	18	24
Anti-holocytochrome c	3-H	260	944
	35-S	4420	4320

a small amount of in vitro synthesized holocytochrome c was precipitated which increased during incubation in the presence of cycloheximide (from about 5 to 10% of total 3-H-holocytochrome c in the homogenate). From the mitochondrial fraction the antibody against holocytochrome c brought down preexistent 35-S-holocytochrome c. Also, 3-H-labelled holocytochrome c was found in the precipitate. Its amount increased during incubation in the absence of protein synthesis (Fig. 1C and D).

From the microsomal fraction no apocytochrome c could be immunoprecipitated, however a small amount of 35-S-holocytochrome c (3-4% of total). Also, 3-H-labelled holocytochrome c was detected in this fraction (10-15% of total) which did not show a significant increase or decrease during incubation in the presence of cycloheximide.

These data suggest that apocytochrome c in the post-ribosomal supernatant acts as a precursor of holocytochrome c in the mitochondria.

Transfer of cytochrome c was further investigated in a re-constituted system, in which a postribosomal supernatant containing in vitro synthesized apocytochrome c was incubated with isolated mitochondria from 35-S-labelled cells. Table II shows that holocytochrome c appears in the mitochondria which were reisolated after incubation. When unlabelled holo-

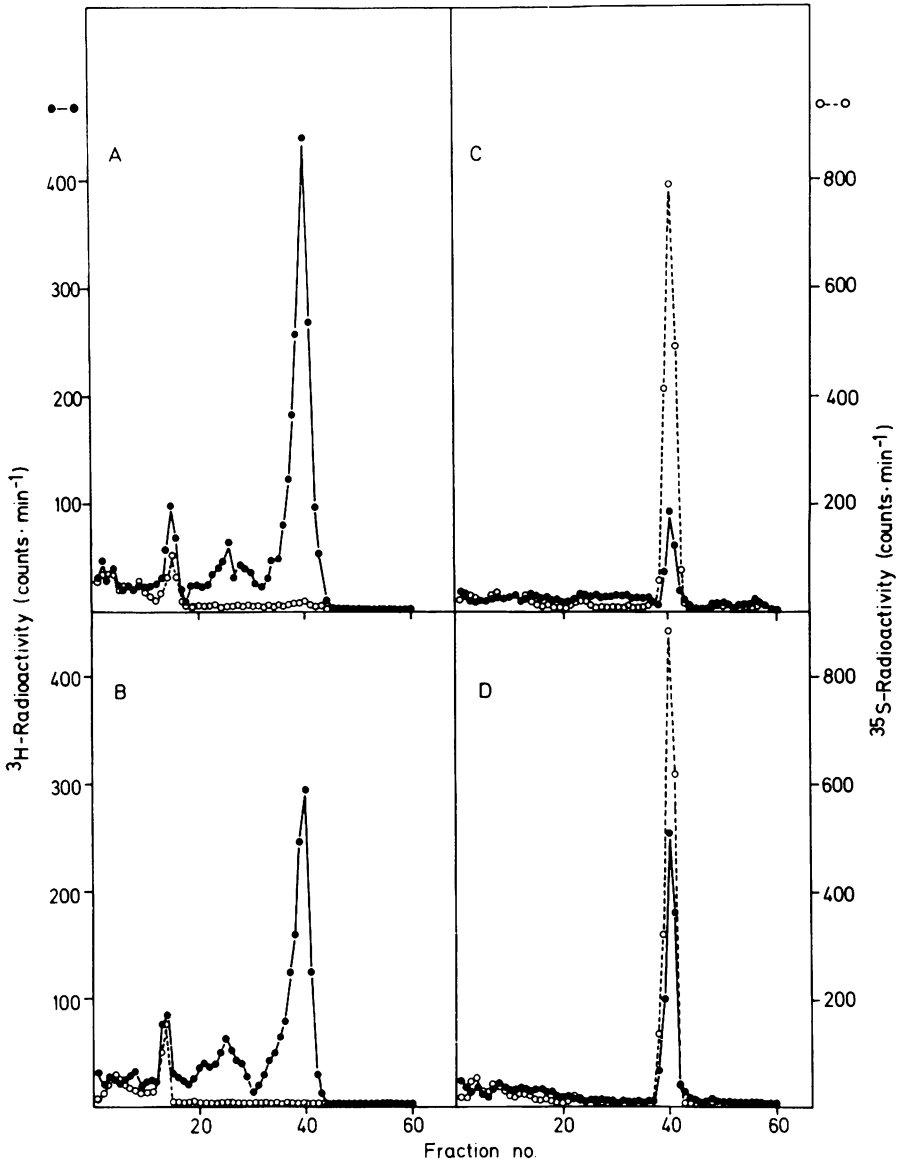


FIGURE 1. Immunoprecipitation with antibodies against apo-cytochrome c from the postribosomal fraction and with antibodies against holocytochrome c from the mitochondrial fraction. The immunoprecipitates were analysed by SDS gel electrophoresis. A,B: Ip with anti-apocytochrome c from the postribosomal supernatant; C,D: Ip with anti-holocytochrome c from the mitochondria; A,C: 10 min incubation; B,D: 10 min incubation plus 30 min in the presence of cycloheximide.

TABLE II. Transfer of In Vitro Synthesized Apocytochrome c into Mitochondria and Conversion to Holo-cytochrome c

A postmitochondrial homogenate was incubated for 10 min with 3-H-leucine and a postribosomal supernatant was prepared. Mitochondria were isolated from cells grown in the presence of 35-S-sulfate and resuspended with this postribosomal supernatant. The suspension was divided into three equal portions. One served as a control, to the second portion holo-cytochrome c (1 nmol/ml) was added, to the third portion apocytochrome c (1 nmol/ml). One half of each portion was immediately withdrawn after resuspension and kept at 0°C, the other half was incubated for 30 min at 25°C. Then mitochondria were collected from each sample by centrifugation and washed with sucrose/EDTA medium. The mitochondrial pellets were dissolved with Triton X-100 containing buffer and immunoprecipitation with anti-holo-cytochrome c was carried out. The immunoprecipitates were subjected to SDS gel electrophoresis. The 3-H- and 35-S-radioactivities in the cytochrome c peaks were determined after slicing the gels.

		0 min	30 min
		Radioactivity (counts x min ⁻¹)	
Control	3-H	26	440
	35-S	3836	4224
+ Holo-cytochrome c	3-H	72	380
	35-S	4016	4112
+ Apocytochrome c	3-H	20	78
	35-S	4720	4048

cytochrome c was added to the incubation mixture, no significant change of the amount of holo-cytochrome c appearing in the mitochondria was observed. This should be expected since the postribosomal supernatant already contains appreciable amounts of holo-cytochrome c which leaked out from the mitochondria during cell breakage (up to 40% of total cytochrome c). In contrast, addition of apocytochrome c leads to a marked reduction of the amount of holo-cytochrome c appearing in the mitochondria.

On the basis of these results the following mechanism of synthesis and assembly of cytochrome c is proposed. Apocytochrome c is synthesized on cytoplasmic ribosomes and released

into the cytosol. It seems reasonable to assume that apocytochrome c found in the postribosomal supernatant is actually present in vivo in the cytosol in free form. It can however not definitely be excluded that it is derived from some unknown very fragile structures which are destroyed during cell fractionation. It is further proposed that apocytochrome c diffuses to the mitochondrial membrane where the heme group is covalently linked to the apoprotein. According to our experiments, kinetic data argue against a possible role of microsomal apo- or holocytochrome c as precursors of mitochondrial holocytochrome c. The conversion of apocytochrome c to holocytochrome c involves a drastic change in the conformation of the molecule (Fisher et al., 1973). It is proposed that this conformational change leads to the trapping of newly formed holocytochrome c in the mitochondrial membrane.

Clearly, our hypothesis raises a number of questions. They concern the permeability of the outer mitochondrial membrane for apocytochrome c, the mechanism by which the heme group is covalently linked to the apoprotein and the problem, why newly synthesized cytochrome c is bound to the membrane in preference to the preexistent cytochrome c which leaked out from the mitochondria.

It should be pointed out that in a number of studies on the biogenesis in rat liver it was claimed that holocytochrome c is synthesized as a whole by the microsomes and eventually transferred into mitochondria (for reviews see Sherman and Stewart, 1971; Gonzalez-Cadauid, 1974). It was however not possible in these studies to discriminate between newly synthesized and preexistent cytochrome c; furthermore, apocytochrome c could not be separately determined. Moreover, the possible precursor role of microsomal cytochrome c was recently ruled out by Robbi et al., (1978) (see also Kadenbach, 1970).

B. Carboxyatractyloside Binding Protein

The carboxyatractyloside binding protein (CAT-protein) or ATP/ADP carrier represents an integral protein of the inner mitochondrial membrane which is characterized by its hydrophobic properties and an apparent molecular weight of about 32,000 (Klingenberg et al., 1975). Its synthesis and transfer were studied in essentially the same experimental systems as described for cytochrome c.

A cell free homogenate derived from Neurospora cells grown in the presence of 35-S-sulfate was incubated with 3-H-leucine for 10 min; mitochondria were isolated and the CAT-protein was immunoprecipitated. In vitro synthesized CAT-protein was found in the mitochondria (Fig. 2A). In order to detect possible

extramitochondrial precursors, the various subcellular fractions were subjected to immunoprecipitation, adding mitochondria from unlabelled cells as a source of carrier protein or by double immunoprecipitation. With the ribosomal and microsomal fractions no clear indication for the presence of *in vitro* synthesized CAT-protein was found. However, from the postribosomal supernatant, 3-H-radioactivity was immunoprecipitated which upon SDS gel electrophoresis yielded a peak with identical electrophoretic mobility as the functional CAT-protein. No 35-S-radioactivity was present in this peak, demonstrating absence of contaminating mitochondrial membrane fragments.

In order to verify the identity of the 3-H-labelled putative CAT-protein from the postribosomal supernatant, an immunoprecipitate obtained from this fraction was mixed with an immunoprecipitate obtained from mitochondria which were derived from cells labelled *in vivo* with 14-C-leucine. The mixture was subjected to cyanogen bromide cleavage and the

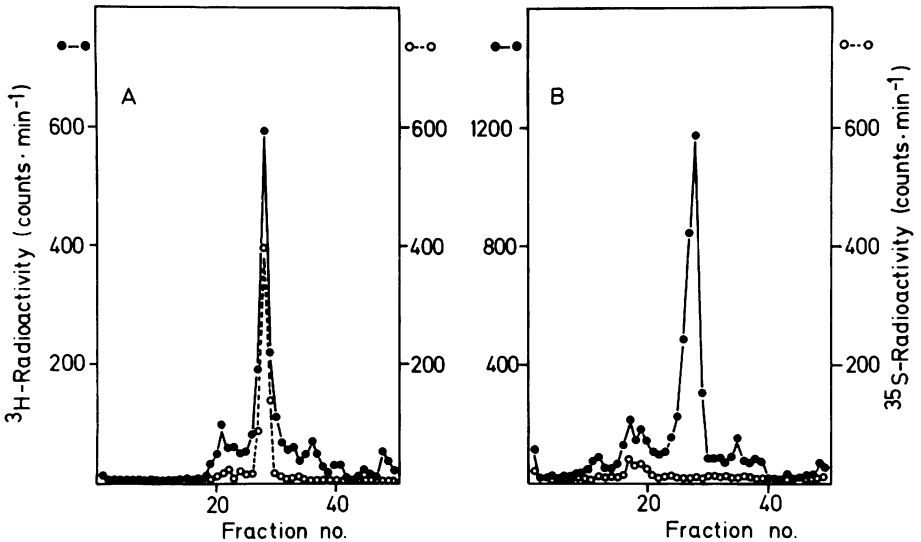


FIGURE 2. Immunoprecipitation of CAT-protein from mitochondria and from a postribosomal pellet. A: Mitochondria; immunoprecipitate obtained directly with antibodies against CAT-protein. B: Pellet obtained by 2 h ultracentrifugation of a postribosomal supernatant; immunoprecipitate obtained by double precipitation with antibodies against CAT-protein and antibodies against rabbit immunoglobulins.

The immunoprecipitates were analysed by SDS gel electrophoresis.

radioactive cleavage products were analysed on phenol/formic acid polyacrylamide gels. The 3-H and 14-C patterns showed satisfactory coincidence.

Double immunoprecipitation employing anti-rabbit-immunoglobulin-antibodies from sheep lead also to the precipitation of 3-H-labelled CAT-protein from the postribosomal supernatant, however only when Triton X-100 was added. This indicated that the *in vitro* synthesized component does not exist in a free form in the supernatant, but rather that its antigenic sites are not available to the antibody. When the postribosomal supernatant was centrifuged for 2 h at 165,000 x g, all the 3-H-labelled CAT-protein was found in the pellet (Fig. 2B).

The question arises whether the *in vitro* synthesized CAT-protein in this fraction is a precursor of the mitochondrial CAT-protein. Actually it was found that the amount of 3-H-labelled CAT-protein in this fraction decreased when after the 10 min labelling period translation was blocked with cycloheximide and the cell free homogenate incubated further. On the other hand, 3-H-labelled CAT-protein in the mitochondrial fraction increases under these conditions (Harmey et al., 1977). It was however not possible to quantitatively correlate these two processes. The main difficulties in these experiments are the exceptional lability of the CAT-protein and the inability to demonstrate transfer into functional positions. The newly synthesized protein appears to be easily subject to proteolytic degradation.

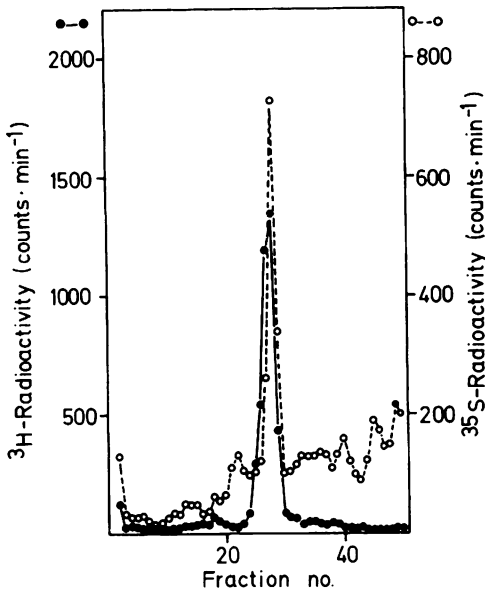


FIGURE 3. Translation of *Neurospora* CAT-protein in a rabbit reticulate cell free system. Coelectrophoresis of immunoprecipitates from the heterologous system (^{35}S) and from mitochondria labelled *in vivo* (^3H).

It is of interest in this context whether CAT-protein is translated as a precursor with identical or higher molecular weight as compared to the functional protein in the mitochondria. Poly-A-containing RNA was isolated from *Neurospora* and translated in a rabbit reticulocyte lysate in the presence of 35-S-methionine. Double immunoprecipitation was carried out after lysis with Triton and the resulting precipitate was mixed with an immunoprecipitate obtained from mitochondria isolated from cells labelled in vivo with 3-H-leucine. The mixture was analysed by SDS gel electrophoresis. Fig. 3 shows that the 35-S peak and the 3-H peak with apparent molecular weights of about 32,000 coincide. This indicated that CAT-protein is not translated as a larger molecule, at least not as a precursor differing in its apparent molecular weight by more than about 500 from the authentic protein.

Further experiments will have to clarify the precursor role of a postribosomal CAT-protein. As a working hypothesis it is proposed that CAT-protein is not translocated as a freely soluble precursor but rather that binding to lipid containing structures is involved.

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