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Biogenesis of Cytochrome *c* in *Neurospora crassa*

Synthesis of Apocytochrome *c*, Transfer to Mitochondria and Conversion to Holocytochrome *c*

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1. Precipitating antibodies specific for apocytochrome *c* and holocytochrome *c*, respectively, were employed to study synthesis and intracellular transport of cytochrome *c* in *Neurospora in vitro*.

2. Apocytochrome *c* as well as holocytochrome *c* were found to be synthesized in a cell-free homogenate. A precursor product relationship between the two components is suggested by kinetic experiments.

3. Apocytochrome *c* synthesized *in vitro* was found in the post-ribosomal fraction and not in the mitochondrial fraction, whereas holocytochrome *c* synthesized *in vitro* was mainly detected in the mitochondrial fraction. A precursor product relationship between postribosomal apocytochrome *c* and mitochondrial holocytochrome *c* is indicated by the labelling data. In the microsomal fraction both apocytochrome *c* and holocytochrome *c* were found in low amounts. Their labelling kinetics do not suggest a precursor role of microsomal apocytochrome *c* or holocytochrome *c*.

4. Formation of holocytochrome *c* from apocytochrome *c* was observed when postribosomal supernatant containing apocytochrome *c* synthesized *in vitro* was incubated with isolated mitochondria, but not when incubated in the absence of mitochondria. The cytochrome *c* formed under these conditions was detected in the mitochondria.

5. Conversion of labelled apocytochrome *c* synthesized *in vitro* to holocytochrome *c* during incubation of a postribosomal supernatant with isolated mitochondria was inhibited when excess isolated apocytochrome *c*, but not when holocytochrome *c* was added.

6. The data presented are interpreted to show that apocytochrome *c* is synthesized on cytoplasmic ribosomes and released into the supernatant. It is suggested that apocytochrome *c* migrates to the inner mitochondrial membrane, where the heme group is covalently linked to the apoprotein. The hypothesis is put forward that the concomitant change in conformation leads to trapping of holocytochrome *c* in the membrane. The problems of permeability of the outer mitochondrial membrane to apocytochrome *c* and the site and nature of the reaction by which the heme group is linked to the apoprotein are discussed.

Mitochondrial proteins synthesized on cytoplasmic ribosomes and transported into the mitochondria are rather heterogeneous with respect to their structure, function and submitochondrial location. Detailed investigations on the mechanism of synthesis and translocation of these proteins must therefore be carried out with a variety of individual mitochondrial proteins.

The present study is concerned with cytochrome *c*. It is well known from a number of investigations that this protein is a nuclear gene product and that it is translated on cytoplasmic ribosomes [1–11]. In terms of the complexity of the transport reaction, cytochrome *c* seems to be a relatively simple protein. There is ample evidence that this component of the respiratory chain is a peripheral membrane protein which is located on the cytoplasmic side of the inner mitochondrial membrane [12–15]. Thus, in contrast to integral inner membrane proteins and to matrix proteins, cytochrome *c* has not to be inserted into or translocated across the lipid phase of the inner mitochondrial membrane. On the other hand, the study of the biogenesis of cytochrome *c* obtains a degree of complexity by the fact that a heme group must be covalently linked to a precursor apoprotein at some step in the biogenetic process [16].

Quite a number of investigations have been devoted to the biogenesis of cytochrome *c*. However, the results obtained on a variety of questions are ambigu-

ous and conflicting in several respects. Especially, different opinions exist whether apocytochrome *c* or holocytochrome *c* is transferred to the mitochondria (for reviews see [1, 17]). Most of these studies were carried out with rat liver, where very low rates of cytochrome *c* formation are observed, and they are based on techniques which require the isolation of large amounts of cytochrome *c*. Moreover, it was not possible to discriminate between apocytochrome *c* and holocytochrome *c*.

In this study we report on the biogenesis of cytochrome *c* in various cell free systems from *Neurospora crassa*. Antibodies specific for apocytochrome *c* and cytochrome *c* were used. Dual labelling was employed to enable discrimination between preexistent and protein synthesized *in vitro*. The results show that apocytochrome *c* is synthesized *in vitro* and that it is located in the postribosomal fraction. Evidence is presented to indicate that the apoprotein is transferred to the mitochondria, where it is converted to holocytochrome *c*.

MATERIALS AND METHODS

Growth of Neurospora *Cells, Preparation of a Cell-Free Homogenate and Cell Fractionation*

Neurospora crassa (wild type, 74A) was grown in Vogel's minimal medium [18], supplemented with 2% sucrose, at 25 °C for 12–14 h under vigorous aeration and illumination with fluorescent lights. When indicated, cells were labelled with ^{35}S by growing them under reduced concentration of sulfate (0.08 mM) in the presence of 50 μCi/l sodium [^{35}S]sulfate (specific radioactivity 10–1000 Ci/mol, New England Nuclear Corp., Boston, Mass.) for 10–12 h. A chase of unlabelled sulfate (1 mM) was added and cells were grown for further 2 h.

Cell-free homogenates were prepared by sand grinding. 1 g of cells (wet weight) was mixed with 2 g of sterile quartz sand (Riedel de Haën, Hannover), 4 ml of medium A were added and the mixture ground in a sterile mortar for 6 min. Medium A contained 0.30 M sucrose, 0.02 M KH_2PO_4, 0.01 M $MgCl_2$, 0.01 M mercaptoethanol, a mixture of all L-configurated amino acids (0.00025 M) except leucine, pH 7.4. In order to obtain a cell-free homogenate, the resulting slurry was centrifuged twice for 5 min at $3500 \times g$ and the pellets were discarded.

A crude mitochondrial pellet was obtained by centrifuging the supernatant for 12 min at $14000 \times g$. Mitochondria were purified by two washing steps. They were resuspended in SET medium (0.44 M sucrose, 0.002 M EDTA, 0.03 M Tris-HCl, pH 7.2) and recentrifuged for 12 min at $14000 \times g$.

A homogenate depleted of mitochondria was obtained by recentrifuging the first supernatant from the first 12 min, $14000 \times g$ centrifugation under the same conditions. The resulting supernatant was centrifuged for 20 min at $48000 \times g$. The pellet was taken as the crude microsomal fraction. This was washed twice with 0.44 M sucrose, 0.01 $MgCl_2$, 0.01 M Tris-HCl, pH 7.5, to obtain a microsomal fraction largely devoid of free cytoplasmic ribosomes. Cytoplasmic ribosomes were sedimented from the supernatant from centrifugation for 20 min at $48000 \times g$ by a centrifugation for 60 min at $144000 \times g$. To obtain the postribosomal fraction the upper two thirds of the supernatant were collected. The colourless ribosomal pellet was covered by yellow layer, which was removed by gently shaking twice with 0.5 ml 0.44 M sucrose, 0.01 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.5.

Incorporation of [^3H]Leucine into Protein

ATP, GTP and creatine phosphate (Boehringer, Mannheim) were added to the cell-free homogenate in final concentrations of 0.004 M, 0.001 M and 0.008 M, respectively, then 50 μg/ml creatine kinase (Sigma Chemical Co., St Louis, Mo.) and chloramphenicol in a final concentration of 500 μg/ml. Chloramphenicol was a gift from Bayer (Leverkusen). 10 nmol [^3H]leucine (specific radioactivity 50 Ci/mmol, New England Nuclear Corp., Boston, Mass.) were added to 1 ml homogenate in a volume of 0.5 ml, containing 0.01 M KCl. Samples were incubated at 25 °C for periods indicated in the various experiments. Incorporation was terminated by cooling samples to 0 °C.

For the determination of protein synthesis by the microsomal and ribosomal fractions, the respective sediments were resuspended in separately prepared postribosomal supernatant. An energy regenerating system was added as described for the cell-free homogenate and incubation with [^3H]leucine was carried out under the same conditions.

Isolation of Holocytochrome c *and of Apocytochrome* c

Holocytochrome *c* was isolated according to the method of Heller and Smith [19] with several modifications. 3 kg of *Neurospora* cells (wet weight) were suspended in 12 l of distilled water in a kitchen homogeniser and then passed through a corundum disc mill especially designed for large scale preparations [20]. The effluent was adjusted to pH 10 with conc. ammonia and stirred for 2 h at 4 °C. Then glacial acetic acid was added until a pH of 8.0 was reached. The suspension was centrifuged for 20 min at $8000 \times g$ and the pellet was discarded. The supernatant was diluted with water to 100 l. Biorex 70 (400 mesh, Biorad Lab., Richmond, Calif.) equili-

brated with 0.005 M ammonium phosphate, pH 7.4, was added (4 g dry weight/l). The mixture was stirred overnight at 4 °C. The ion-exchange resin was collected by filtration and the extraction with Biorex 70 was repeated twice. The collected ion-exchange resin was treated with 0.25 M ammonium phosphate, pH 7.4, to elute the cytochrome *c*. The red supernatant was diluted with water 1 : 10 and rebound to fresh Biorex 70, equilibrated with 0.005 M ammonium phosphate. The resin was loaded on a column of Biorex 70, and washed successively with 400 ml 0.025 M ammonium phosphate buffer and 200 ml 0.05 M ammonium phosphate buffer, pH 7.4. Cytochrome *c* was then eluted as a sharp band with 0.25 M ammonium phosphate buffer, pH 7.5. The eluted cytochrome *c* was diluted and rechromatographed on Biorex 70. The finally eluted cytochrome *c* was dialysed against water, lyophilized, taken up in 2 ml 0.005 M ammonium phosphate, applied to a Sephadex G-50 column (20 × 2.5 cm) and eluted with the same buffer. The fractions containing cytochrome *c* were pooled and ammonium sulfate was added to a saturation of 90%. The pH was kept at 7.4 by addition of ammonia. The resulting small precipitate was removed by centrifugation. The supernatant was dialysed against water and then lyophilized. The purified cytochrome *c* was taken up in distilled water. Upon gel electrophoresis in the presence of dodecylsulfate it revealed one single band and the ratio $A_{550\,red}/A_{280\,ox}$ was 1.3 – 1.4. The yield was 4 – 6 µmol.

Apocytochrome *c* was prepared from cytochrome *c* according to the method of Fisher et al. [21], involving cleavage of the thioether bonds with Ag_2SO_4 in acetic acid and successive removal of Ag by treatment with dithiothreitol. The final preparation of apocytochrome *c* was taken up in distilled water and stored at – 20 °C.

Immunoprecipitation of Holocytochrome c *and Apocytochrome* c

Antibiodies against holocytochrome *c* and apocytochrome *c* were obtained in the following way: 0.5 ml of solution containing 1 mg of the respective protein was mixed with 0.5 ml of complete Freund's adjuvant (Behringwerke, Marburg) and injected into the neck region of rabbits. This procedure was repeated thrice at weekly intervals. Blood was drawn from the ear vein one week after the last injection, and the immunoglobulin fraction was prepared by ammonium sulfate precipitation.

Immunoprecipitation was carried out under the following conditions. Mitochondria and microsomes were resuspended in SET medium. To 0.5 ml of the various cell fractions 0.5 ml of 0.6 M KCl, 0.02 M Tris-HCl, pH 7.5, and 0.05 ml of 20% Triton X-100 were added. After standing for 10 min the samples were subjected to a clarifying spin (5 min, 5000 × *g*). Then holocytochrome *c* or apocytochrome *c* (10 µg) were added to the supernatant as carrier proteins. Appropriate amounts of immunoglobulin preparations, as determined by titration (usually in 20 – 50 µl) were added. Samples were kept at 2 – 4 °C for 12 h. The precipitates were collected by centrifugation. Pellets were washed by resuspension and centrifugation thrice with 1 ml of 0.3 M KCl, 0.01 M Tris-HCl, 1% Triton, pH 7.5 and twice with 1 ml 10 mM Tris-HCl, pH 7.5. The immunoprecipitates were dissolved in 40 µl of 0.1 M Tris-HCl, 2.5% 2-mercaptoethanol, 2% sodium dodecylsulfate, pH 7.5 for 2 h at room temperature. Aliquots of 20 µl were subjected to gel electrophoresis in the presence of dodecylsulfate.

Specificity of antibodies was determined on Ouchterlony plates [22]. The plates were stained with Coomas-

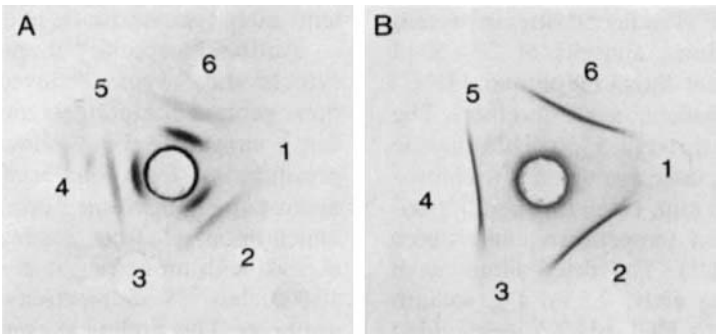

Fig. 1 *Reaction of anti-apocytochrome* c *and anti-holocytochrome* c *with apocytochrome* c *and holocytochrome* c *in the Ouchterlony double-immunodiffusion test.* Antisera obtained against apocytochrome *c* and holocytochrome *c* were placed in the center wells. (A) Antiserum against apocytochrome *c* (20 µl). Outer wells: 1,3,5 holocytochrome *c*, 0.5, 1 and 2 µg, respectively; 2,4,6 apocytochrome *c*, 5 µg. (B) Antiserum against holocytochrome *c* (20 µl). Outer wells: 1,3,5 apocytochrome *c*, 0.5, 1 and 5 µg, respectively; 2,4,6 holocytochrome *c*, 0.5 µg. The immunoprecipitation of apocytochrome *c* with anti-apocytochrome *c* yielded in most cases two lines. These may be due to the monomeric and dimeric forms of apocytochrome *c*, which are usually seen upon gel electrophoresis of preparations of apocytochrome *c* in the presence of dodecylsulfate in varying amounts

sie blue. Fig. 1 shows that the antibody obtained against apocytochrome *c* is able to precipitate isolated apocytochrome *c*, but not holocytochrome *c*. On the other hand, under the same conditions, the antibody against holocytochrome *c* precipitates isolated holocytochrome *c* but not apocytochrome *c*. However, when very high concentrations of apocytochrome *c* were applied, a slight precipitation line with anti-holocytochrome *c* was observed.

Gel Electrophoretic Analysis

Polyacrylamide gel slabs were prepared in the following way. 12 g acrylamide, 0.32 g *N'*,*N'*-methylenebisacrylamide and 100 µl tetramethylethylenediamine (Serva, Heidelberg) were dissolved in a buffer containing 0.1 M Tris-HCl, pH 8.0, and 0.5% sodium dodecylsulfate, in a final volume of 60 ml (solution A). 0.2 g ammonium peroxodisulfate were dissolved in 0.1 M Tris-HCl, pH 8, 0.5% sodium dodecylsulfate, in a final volume of 20 ml (solution B). Solutions A and B were mixed at 0 °C and placed in dismountable plastic moulds (3 × 75 × 150 mm) with 3 slot formers (20 µl volume). After polymerisation for 1 h at room temperature, the gels were removed from the forms and equilibrated with 0.1 M Tris-HCl, pH 8.0, 0.5% sodium dodecylsulfate at 4 °C for at least 24 h before use. The gels were then placed in a horizontal electrophoresis chamber and samples (up to 20 µl) were applied to the slots. The chamber had water cooled base and covering plates. The voltage was first set to 5 V/cm until proteins had entered the gel and then rised to 15 V/cm. Horse heart cytochrome *c* (Boehringer, Mannheim) was run in parallel as a marker and electrophoresis was stopped when the marker had migrated 50 to 60 mm. The gel strips were then cut with an automatic gel slicer into 1-mm slices.

Determination of Radioactivity

For measuring ^{35}S and ^{3}H radioactivities in protein of the various cell fractions, aliquots of 20–50 µl were pipetted on glass fiber filters (Whatman GF/C) presoaked with trichloroacetic acid in ether. The filters were passed twice through 5% trichloroacetic acid at room temperature, once through 5% trichloroacetic acid at 90 °C for 10 min, twice through 5% trichloroacetic acid at room temperature, and thrice through ethanol/ether (1/1). The dried filters were placed in plastic counting vials; 0.5 ml 1% sodium dodecylsulfate, 0.01 M Tris-HCl, pH 7.5 were added and after 2 h 12 ml scintillation fluid [600 ml toluene, 400 ml methoxyethanol, 6 g Permablend III (Packard Instruments, Frankfurt)]. Radioactivity in gel slices was determined after keeping the slices in 0.2 ml 1% sodium dodecylsulfate, 0.01 M Tris-HCl, pH 7.5, for 12 h at 60 °C in 1.7 ml polyethylene tubes. 1.2 ml scintillation fluid (700 ml toluene, 300 ml Triton X-100, 6 g Permablend III) was added.

Samples were counted in a Packard Tricarb 2425 liquid scintillation spectrometer. Yields of ^{35}S and ^{3}H radioactivities were 40% and 20%, respectively, in double labelled samples.

Conditions of Cyanogen Bromide Cleavage

Samples of isolated holocytochrome *c* or apocytochrome *c* or of immunoprecipitated holocytochrome *c* or apocytochrome *c* were dissolved in 90% formic acid and BrCN in a 100-fold excess with respect to methionine was added. The mixtures were incubated at room temperature for 12 h. Then formic acid and unreacted BrCN were removed in a rotary evaporator. The dry residues were taken up in 40 µl 1% sodium dodecylsulfate, 0.01 M Tris-HCl, pH 7.5 and subjected to gel electrophoresis in the presence of dodecylsulfate. Cytochrome *c* from horse heart and its large BrCN cleavage product were used as marker polypeptides.

RESULTS

Synthesis of Apocytochrome c *and Holocytochrome* c *in a Cell-Free System from* Neurospora

A cell-free homogenate prepared from *Neurospora* cells which were grown in the presence of [^{35}S]sulfate was incubated with [^{3}H]leucine for 10 min. Then cycloheximide was added to block further translation. One portion of the homogenate was immediately withdrawn and cooled to 0 °C. A second portion was incubated for further 30 min. Then also this portion was cooled to 0 °C. Incorporation of [^{3}H]leucine into the protein of the homogenate and of the various cell fractions was determined. The results are presented in Table 1. They show complete inhibition of [^{3}H]leucine incorporation into the homogenate proteins after cycloheximide poisoning.

Antibodies specific for apocytochrome *c* and holocytochrome *c* were employed to determine whether these protein components are synthesized in the cell-free homogenate. Fig. 2 shows the result of immunoprecipitation from the homogenate. The antibody against apocytochrome *c* precipitates ^{3}H radioactivity, which upon gel electrophoretic analysis is detected in a peak with an apparent molecular weight of about 12000. No ^{35}S radioactivity is precipitated by this antibody. This finding suggests that apocytochrome *c* is formed in the cell-free homogenate and that holocytochrome *c* is not recognized by the antibody.

Antibodies against holocytochrome *c* precipitate the holocytochrome *c* present in the cells (Fig. 2C). About 0.2% of the total ^{35}S radioactivity of the homogenate is precipitated by the latter antibody and

Table 1. *Incorporation of [^{3}H]leucine into the proteins of a cell-free homogenate and of various cell fractions*
A cell-free homogenate was incubated for 10 min with [^{3}H]leucine (see Methods). Then cycloheximide (100 µg/ml) was added. One half of the preparation was immediately cooled to 0 °C, the other half was further incubated for 30 min and then also cooled to 0 °C. The homogenate was subjected to differential centrifugation to obtain the various cellular fractions and incorporation of [^{3}H]leucine into protein was determined. Incorporated radioactivity is related to 1 ml of homogenate, or to the amount of the individual cell fractions derived from 1 ml of homogenate, respectively. About 50–60% of mitochondria and microsomes present in the cell-free homogenate were lost during isolation and washing by repeated differential centrifugation.

Fraction	Radioactivity after incubation for	
	10 min	10 min + cycloheximide 30 min
	counts × min^{-1}	
Total homogenate	4879655	4862907
Mitochondria	175980	230132
Microsomal fraction	453992	438312
Postribosomal supernatant	796131	660788
Cytoplasmic ribosomes	1480500	1468950

this radioactivity is found in a peak with the apparent molecular weight of about 12000. Also, ^{3}H radioactivity is found in the immunoprecipitate obtained with anti-holocytochrome *c*. Under appropriate conditions (low concentration of apocytochrome *c*) this antibody does not precipitate isolated apocytochrome *c*, as demonstrated by the Ouchterlony test (see Methods). As will be detailed later (see Table 2), it appears that also under the conditions of the experiment anti-holocytochrome *c* does not co-precipitate apocytochrome *c* synthesized *in vitro*. This is in agreement with the finding in the Ouchterlony test, since the amount of apocytochrome *c* synthesized *in vitro* in the cell homogenate is extremely small. ^{3}H radioactivity immunoprecipitated from the homogenate with antiholocytochrome *c* is found in a peak comigrating with the ^{35}S radioactivity. A large part of the ^{3}H radioactivity does not represent holocytochrome *c*. The presence of cytoplasmic ribosomes in the cell homogenate leads to coprecipitation of other material than newly synthesized holocytochrome *c*. In contrast, from the mitochondrial fraction (and the postribosomal fraction) newly synthesized holocytochrome *c* can be immunoprecipitated in a highly specific manner [23].

In order to further substantiate the identity of the immunoprecipitated components the following experiments were carried out. Isolated holocytochrome *c* and apocytochrome *c* were analysed on dodecyl-sulfate-containing gels and a difference in electrophoretic mobility was not observed (not shown here). Furthermore, an immunoprecipitate obtained as in Fig. 2A was mixed with an immunoprecipitate obtained from mitochondria from ^{35}S-labelled cells with antibodies against holocytochrome *c*. In the gel electrophoretic profile (Fig. 3A), the ^{3}H radioactivity and the ^{35}S radioactivity coincide completely.

An immunoprecipitate obtained as described in Fig. 2A was subjected to cyanogen bromide cleavage and then mixed with an immunoprecipitate of ^{35}S-labelled holocytochrome *c*. The gel electrophoretic separation is shown in Fig. 3B. Besides the ^{35}S peak representing holocytochrome *c*, one major peak with an apparent molecular weight of about 9000 is seen in the ^{3}H profile. *Neurospora* cytochrome *c* contains two methionine residues and upon cyanogen bromide cleavage yields fragments with 84, 18 and 5 amino acid residues [24]. Obviously, the ^{3}H peak in Fig. 3B represents the large fragment. Cyanogen bromide cleavage products of isolated apocytochrome *c* display upon gel electrophoresis and staining with Coomassie brilliant blue a band in the same position. In order to also demonstrate the identity of the holocytochrome *c* synthesized *in vitro*, immunoprecipitates were subjected to cyanogen bromide cleavage and the fragments analysed by gel electrophoresis (Fig. 3C). Both the ^{35}S and ^{3}H radioactivity are mainly found in a peak with an apparent molecular weight of about 9000, in this case representing the 84-amino-acid residue fragment with the attached heme.

It is concluded from the above findings that apocytochrome *c* and holocytochrome *c* are synthesized in the cell-free homogenate. It seems reasonable to assume that the detected apocytochrome *c* is a precursor of holocytochrome *c*. This assumption is supported by the second part of the experiment described in Fig. 2. When after 10 min of leucine incorporation translation was blocked by cycloheximide and the system incubated for further 30 min, the amount of ^{3}H-labelled apocytochrome *c* which can be precipitated is decreased, whereas the amount of ^{3}H-labelled holocytochrome *c* is increased. This indicates that apocytochrome *c* synthesized during the 10-min period is converted to holocytochrome *c* during the following 30-min period in the absence of protein synthesis.

Subcellular Localisation of Apocytochrome c *and Holocytochrome* c *Synthesized in the Cell-Free Homogenate*

A cell-free homogenate incubated with [^{3}H]leucine as described for Fig. 2 was subfractionated into mitochondria, microsomes, free ribosomes and postribosomal supernatant. Incorporation of [^{3}H]leucine into the proteins of various cell fractions was determined

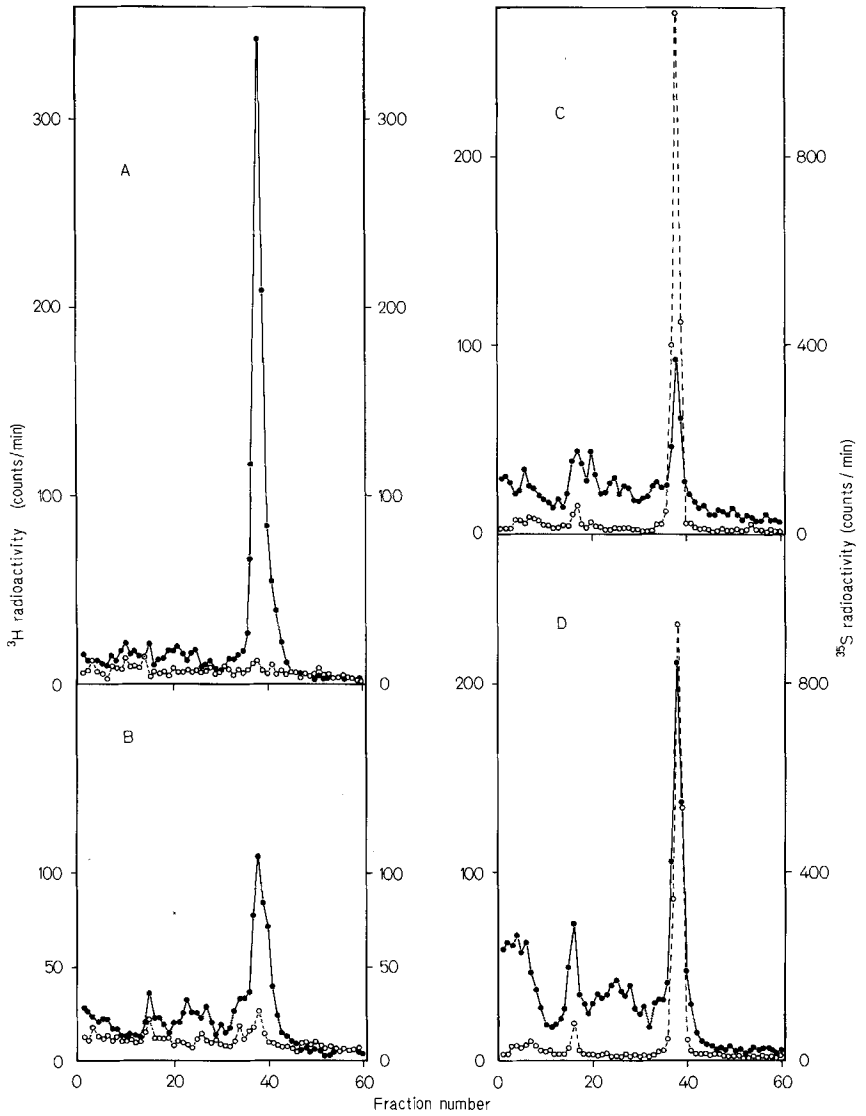

Fig. 2. *Immunoprecipitation with anti-apocytochrome* c *and anti-holocytochrome* c *from a cell-free homogenate after labelling with [^{3}H]leucine.* A cell-free homogenate was prepared from cells grown in the presence of [^{35}S]sulfate. The homogenate was incubated with [^{3}H]leucine for 10 min; one half was withdrawn and cooled, the other half was incubated in the presence of cycloheximide for further 30 min. Then immunoprecipitation with anti-apocytochrome *c* and anti-holocytochrome *c* was carried out with both portions. The immunoprecipitates were subjected to gel electrophoresis in the presence of dodecylsulfate. ^{3}H and ^{35}S radioactivities were determined after slicing the gels. (A, B) Immunoprecipitates obtained with anti-apocytochrome *c*; (C, D) immunoprecipitates obtained with anti-holocytochrome *c*; (A, C) incubation for 10 min; (B, D) incubation for 10 min, followed by 30 min in the presence of cycloheximide. (●——●) ^{3}H; (O---O) ^{35}S

(Table 1). The ^{3}H radioactivity of the mitochondrial fraction increases, when after 10 min incubation the homogenate is further incubated for 30 min in the presence of cycloheximide. This is interpreted as a continued uptake of newly synthesized protein into the mitochondrial fraction after the end of the period of protein synthesis. The microsomal fraction and free ribosome fraction remain practically unchanged after cycloheximide addition whereas the ^{3}H radioactivity of the postribosomal supernatant decreases. This is in agreement with the results presented in a previous paper [23].

Apocytochrome *c* as well as holocytochrome *c* were immunoprecipitated from the various cell fractions after 10 min and 40 min incubation. Table 2 shows the ^{3}H and ^{35}S radioactivities detected in the peak fractions after electrophoretic analysis of the immunoprecipitates.

From mitochondria, holocytochrome *c* synthesized *in vitro* can be immunoprecipitated, but not apocytochrome *c*. The amount of preexistent holocytochrome *c* is the same after 10 min and 40 min, as shown by the ^{35}S radioactivity. The ^{3}H-labelled holocytochrome *c* however increases considerably during the 30-min

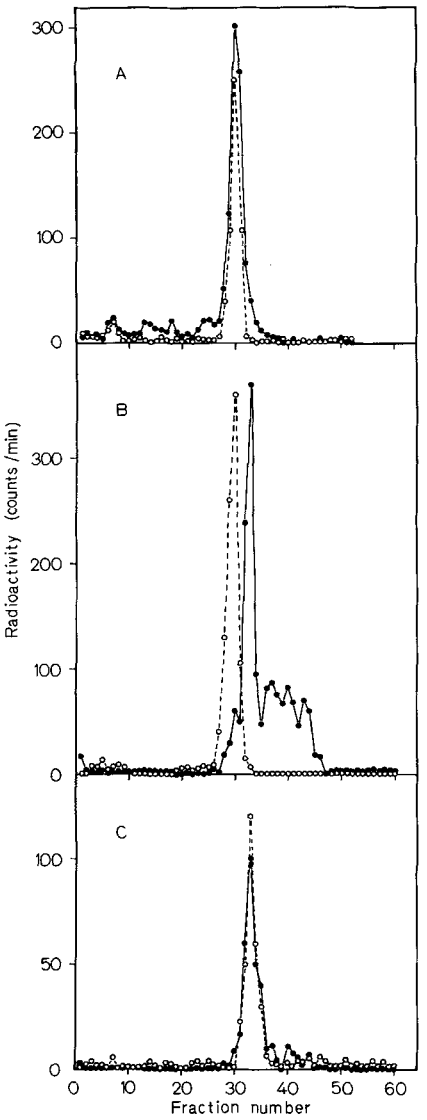

Fig. 3. *Cyanogen bromide cleavage of immunoprecipitated apocytochrome* c *and holocytochrome* c. (A) An immunoprecipitate obtained with anti-apocytochrome *c* from a cell-free homogenate incubated for 10 min with [^{3}H]leucine was mixed with an immunoprecipitate obtained with anti-holocytochrome *c* from mitochondria isolated from cells grown in the presence of [^{35}S]sulfate. (B) An immunoprecipitate obtained with anti-apocytochrome *c* from a cell-free homogenate incubated for 10 min with [^{3}H]leucine was subjected to BrCN cleavage and then mixed with an immunoprecipitate obtained with anti-holocytochrome *c* from mitochondria isolated from cells grown in the presence of [^{35}S]sulfate. (C) An immunoprecipitate obtained with anti-holocytochrome *c* from a homogenate prepared from cells grown in the presence of [^{35}S]sulfate, and an immunoprecipitate from mitochondria obtained from a cell-free homogenate which was incubated for 40 min with [^{3}H]leucine were mixed. The mixture was subjected to BrCN cleavage. Samples were dissolved in dodecylsulfate-containing buffer and subjected to gel electrophoresis in the presence of dodecylsulfate. (●——●) ^{3}H; (○---○) ^{35}S

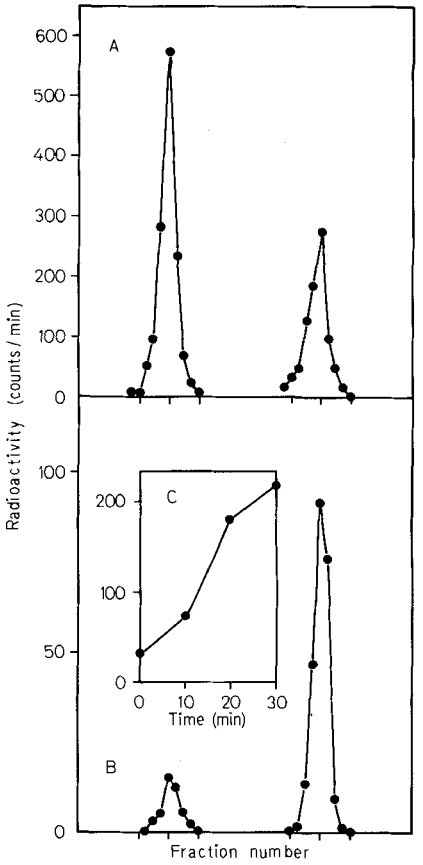

Fig. 4. *Conversion of apocytochrome* c *synthesized* in vitro *to holocytochrome* c *upon incubation of a postribosomal supernatant with isolated mitochondria.* A postmitochondrial supernatant from a cell-free homogenate was obtained and incubated with [^{3}H]leucine for 10 min. Then cycloheximide was added, and a postribosomal supernatant prepared. Mitochondria which were isolated from 1 ml of a cell-free homogenate were resuspended in 1 ml of this postribosomal supernatant. After resuspension, 0.5 ml were immediately withdrawn and kept at 0 °C, the other 0.5 ml were incubated for 30 min at 25 °C. Then both portions were separated into the mitochondrial fraction and the supernatant fraction by centrifugation for 20 min at 20000 × *g*. Immunoprecipitation was carried out with anti-apocytochrome *c* and anti-holocytochrome *c* and the immunoprecipitates were analysed by gel electrophoresis in the presence of dodecylsulfate. (A) Immunoprecipitation with anti-apocytochrome *c* from the supernatant fraction; (B) immunoprecipitation with anti-holocytochrome *c* from the mitochondrial fraction. The peaks on the left represent the 0 °C control sample, the peaks on the right represent the sample incubated at 25 °C. (C, insert) In a separate experiment mitochondria were obtained after incubating postribosomal supernatant with mitochondria for 0, 10, 20 and 30 min, respectively; immunoprecipitation with anti-holocytochrome *c* was carried out from the four mitochondrial fractions. The radioactivity in the gel electrophoretic peak fraction was determined and plotted versus time of incubation

period in the presence of cycloheximide. This suggests that the amount of apocytochrome *c* in the mitochondria is below the level of detection and that apocytochrome *c* or holocytochrome *c* in the absence of protein synthesis is transferred to the mitochondrial fraction.

From the microsomal fraction a small amount of preexistent holocytochrome *c* can be precipitated, indicating contamination of this fraction with mitochondria or artifactual adsorption to this fraction after leakage from the mitochondria. ^{3}H-labelled apo-

Table 2. *Immunoprecipitation of apocytochrome* c *and holocytochrome* c *from various cell fractions after labelling of a cell-free homogenate with [^{3}H]leucine*

A cell-free homogenate was obtained from cells grown in the presence of [^{35}S]sulfate. The homogenate was labelled for 10 min with [^{3}H]leucine. Then one half was withdrawn and cooled to 0 °C, the other half was further incubated for 30 min in the presence of cycloheximide and then cooled to 0 °C. The homogenate was fractionated by differential centrifugation to obtain mitochondria, microsomes and the postribosomal supernatant. Immunoprecipitation with antisera against apocytochrome *c* and holocytochrome *c* was carried out. The immunoprecipitates were subjected to gel electrophoresis in the presence of dodecylsulfate. ^{3}H and ^{35}S radioactivities were determined in gel slices and the radioactivities of the peak fractions corresponding to holocytochrome *c* or apocytochrome *c* were calculated. The radioactivities are related to those amounts of the various cell fractions which were derived from 1 ml homogenate

Fraction	Incubation	Radioactivity after precipitation with			
		anti-apocytochrome *c*		anti-holocytochrome *c*	
		^{3}H	^{35}S	^{3}H	^{35}S
		counts/min			
Mitochondria	10 min	13	11	1204	4607
Mitochondria	10 min + cycloheximide 30 min	14	3	1852	4624
Microsomes	10 min	26	12	249	245
Microsomes	10 min + cycloheximide 30 min	13	0	328	317
Postribosomal supernatant	10 min	2862	46	70	4004
Postribosomal supernatant	10 min + cycloheximide 30 min	1874	23	263	4191

cytochrome *c* is not found in this fraction. The amount of ^{3}H-labelled holocytochrome *c* is low as compared to the mitochondrial fraction, however the ^{3}H/^{35}S ratio is relatively high. On the other hand a precursor function of the microsomal holocytochrome *c* is clearly not suggested by the change of ^{3}H and ^{35}S radioactivities.

From the postribosomal fraction holocytochrome *c* can be precipitated (about 30–40% of total preexistent cytochrome *c* in the homogenate). This is obviously due to the well known fact that cytochrome *c* upon breakage of cells easily leaks out of the mitochondria. However, only a small amount of holocytochrome *c* synthesized *in vitro* is found in this fraction, which increases during incubation in the presence of cycloheximide. This indicates that holocytochrome *c* in the postribosomal supernatant is not a precursor of mitochondrial holocytochrome *c* and that the holocytochrome *c* synthesized *in vitro* leaks out from the mitochondria only to a low degree. On the other hand, apocytochrome *c* synthesized *in vitro* is clearly present in the postribosomal supernatant. The amount of apocytochrome *c* in this fraction decreases after blocking protein synthesis with cycloheximide.

The inability of the antibody against holocytochrome *c* to precipitate significant amounts of ^{3}H radioactivity from the postribosomal fraction after 10 min incubation demonstrates absence of cross-reaction with apocytochrome *c*.

In summary these data show that within the limits of experimental error, apocytochrome *c* synthesized *in vitro* is located in the postribosomal fraction, whereas holocytochrome *c* synthesized *in vitro* is essentially located in the mitochondrial fraction. The low amounts of holocytochrome *c* in the postribosomal and microsomal fractions are probably due to leakage from mitochondria. It should be noted that apocytochrome *c* can be precipitated from the postribosomal fraction in the absence of Triton X-100, suggesting that it is not embedded in membranes, or in the lumen of vesicles, or associated with other components, which mask its antigenic sites. Furthermore, there appears to exist a precursor product relationship between apocytochrome *c* in the postribosomal fraction and holocytochrome *c* in the mitochondrial fraction.

Precursor Product Relationship between Apocytochrome c *and Holocytochrome* c

A cell-free homogenate from which mitochondria were removed by centrifugation was incubated with [^{3}H]leucine. After 15 min cycloheximide was added and the homogenate was cooled and subjected to ultracentrifugation. Mitochondria isolated from cells prelabelled with [^{35}S]sulfate were resuspended in this postribosomal supernatant. One portion of the mixture was kept at 0 °C, a second portion was incubated without further addition for 30 min. Then mitochondria and postribosomal supernatant were separated again by centrifugation. Holocytochrome *c* was immunoprecipitated from mitochondria and apocytochrome *c* from the postribosomal supernatants. Fig. 4 shows that ^{3}H-labelled holocytochrome *c* is found in the mitochondria of the 0 °C control in a very low amount, and in much higher amounts after 30 min incubation at 25 °C. At the same time, part of the apocytochrome *c* disappears from the postribosomal

supernatant. From the supernatant no holocytochrome *c* and from the mitochondrial fraction no apocytochrome *c* could be precipitated.

This demonstrates that apocytochrome *c* is converted to holocytochrome *c* during incubation of the postribosomal supernatant with the isolated mitochondria. Extramitochondrial apocytochrome *c* therefore appears to be a precursor of mitochondrial holocytochrome *c*.

In order to determine whether apocytochrome *c* or holocytochrome *c* is transferred into the mitochondria, i.e. whether the heme group is linked to the apoprotein in the mitochondria or outside the mitochondria, the following experiments were carried out.

A cell-free homogenate from which mitochondria were removed by differential centrifugation, was incubated with [^{3}H]leucine for 15 min. Then cycloheximide was added and the postribosomal fraction was prepared. This latter fraction was incubated for 30 min at 25 °C under the following conditions: without further addition; after resuspension with isolated mitochondria; after addition of hemin; after addition of mitochondria lysed with Triton X-100. Furthermore, as a control, the postmitochondrial fraction was kept at 0 °C for 30 min. Holocytochrome *c* was immunoprecipitated from the various samples after addition of Triton X-100. The immunoprecipitates were subjected to gel electrophoresis and the ^{3}H radioactivity in the peaks corresponding to apocytochrome *c* and holocytochrome *c*, respectively, determined. Table 3 shows, that in the postribosomal supernatant incubated at 25 °C no ^{3}H-labelled holocytochrome *c* was detected. This demonstrates that the postribosomal supernatant was incapable by itself of synthesizing holocytochrome *c* from apocytochrome *c*. Addition of heminchloride to the postribosomal supernatant did not promote the formation of holocytochrome *c*. When isolated mitochondria were added to the supernatant, apocytochrome *c* decreased and holocytochrome *c* was formed. Upon incubation at 0 °C conversion of apocytochrome *c* was not observed. When mitochondria were solubilized with Triton X-100 formation of holocytochrome *c* was abolished. Also in this case, added hemin did not promote the formation of holocytochrome *c*.

In a further experiment, a postribosomal supernatant prelabelled with [^{3}H]leucine as described in the previous paragraph was resuspended with mitochondria isolated from ^{35}S-labelled cells. The suspension was divided into three equal portions. One portion served as a control; to the second portion holocytochrome *c* (1 nmol/ml), and to the third portion apocytochrome *c* (1 nmol/ml) was added. From all three samples aliquots were withdrawn immediately after resuspension at 0 °C, and after incubation at 25 °C for 30 min. Mitochondria were reisolated; holocytochrome *c* was immunoprecipitated after addition of Triton X-100 and analysed by gel electrophoresis. The peak fractions are shown in Fig. 5. In the control, the formation of ^{3}H-labelled holocytochrome *c* after 30 min incubation is clearly seen. When holocytochrome *c* was added to the suspension, no difference as compared to the control was observed. In contrast, when apocytochrome *c* was added, formation of ^{3}H-labelled holocytochrome *c* was practically completely inhibited. It appears that the added apocytochrome *c*, but not holocytochrome *c*, could compete with the endogenous apocytochrome *c* synthesized *in vitro* for uptake into the mitochondria.

Table 3. *Conversion of apocytochrome* c *synthesized* in vitro *to holocytochrome* c

A cell-free homogenate was centrifuged twice for 10 min at 14000 × *g* and then incubated for 10 min with [^{3}H]leucine. Cycloheximide was added and the homogenate was cooled to 0 °C. The postribosomal supernatant was prepared. When indicated, mitochondria isolated from 1 ml homogenate and/or hemin chloride (30 nmol/ml) were added. Samples of 1 ml were incubated under various conditions for 30 min at 25 °C. Then Triton X-100 was added in a final concentration of 1% and immunoprecipitation with anti-holocytochrome *c* was carried out. The immunoprecipitates was subjected to gel electrophoresis in the presence of dodecylsulfate and the radioactivity in the holocytochrome *c* peaks was determined

Addition to postribosomal supernatant	^{3}H radioactivity in immunoprecipitated holocytochrome *c*
	counts × min^{-1}
No addition	20
Mitochondria	592
Mitochondria (incubation at 0 °C)	22
Hemin-chloride	20
Mitochondria Hemin-chloride	536
Mitochondria Lysis with 1% Triton X-100	18
Mitochondria Lysis with 1% Triton X-100 Hemin-chloride	0

DISCUSSION

In a previous report we have shown that in a cell-free protein-synthesizing system of *Neurospora crassa*, cytochrome *c* is synthesized and integrated into the mitochondria [23]. The data presented here demonstrate that in this system apocytochrome *c* can be detected and that this apocytochrome *c* is a precursor to holocytochrome *c*. The data furthermore suggest that it is apocytochrome *c* which is transferred into the mitochondria and that the heme group is linked to the apoprotein in a mitochondrial reaction. The following route by which cytochrome *c* reaches its functional site in the mitochondrial mem-

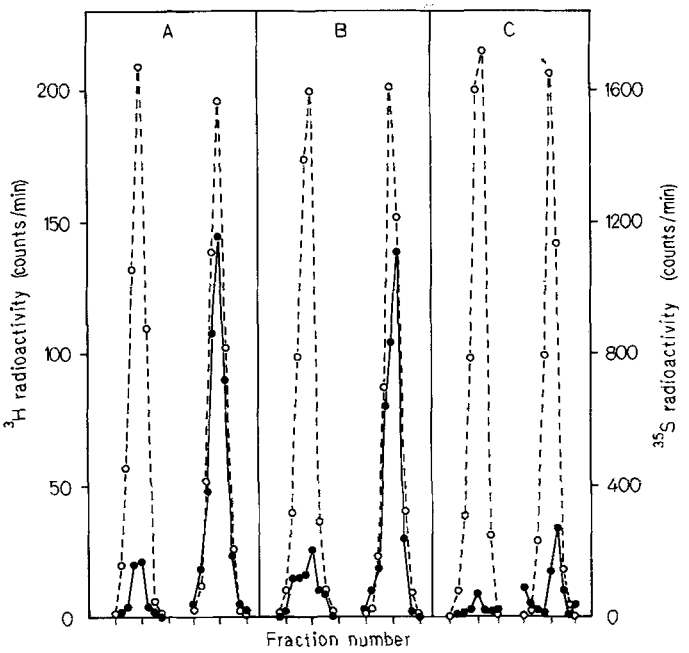

Fig. 5. *Competition of unlabelled holocytochrome* c *and apocytochrome* c *with labelled apocytochrome* c *synthesized* in vitro *for conversion into holocytochrome* c *and uptake into mitochondria.* A post-mitochondrial supernatant was incubated with [^{3}H]leucine for 10 min and cycloheximide was added. A postribosomal supernatant was prepared. Mitochondria were isolated from cells grown in the presence of [^{35}S]sulfate and resuspended in the postribosomal supernatant. The suspension was divided into three equal portions. One served as a control, to the second unlabelled holocytochrome *c* was added, to the third unlabelled apocytochrome *c*. One half of each portion was immediately withdrawn and kept at 0 °C, the other half was incubated at 25 °C for 30 min. Then these portions were cooled to 0 °C. Mitochondria were isolated from all samples. Then immunoprecipitation with anti-holocytochrome *c* was carried out. The immunoprecipitates were subjected to gel electrophoresis in the presence of dodecylsulfate. ^{3}H and ^{35}S radioactivities were determined in gel slices. Peak fractions corresponding to cytochrome *c* are shown. (A) Control; (B) addition of holocytochrome *c* (1 nmol/ml); (C) addition of apocytochrome *c* (1 nmol/ml). (●——●) ^{3}H; (O---O) ^{35}S. The peaks on the left in A, B and C represent the 0 °C control samples, the peaks on the right represent the samples incubated at 25 °C

brane may be suggested on the basis of the experiments described here.

Apocytochrome *c* is synthesized on free or membrane-bound cytoplasmic ribosomes. In preliminary experiments both ribosome fractions were found to be active in synthesizing apocytochrome *c* (unpublished results). Since, however, practically nothing is known about the nature of membrane-bound ribosomes in *Neurospora*, about possible interaction of free ribosomes with membranes or release of bound ribosomes from membranes, it is not possible at present to decide whether actually both types of ribosomes are active or whether only one type is active *in vivo*.

Apocytochrome *c* is released into the cytosol. Apocytochrome *c* is detected in the postribosomal fraction after incubation of a cell-free homogenate or of partial systems containing only free or membrane-bound ribosomes. The apocytochrome *c* formed *in vitro* can be immunoprecipitated without prior treatment of the cell-free system with detergent, indicating the presence of apocytochrome *c* in free form. The ability of extramitochondrial apocytochrome *c* to function as a precursor of mitochondrial cytochrome *c* may indicate that the cytosol is *in vivo* the site of an apocytochrome *c* pool. Support for this indication comes from two lines of evidence. Reconstitution experiments described in this report, show that prelabelled apocytochrome *c* present in the isolated postribosomal fraction is converted to holocytochrome *c* after addition of isolated mitochondria. Excess unlabelled apocytochrome *c* but not holocytochrome *c* acts as a competitor for uptake of labelled apocytochrome *c* into the mitochondria.

It must certainly be taken into consideration that the apocytochrome *c* detected in the postribosomal supernatant may derive from a cellular compartment other than the cytosol. Taking together all the available information, however, a localisation of an apocytochrome *c* pool in the cytosol appears the most reasonable interpretation.

On its way to the inner membrane apocytochrome *c* has to pass the outer mitochondrial membrane. This appears to be a problem of major and general importance for protein transport into mitochondria. The outer membrane has been described to have pores for molecules with molecular weights up to 6000 to 10000 [25,26]. On the other hand, impermeability of the outer membrane to holocytochrome *c* has been observed with isolated mitochondria and outer membranes from rat liver [27,28]. It is however not excluded that the suggested pores with diameters of about 2 nm corresponding to the above mentioned molecular weights, result from the rather crude procedures involved in the isolation of mitochondria and outer mitochondrial membranes.

It should be noted in this context that in the cell-free homogenate the outer mitochondrial membranes are frequently ruptured, as revealed by electron-microscopic examination of the preparations. Therefore it is by no means excluded that in the experiments described here, apocytochrome *c* synthesized *in vitro* can reach the inner membrane by direct diffusion.

Experiments presented here suggest that the addition of the heme group occurs only in the mitochondria but not in the cytosol. Conversion of apocytochrome *c* to holocytochrome *c* could not be observed in the cytosolic fraction, also not after the addition of hemin. On the other hand, nothing is known about the reaction in which the thioether bridges between heme and apoprotein are formed. It might be expected to be an enzymatically catalysed

reaction. It has been reported that this reaction takes place without enzymatic catalysis [29]. However, the conditions required were far from conditions *in vivo*. The submitochondrial site of the reaction remains to be determined.

The hypothesis is put forward that the conformational change involved in the transition from apocytochrome *c* to holocytochrome *c* leads to trapping of holocytochrome *c* in the mitochondrial membrane. It remains to be explained why holocytochrome *c*, released from the mitochondria during cell fractionation, is not rebound to the mitochondrial membrane. Such an explanation would require a detailed knowledge on the alterations of the mitochondrial membranes during cell fractionation, on the factors which govern the binding of cytochrome *c*, and on the question whether there exist heterogenous pools of cytochrome *c* which are differently bound to the mitochondrial membrane.

There is a large body of literature on the synthesis and transfer of cytochrome *c* in rat liver. However, the conclusions reached by the individual authors are at variance. Summaries on the developments in the field of biosynthesis of cytochrome *c* were presented by Sherman and Stewart [1] and by Gonzalez-Cadavid [17]. The main reasons for the ambiguous results and explanations come from a number of experimental difficulties such as: unsatisfactory purification and identification of cytochrome *c*; need for large samples for the isolation of cytochrome *c*; inability to differentiate between preexistent and newly synthesized cytochrome *c*; artifactual redistribution of cytochrome *c* during cell fractionation; inability to separate and determine precursors of cytochrome *c*; extremely low rates of incorporation of radioactive amino acids into cytochrome *c*.

Despite these difficulties, on the basis of kinetic studies *in vivo* and of studies with subcellular systems *in vitro* it was concluded that in liver, cytochrome *c* is synthesized by the microsomal fraction, i.e. that not only apocytochrome *c* is translated on membrane-bound ribosomes but that also the heme group is linked to the apoprotein at the microsomes [3–5, 7, 8]. It was however contended by Kadenbach, reinterpreting earlier results [7, 30] that in rat liver, apocytochrome *c* is synthesized by the microsomal fraction and then transferred into the mitochondria [31]. A chromatographic fraction with similar elution characteristics as cytochrome *c* was suspected to represent apocytochrome *c*, although its identity was not verified, A precursor product relationship between this suspected apocytochrome *c* and cytochrome *c* was also suggested, however this was based on extremely low rates of radioactivities. This suggestion was questioned by several authors [32–34] and it was claimed that even preparations of free and detergent-treated ribosomes from rat liver are able to incorporate radioactive leucine into cytochrome *c* and the heme precursor δ-aminolevulinic acid into cytochrome *c* bound heme [34]. Recently, a careful study on the distribution of cytochrome *c* in various subcellular fractions of rat liver homogenates was published [35]. By kinetic labelling studies the authors ruled out the possibility that microsomal cytochrome *c* might act as precursor of mitochondrial cytochrome *c* [36].

In summary, it seems not possible at present to decide whether in the rat liver system apocytochrome *c* or holocytochrome *c* is transported into the mitochondria.

Synthesis of cytochrome *c* from yeast in a wheat germ system programmed with yeast poly(A)-containing RNA was recently reported [37]. It is interesting in this context whether apocytochrome *c* is possibly synthesized as a precursor with a higher molecular weight. In this study *in vitro* the translation product was isolated by double immunoprecipitation and identified by analysis of the cyanogen-bromide-cleavage products. On dodecylsulfate containing gels no difference in size compared to authentic cytochrome *c* was observed. This agrees with our own observation on apocytochrome *c* translated in a wheat-embryo cell-free system programmed with *Neurospora* polyA-RNA. The immunoprecipitated apocytochrome *c* had the same apparent molecular weight as the isolated protein (unpublished results).

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