CELL-FREE SYNTHESIS OF CYTOCHROME *c*

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1. Introduction

Cytochrome *c* is a peripheral membrane protein, attached to the cytoplasmic surface of the inner mitochondrial membrane [1–4]. It is coded for by a nuclear gene and translated on cytoplasmic ribosomes (reviewed [5]). We have reported that in *Neurospora*, cytochrome *c* is synthesized as apocytochrome *c* on cytoplasmic ribosomes, released into the cytosolic compartment and then transferred to mitochondria where it is converted to holocytochrome *c* [6]. Here we analyse the primary translation product and demonstrate that apocytochrome *c* is synthesized not as a larger precursor but with its authentic size. It is further demonstrated that apocytochrome *c* synthesized in a heterologous cell-free system can be converted to holocytochrome *c* by addition of mitochondria isolated from *Neurospora* cells. Free ribosomes were found to be the main site of synthesis of apocytochrome *c*.

2. Materials and methods

Most methods applied were described in [6–9].

2.1. *Conversion of apocytochrome* c *to holocytochrome* c

For transfer experiments of apocytochrome *c*, synthesized in the reticulocyte lysate, protein synthesis directed by *Neurospora* poly(A)-RNA was carried out for 60 min [9]. Then the incubation mixture was centrifuged for 60 min at 144 000 × *g*. The supernatant was collected and methionine and sucrose were added to final concentrations of 1 mM and 0.3 M, respectively. To this mixture, mitochondria isolated from *Neurospora* cells [10] were added (1.0–1.5 mg mitochondrial protein/ml). After resuspension, the mixture was incubated for 30 min at 25°C. Then mitochondria were re-isolated and lysed in buffer containing 1% Triton X-100 and 0.3 M KCl [6]. Immunoprecipitation with antibody to holocytochrome *c* was carried out as in [6].

2.2. *Protein synthesis by isolated free and membrane-bound ribosomes*

For preparation of free and bound ribosomes, *Neurospora* cells (8 g wet wt) were ground with 12 g quartz sand and 16 ml 'medium A' as in [8]. The mixture was centrifuged twice for 5 min at 3500 × *g*, and twice for 12 min at 14 000 × *g*. The supernatant was placed on top of sucrose step density gradients constructed and centrifuged according to [11], with the exceptions that medium A was used to make up the sucrose solutions, and that a Beckman rotor 50 Ti with 13.5 ml tubes was used. Further purification of membrane-bound ribosomes was performed in the same rotor and using medium A but otherwise following the procedure in [11].

Postribosomal supernatant was prepared from cells homogenized as above by centrifugation of the 5 min, 3500 × *g* supernatant for 60 min at 144 000 × *g*. The upper 2/3rds of the resulting supernatant were collected.

Each of the two ribosome preparations was taken up in 2 ml of this supernatant. Then [^{35}S]methionine, ATP, GTP, creatine phosphate, creatine kinase were added at final concentrations described for protein synthesis in cell-free homogenates [6,8] and incubation was carried out for 30 min.

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3. Results

3.1. *Synthesis of apocytochrome* c *in heterologous cell-free systems*

Poly(A)-RNA isolated from *Neurospora* was used to direct the synthesis of cytochrome *c* in cell-free extracts from rabbit reticulocytes and of wheat germ. Antibodies against *Neurospora* apocytochrome *c* were employed to precipitate the product. The immunoprecipitates were analysed by SDS–electrophoresis and autoradiography. Figure 1 shows that in both systems a single component was immunoprecipitated, which

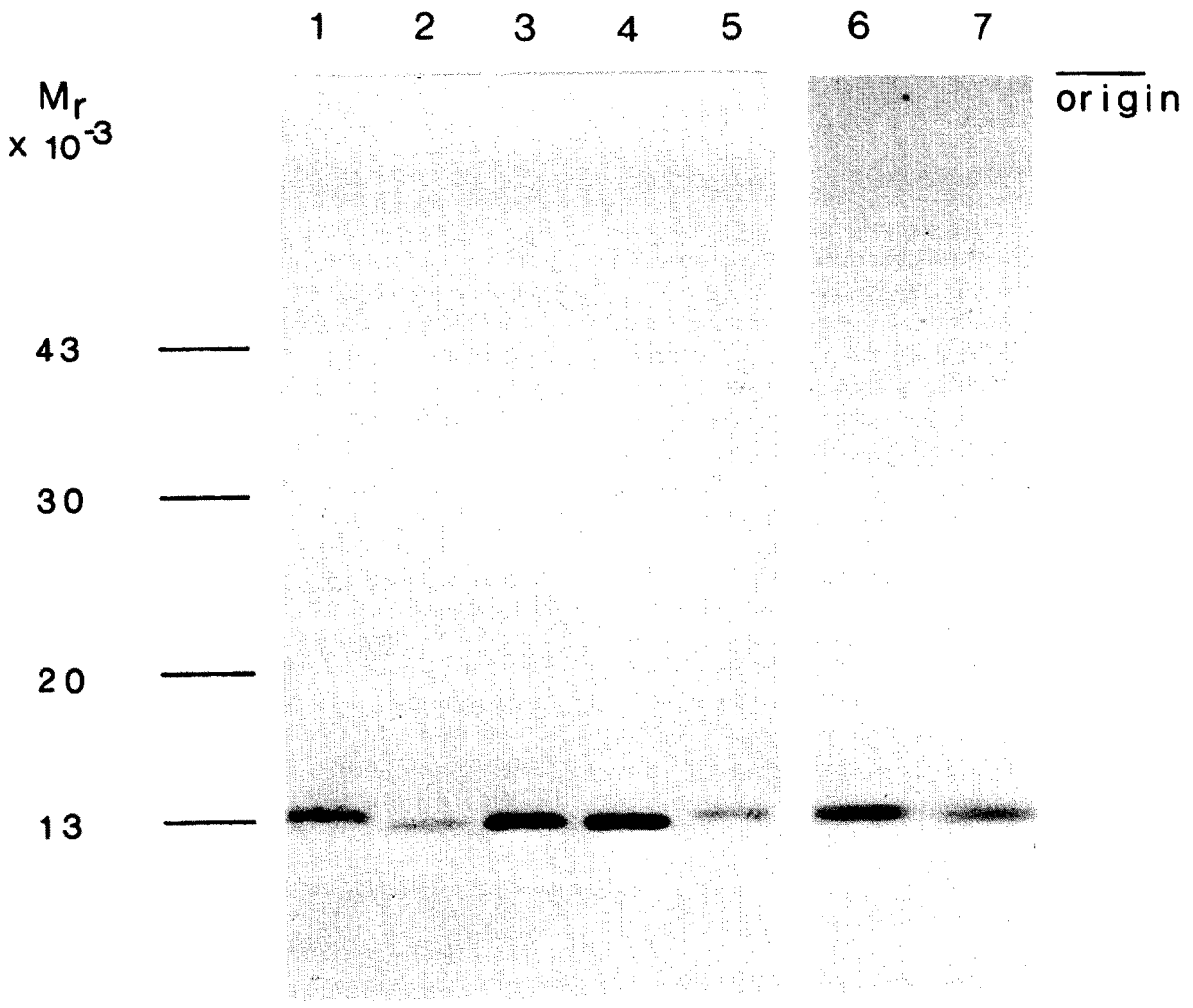

Fig.1. Synthesis of apocytochrome *c* in heterologous systems directed by *Neurospora* messenger-RNA. Rabbit reticulocyte lysates and wheat embryo extract were supplemented with *Neurospora* poly(A)-RNA. Radioactive labelling was performed with either [^{35}S]methionine or f-[^{35}S]met-tRNA. Apocytochrome *c* was immunoprecipitated with specific antibodies and the immunoprecipitate analysed by SDS–gel electrophoresis and autoradiography. For comparison, holocytochrome *c* was immunoprecipitated with specific antibody from mitochondria isolated from cells grown in the presence of [^{35}S]sulfate. Isolated apo- and holocytochrome *c* were also separated on the gel and stained with Coomassie brilliant blue. (1–5) Autoradiograph; (6,7) protein staining; (1,5) holocytochrome *c* from mitochondria; (2) apocytochrome *c* from wheat germ extract, labelled with [^{35}S]methionine; (3) apocytochrome *c* from rabbit reticulocyte lysate, labelled with [^{35}S]methionine; (4) apocytochrome *c* from rabbit reticulocyte lysate, labelled with f-[^{35}S]met-tRNA; (6) isolated holocytochrome *c*; (7) isolated apocytochrome *c*.

had a slightly higher electrophoretic mobility than *Neurospora* holocytochrome *c* and the same mobility as chemically prepared *Neurospora* apocytochrome *c* [12]. Antibodies against holocytochrome *c* which were shown not to react with apocytochrome *c* under the pertaining conditions did not lead to precipitation of a corresponding band (not shown). This finding suggests that apocytochrome *c* but not holocytochrome *c* is formed in the heterologous system and that the original translation product has the same molecular size as apocytochrome *c* prepared from holocytochrome *c*.

In order to confirm that cytochrome *c* is not synthesized as a larger precursor, protein synthesis in a reticulocyte lysate was carried out in the presence of formyl-[^{35}S]methionyl-transfer RNA. Charging of bulk calf liver tRNA with [^{35}S]methionine was carried out with enzyme from *Escherichia coli*, which leads to selective aminoacylation of initiator tRNA and this product was formylated with *E. coli* transformylase [9,13,14]. Formyl-methionine is incorporated in the cell-free system into the amino terminal position selectively and is not removed by methionine aminopeptidase [15,16]. Therefore, labelling of the product indicates an intact amino terminus. Figure 1 shows that the immunoprecipitated apocytochrome *c* labelled by this procedure displays the same apparent molecular weight as the product labelled with [^{35}S]-methionine. After deformylation of the product some 39% of the incorporated radioactivity was released in the first step of Edman degradation (table 1). Since a deformylation rate of the same order under the

Table 1
Edman degradation of *Neurospora* apocytochrome *c* synthesized in rabbit reticulocyte lysates

A. Labelling with f-[^{35}S]met -tRNA

Fraction	^{35}S radioactivity (cpm)	
	After deformylation	Without deformylation
Ethylene chloride extract	581	100
Residual protein	909	1907

B. Labelling with [^{3}H]phenylalanine and [^{35}S]methionine

Fraction	^{3}H radioact. (cpm)	^{35}S radioact. (cpm)
1. Ethylene chloride extract	18	251
2. Ethylene chloride extract	299	229
3. Ethylene chloride extract	24	161
Residual protein	1114	5133

C. Labelling with [^{3}H]phenylalanine in the presence of 3.5×10^{-7} M f-met-tRNA; deformylation with 0.6 M HCl in methanol

Fraction	^{3}H radioact. (cpm)
1. Ethylene chloride extract	50
2. Ethylene chloride extract	543
3. Ethylene chloride extract	210
Residual protein	2648

Cell free protein synthesis systems were directed by *Neurospora* poly-(A)-RNA; apocytochrome *c* was isolated by immunoprecipitation and treated as indicated

applied conditions is reported for several different proteins, it appears that actually the label was only incorporated into the amino terminal position. These data exclude an amino terminal extension of the size reported for secretory proteins [17] and some cytoplasmically synthesized mitochondrial proteins [18].

In order to determine the amino terminal structure of the in vitro product, [^{3}H]phenylalanine and [^{35}S]-methionine were incorporated in the reticulocyte system simultaneously. The amino terminal sequence of *Neurospora* cytochrome *c* is Gly–Phe–Ser–Ala–Gly– [19,20]. The in vitro product immunoprecipitated with anti-apocytochrome *c* was subjected to Edman degradation [9,21] (table 1). About 18% of the ^{3}H radioactivity appeared in the second step, whereas no significant ^{35}S radioactivity was released in the first 3 steps. The total content of phenylalanine is 6 and of methionine is 2 per 108 amino acids. In a further experiment, in vitro translation was performed in the presence of [^{3}H]phenylalanine and unlabelled f-met-tRNA. Deformylation was then carried out followed by Edman degradation. In this case, ^{3}H radioactivity appeared in step 2, obviously resulting from product which had not initiated with the unlabelled f-met-tRNA, but with endogenous met-tRNA. Also in step 3 radioactivity appeared, which must have resulted from the product with the amino terminus f-met-Gly–Phe–. Taking into account a deformylation rate of ~40%, some 50% of the in vitro product was initiated with the added f-met-tRNA whereas the other 50% were initiated with endogenous reticulocyte met-t-RNA.

It is concluded from these data that the in vitro product has the same amino terminal sequence as the functional protein in mitochondria.

3.2. *Transfer of apocytochrome* c *synthesized in a heterologous cell-free system and conversion to holocytochrome* c

Translation was carried out in a reticulocyte lysate and the postribosomal supernatant was prepared. This fraction was incubated with isolated mitochondria in the presence of 0.3 M sucrose. After incubation, mitochondria were isolated and holocytochrome *c* was immunoprecipitated. Figure 2 demonstrates that this incubation leads to the appearance of holocytochrome *c* in the mitochondria. It is concluded that the in vitro product is transferred to mitochondria, linked to

Fig.2. Formation of holocytochrome *c* after incubation of in vitro synthesized apocytochrome *c* with isolated mitochondria. [^{35}S]Methionine was incorporated into a reticulocyte lysate and the postribosomal supernatant was prepared. Mitochondria isolated from *Neurospora* cells were homogenized with this supernatant. After incubation, mitochondria were re-isolated, holocytochrome *c* was immunoprecipitated and analysed by SDS–gel electrophoresis and autoradiography. For comparison apocytochrome *c* immunoprecipitated as in fig.1, lane 3, was co-electrophoresed. (1) Holocytochrome *c* from mitochondria after incubation with supernatant from reticulocyte lysate; (2) apocytochrome *c*.

heme and bound to the mitochondria.

3.3. *Synthesis of apocytochrome* c *by free and membrane-bound ribosomes*

Free and membrane-bound ribosomes from *Neurospora* were prepared by equilibrium density gradient centrifugation. The totals of both fractions were collected. After addition of a *Neurospora* postribosomal supernatant, readout protein synthesis was carried out with the fractions. Apocytochrome *c* was immunoprecipitated from postribosomal supernatant. Figure 3 shows that only in the case of free ribosomes was newly synthesized apocytochrome *c* detected. The ribosomal RNA in the bound ribosome fraction constitutes 2.5–5% of total ribosomal RNA. On the other hand bound ribosomes were 2–10-times more active in incorporating [^{3}H]leucine into protein as compared to free ribosomes. Therefore, we interpret the data in the following way: Free ribosomes are able to synthesize apocytochrome *c* and are at least the main site of apocytochrome *c* synthesis. It should be borne in mind that these in vitro data must be interpreted with care, since the long procedure involved in the preparation of the two ribosomal fractions may influence their synthetic abilities. On the other hand, separation of free and bound ribosomes was attempted by simple differential centrifugation. In this case ~40% of total ribosomal RNA was recovered in the microsome fraction. Obviously, this fraction was contaminated by free polysomes, as verified by electron microscopic examination. In this case, apocytochrome *c* was formed also by this fraction, but the amount constituted only 33% of the total apocytochrome formed by both free and bound ribosomes. This again suggests that free ribosomes are the preferential site of synthesis of apocytochrome *c*.

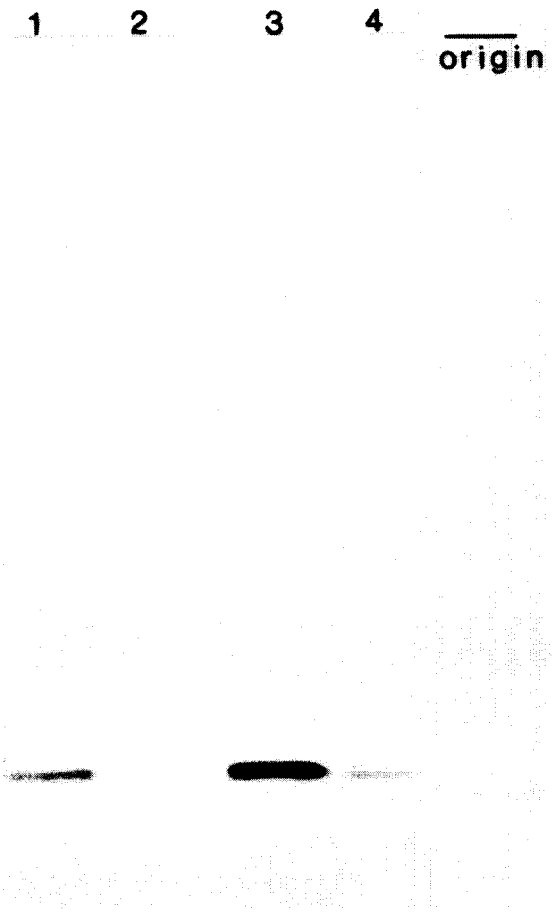

Fig.3. Synthesis of apocytochrome *c* by free ribosomes. Free and membrane-bound ribosomes were isolated and protein synthesis in the presence of [^{35}S]methionine was carried out in a homologous read-out system. Postribosomal supernatants were prepared and apocytochrome *c* immunoprecipitated from both fractions. Immunoprecipitates were analysed by gel electrophoresis and autoradiography. For comparison immunoprecipitated apocytochrome *c* was coelectrophoresed. (1,4) Apocytochrome *c* synthesized in reticulocyte lysate; (2) membrane-bound ribosomes; (3) free ribosomes.

4. Discussion

Transfer of apocytochrome *c* from the cytosol to the mitochondria and covalent binding of the heme group are partial reactions in the biogenesis and assembly of cytochrome *c* about which we have little information. For the understanding of these processes, the structure of the polypeptide chain released from the ribosome must be known. These experiments show that apocytochrome *c* synthesized in heterologous systems is the same size as authentic apoprotein. Moreover, the amino terminal sequence was found to be identical to that of the mature protein. Translocation obviously does not involve the processing of some extra sequence. This finding is in agreement with genetic data on yeast iso-1-cytochrome *c* gene, which have shown that the first base triplet adjacent to the AUG initiation codon specifies the first amino acid

of the authentic protein [22]. They also agree with the DNA sequence of the iso-1-cytochrome *c* gene in yeast [23]. Furthermore, translation in vitro of yeast iso-1-cytochrome *c* has been described and no significant difference in electrophoretic mobility on SDS gels of in vitro product and authentic holoprotein was detected [24].

Post-translational transfer of apocytochrome *c* to mitochondria has been demonstrated in homologous systems from *Neurospora* [6]. It is shown here that apocytochrome *c* synthesized in heterologous systems can be transferred to mitochondria and converted to holocytochrome *c*.

Finally, this report shows that isolated free ribosomes but not membrane-bound ribosomes are able to synthesize apocytochrome *c* in vitro. It is suggested that this reflects the situation in vivo, although redistribution of ribosomes in such experiments cannot definitely be ruled out. With rat liver, it was claimed that microsomes are the major site of synthesis [25]; since the rates of synthesis and the purity of cytochrome *c* were not satisfactory, this conclusion must be met with care.

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