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Genetics and Biogenesis of Mitochondria

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A CELL FREE SYSTEM TO STUDY SYNTHESIS AND TRANSPORT OF CYTOPLASMICALLY TRANSLATED MITOCHONDRIAL PROTEINS

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INTRODUCTION

Previous papers have described synthesis of cytoplasmically synthesized mitochondrial proteins and their transport into the mitochondria of whole cells of *Neurospora crassa* (1,2). These *in vivo* studies have suggested the existence of extra-mitochondrial pools of mitochondrial proteins. However, such studies with intact cells can supply only limited information on the mechanism of the transport of proteins into functional locations. Therefore, an *in vitro* system has been developed to separate the processes of synthesis and transport (3,4). This report deals with further experiments to discriminate between the mechanisms currently proposed for the transport of proteins into mitochondria (2,4-6). The experiments support the earlier view that precursors of mitochondrial proteins are located in the cytosolic fraction. In addition, an attempt is made to characterize these precursors.

RESULTS AND DISCUSSION

1) Synthesis and Transport of Mitochondrial Proteins in the Cell Free System

Neurospora hyphae (wild type 74 *A*) were grown in the presence of ^{35}S-sulphate. After 12 hr of growth a chase of unlabelled sulphate was added for one hr. The cells were then washed with ice-cold distilled water and collected by filtration. The hyphae were broken by grinding with sand in an incubation mixture described previously (7) but supplemented with an ATP

regenerating system and chloramphenicol (0.5 mg/ml).The homogenate was centrifuged twice at 3000 x g for 5 min. The cell free supernatant thus obtained was used for *in vitro* protein synthesis as described in a previous paper (4).

Electronmicroscopic analysis of this homogenate did not show a significant number of cytoplasmic ribosomes bound to the outer mitochondrial membrane.

Incorporation of ^{3}H-leucine into the ^{35}S-prelabelled homogenate reached a plateau within 10-15 min. The individual cellular fractions showed the labelling kinetics given in Fig.1.

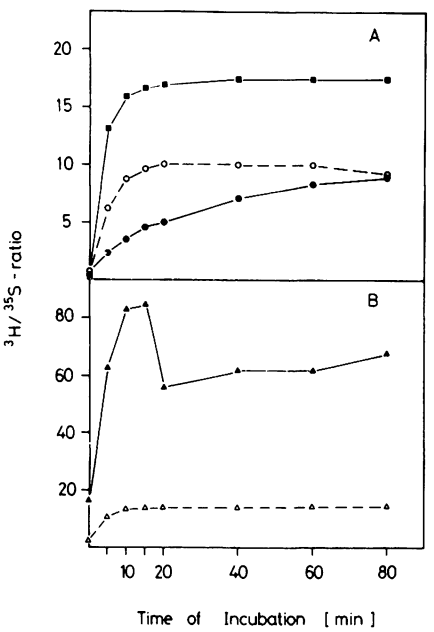

Fig.1 Incorporation of ^{3}H-leucine in a cell free system : specific labelling of the proteins of various cell fractions

A cell free homogenate of ^{35}S-labelled *Neurospora* hyphae was incubated with ^{3}H-leucine in the presence of chloramphenicol. Cell fractions were isolated by differential centrifugation. Specific ^{3}H-radioactivities are expressed by the $^{3}H/^{35}S$-ratio and plotted vs. time of incubation.

A ■——■ Homogenate
○— —○ Cytosol
●——● Mitochondria
B ▲——▲ Microsomes
△— —△ Ribosomes

The microsomal fraction showed very rapid labelling, reaching exceptionally high $^{3}H/^{35}S$-ratios at 10-15 min, which exceeded the final $^{3}H/^{35}S$-ratio of the total homogenate by a factor of 2-6. The free ribosomes showed a lower increase of the $^{3}H/^{35}S$-ratio. Therefore, it appears that in this cell free homogenate membrane bound ribosomes are more active in the synthesis of proteins than are free ribosomes. The mitochondrial fraction also shows an increase in the $^{3}H/^{35}S$-ratio but this increase

is distinctly slower than in the cytosolic fraction. However, the ratio in mitochondria does eventually reach a similar $^{3}H/^{35}S$-ratio. This incorporation into mitochondrial proteins cannot be attributed to mitochondrial protein synthesis since chloramphenicol was present in the reaction medium. Therefore, it appears that proteins synthesized on cytoplasmic ribosomes are transferred into the mitochondrial fraction.

In order to analyze the transferred proteins, gel electrophoretic separation in the presence of SDS was performed on the total mitochondrial protein. Mitochondria were isolated after 10 min and 80 min of incubation. The electrophoretic patterns are presented in Fig.2.

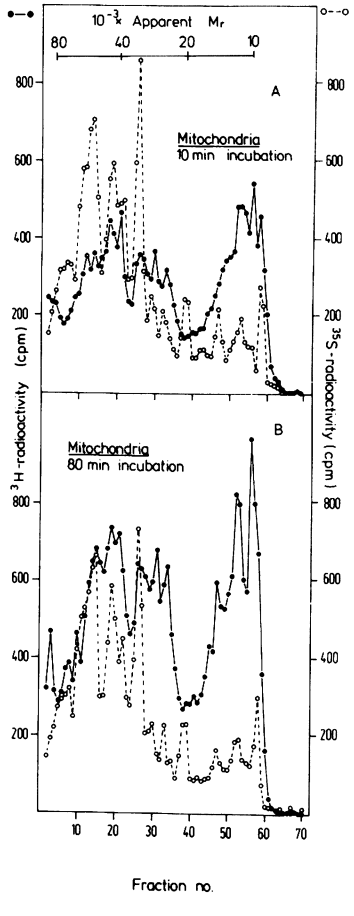

Fig.2 Gel electrophoretic analysis in the presence of SDS of ^{3}H-labelled proteins in the mitochondrial fraction

A cell free homogenate was prepared from ^{35}S-labelled hyphae and incubated with ^{3}H-leucine in the presence of chloramphenicol. Mitochondria were isolated after 10 min (A) and 80 min (B) of incubation, dissolved in SDS-containing buffer, dialysed and subjected to gel electrophoresis.

The ^{3}H- and ^{35}S-radioactivity patterns are not identical, although there is considerable similarity at least after 80 min of incubation. Harmey *et al.* have demonstrated that the *in vitro* synthesized proteins which are associated with the mitochondria do not represent unspecifically attached cytosolic proteins (4). Furthermore, the transferred proteins were characterized as specific mitochondrial proteins by immunoprecipitations from isolated mitochondria using antibodies directed against certain mitochondrial proteins : 1) CAT-binding protein (Fig.3) ++

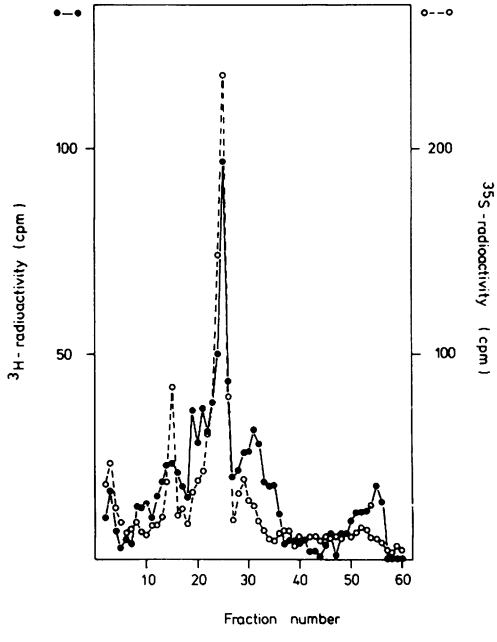

Fig.3 SDS gel electrophoresis of CAT-binding protein immunoprecipitated from the mitochondrial fraction of a cell free homogenate

A ^{35}S-prelabelled homogenate was incubated with ^{3}H-leucine in the presence of chloramphenicol for 20 min.

++*The electrophoretogram shows a major peak with an apparent molecular weight of 32,000. A distinct peak with an apparent molecular weight of 60,000 probably represents the dimeric form. Furthermore, in the molecular weight range of 10,000-30,000 a considerable amount of radioactivity is present. The amount of radioactivity in this region increases with the time of the in vitro incubation period. For this reason, and because these lower molecular weight proteins are not observed when the immuniprecipitation is carried out on mitochondria that have not been incubated in vitro, it seems likely that the radioactivity in this region represents proteolytic degredation products of the CAT-binding protein. Exceptional lability of the CAT-binding protein has also been observed with the beaf-heart protein (8).*

2) cytochrome *c* (see Fig.6) and 3) cytochrome *c* oxidase (Fig.4) In the latter case chloramphenicol was omitted during the incubation to obtain synthesis and labelling of mitochondrially synthesized subunits. In all the above cases the appearance of defined polypeptides in the mitochondria is apparent.

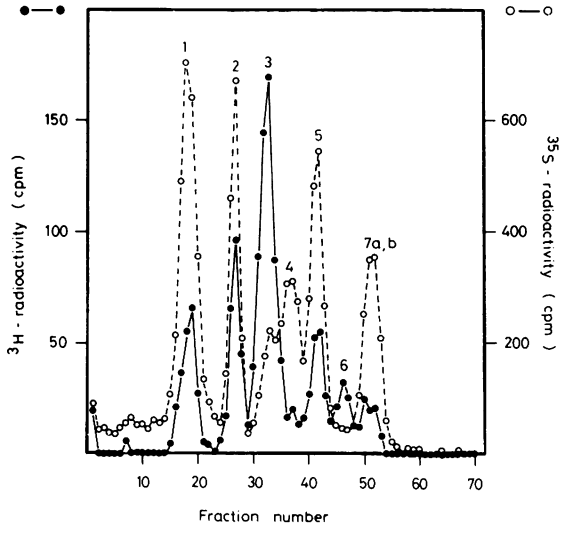

Fig.4 SDS gel electrophoresis of cytochrome *c* oxidase immunoprecipitated from the mitochondrial fraction of a cell free homogenate

A ^{35}S-prelabelled homogenate was incubated with ^{3}H-leucine. In this experiment no chloramphenicol was added. The immunoprecipitation was carried out with antibody directed against holo-cytochrome *c* oxidase.

2) Effect of Cycloheximide and Puromycin on Transfer of Proteins into the Mitochondria

A ^{35}S-prelabelled homogenate was incubated with ^{3}H-leucine for 10 min in the presence of chloramphenicol as described above. Cycloheximide and puromycin were then added to aliquots of the homogenate (final concentrations: 0.36 mM and 0.5 mM)

and incubation was continued.

The $^{3}H/^{35}S$-ratios of the homogenate and the various cell fractions were determined after the intervals shown in Fig.5.

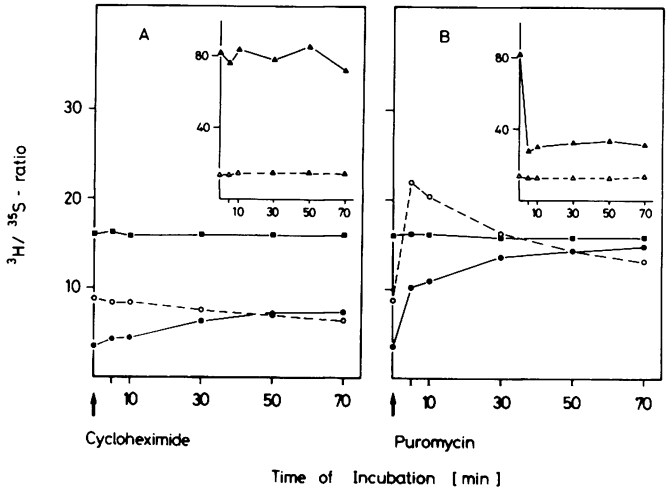

Fig.5 The effect of cycloheximide and puromycin on the transport of mitochondrial proteins into the mitochondria of a cell free homogenate

^{3}H-leucine was incorporated for 10 min in a cell free homogenate prelabelled with ^{35}S. The homogenate was divided into two equal portions and cycloheximide and puromycin were added (zero time) and incubation was continued. The $^{3}H/^{35}S$-ratios of various fractions are plotted vs.time after addition of cycloheximide (A) and puromycin (B)

■——■ Homogenate
O— —O Cytosol ●——● Mitochondria
▲——▲ Microsomes Δ— —Δ Ribosomes

The inhibitory effect of cycloheximide and puromycin on cytoplasmic protein synthesis is complete since the $^{3}H/^{35}S$-ratios of the total homogenates show no increase. When cycloheximide was present during the incubation (Fig.5A) the microsomal and ribosomal fractions showed no significant change in the $^{3}H/^{35}S$

ratio, whereas that of the cytosolic fraction declined slowly. When puromycin was added to an incubation mixture (Fig.5B) the $^{3}H/^{35}S$-ratio in the microsomal fraction dropped sharply but the ratio in free ribosomes was not affected. This rapid release of ^{3}H-label from the microsomes was accompanied by a corresponding increase of the $^{3}H/^{35}S$-ratio in the cytosolic fraction followed by a slow decline of this ratio.

These observations suggest that the release of polypeptides into the cytosolic fraction occurs mainly from the microsomal ribosomes. In the case of both, cycloheximide and puromycin inhibition, the mitochondrial fraction showed a significant increase in the $^{3}H/^{35}S$-ratio suggesting that transport of cytoplasmically synthesized mitochondrial proteins takes place after blocking cytoplasmic protein synthesis. The decline of the $^{3}H/^{35}S$-ratio of the cytosolic fraction indicates that the proteins are transferred into the mitochondria from that fraction. This conclusion is dependent upon the observation that the $^{3}H/^{35}S$-ratio of the total homogenate did not decrease.

In order to demonstrate that completed mitochondrial proteins are transferred after cessation of cytoplasmic protein synthesis the following experiments were carried out : A ^{35}S-prelabelled homogenate was incubated with ^{3}H-leucine for 10 min, cycloheximide or puromycin added (zero time), and aliquots were withdrawn immediately. Incubation was continued for 70 min following which aliquots were again withdrawn. Mitochondria were isolated and matrix proteins, CAT-binding protein, and cytochrome *c* were immunoprecipitated. The immunoprecipitates of CAT-binding protein and of cytochrome *c* were analyzed by SDS gel electrophoresis and the $^{3}H/^{35}S$-ratios determined (electrophoretic analysis of immunoprecipitated cytochrome *c* is shown in Fig.6). In the case of the immunoprecipitated matrix proteins the ratio in the total immunoprecipitates were determined. The $^{3}H/^{35}S$-ratios obtained after 70 min compared to the ratios at zero time are presented in Table 1 for the various proteins. The ratio for total

3) Experiments to Demonstrate the Presence of Precursors in the cytosolic Fraction

In previous papers it was reported that mitochondrial matrix proteins show kinetics characteristic of precursors outside the mitochondria. In experiments with intact cells as well as in *in vitro* experiments putative precursors were detected in the cytosolic fraction (1,3). This work extends these observations with experiments to detect precursors for the CAT-binding protein and for cytochrome *c*.

A ^{35}S-prelabelled homogenate was incubated with ^{3}H-leucine as described above for 10 min. The cytosolic and mitochondrial fraction were then isolated and immunoprecipitations were performed with antibodies against CAT-binding protein.

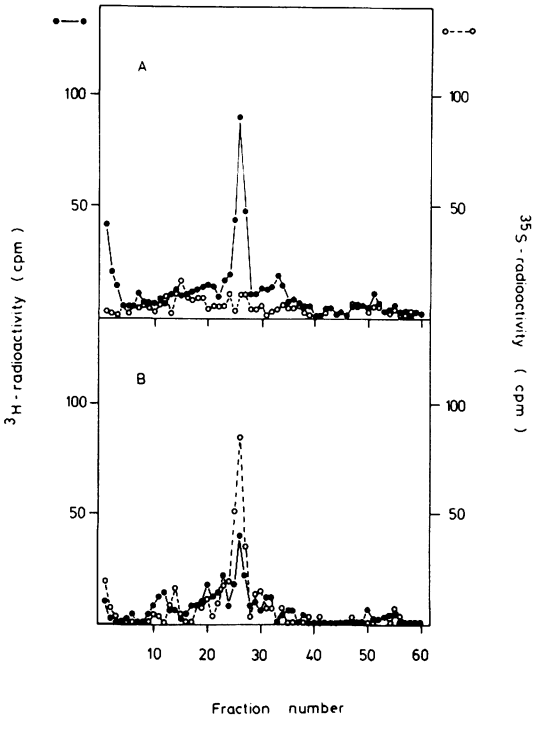

Fig.7 SDS gel electrophoresis of CAT-binding protein immunoprecipitated from the cytosolic fraction (A) and the mitochondrial fraction (B) of a cell free homogenate

A ^{35}S-prelabelled homogenate was incubated with ^{3}H-leucine for 10 min

After 1o min incubation only a small amount of ^{3}H-label co-migrates with the ^{35}S-prelabelled CAT-binding protein from mitochondria (Fig.7B). No ^{35}S-radioactivity could be

mitochondrial proteins is also given in the Table. For comparison the $^{3}H/^{35}S$-ratios determined following incubation with no inhibitor present (control) is included in the Table. The results given in the Table lead to the conclusion that specific and completed polypeptides are transported into the mitochondria when cytoplasmic translation is blocked. This demonstrates the existence of extramitochondrial pools which are exhausted after blocking protein synthesis.

Table 1

Incorporation of ^{3}H-leucine in total mitochondrial protein, matrix proteins, cytochrome *c* and CAT-binding protein after inhibition of cytoplasmic protein synthesis with cycloheximide or puromycin (values were determined as described in the text)

$\frac{^{3}H/^{35}S\ (70\ min)}{^{3}H/^{35}S\ (10\ min)}$	*Mitochondria*	*Matrix Proteins*	*Cytochrome c*	*CAT Protein*
Control	2.2	6.5	2.3	3.2
Cycloheximide	1.6	1.7	1.8	2.5
Puromycin	4.1	4.5	1.8	---

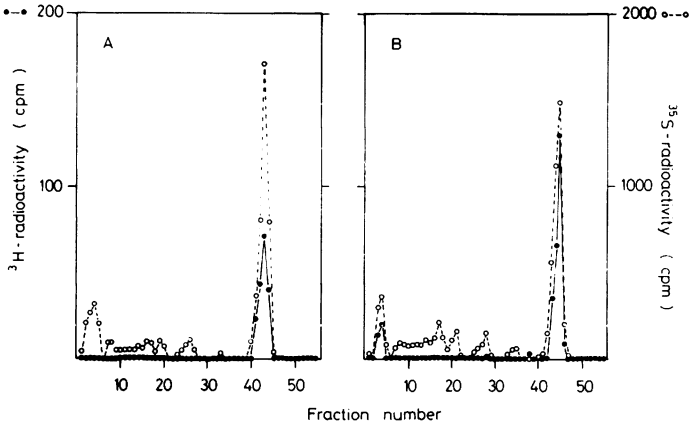

Fig.6 Gel electrophoretic analysis in the presence of SDS of cytochrome *c* immunoprecipitated from mitochondria

A cell free homogenate prelabelled with ^{35}S was incubated with ^{3}H-leucine for A) 10 min B) 10 min, then in the presence of cycloheximide for a further 70 min.

precipitated from the cytosolic fraction demonstrating the specific localisation of the CAT-binding protein in the mitochondria. However, a distinct peak of ^{3}H-radioactivity with the same electrophoretic mobility as the CAT-binding protein was apparent in the cytosol (Fig.7A). We consider this ^{3}H-labelled material to be a putative extramitochondrial precursor of the CAT-binding protein. However, precursor-product kinetic studies and chemical comparison of the putative precursor with the mitochondrial membrane component must be carried out before definite conclusions can be drawn.

In a comparable experiment, in which cells were not prelabelled with ^{35}S, the cytosolic fraction was immunoprecipitated with a specific antibody against apo-cytochrome *c* isolated from holo-cytochrome *c* according to Fisher *et al.*(9).

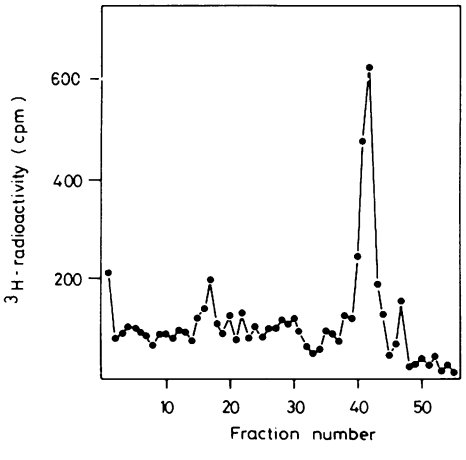

Fig.8 SDS gel electrophoresis of apo-cytochrome *c* immunoprecipitated from the cytosolic fraction of a cell free homogenate

A ^{35}S-prelabelled cell free homogenate was incubated with ^{3}H-leucine for 10 min in the presence of chloramphenicol.

The electrophoretic pattern of the immunoprecipitation displays a single band comigrating with cytochrome *c* (Fig.8). In separate experiments it was shown that this antibody did not cross react with holo-cytochrome *c*. Immunoprecipitates obtained with anti-apo-cytochrome *c* using mitochondria isolated from ^{35}S-prelabelled homogenates incubated *in vitro* with ^{3}H-leucine contained neither ^{35}S- nor ^{3}H-radioactivity. Preliminary experiments, in which apo-cytochrome *c* was precipitated from

the cytosol and holo-cytochrome *c* from the mitochondria showed that following cycloheximide inhibition the ^{3}H-radioactivity disappeared from the cytosolic fraction and increased in the mitochondrial fraction. These data suggest that apo-cytochrome *c* is transported into the mitochondria *in vitro* . However, as in the case of matrix proteins the putative cytosolic precursor has not been chemically shown to be identical with the apo-protein of integrated cytochrome *c* .

FINAL REMARKS

The data we have presented here and in previous papers (1-4) lead us to propose a working hypothesis to describe synthesis and transport of cytoplasmically translated mitochondrial proteins : Mitochondrial proteins are synthesized as precursors on cytoplasmic ribosomes (free or bound) with subsequent release into the cytosol. These precursors differ in their structure and/or conformation from the integrated functional proteins in the mitochondria. Only precursors are transported into the mitochondria (see also ref.4). The outer mitochondrial membrane may not present a barrier for these precursors. Precursors of proteins of the inner mitochondrial membrane may be trapped by structural or conformational changes (a) in the course of assembly reactions which lead to phospholipid containing supermolecular structures and (b) by attachement of prosthetic groups to the apo-proteins. Precursors for proteins of the mitochondrial matrix proteins may be bound to acceptor sites on the inner membrane and subsequently translocated.

The molecular mechanisms underlying these proposed reactions remain to be determined. Comparison with other cellular reactions in which protein translocation across membranes takes place may be helpful to put questions which can be experimentally tested. Such translocation reactions are (a) the transfer of nascent chains of secretory proteins across the microsomal membrane which appears to involve the clipping of a " signal sequence " (10,11) and (b) the trans-

location of fragment *A* of diphtheria toxin across the cellular membrane, in which a second polypeptide chain (fragment *B*) seems to play an essential role in binding and transfer of the toxic fragment *A* (12).

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