

GENETICS AND BIOGENESIS OF CHLOROPLASTS AND MITOCHONDRIA

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IN VITRO SYNTHESIS AND TRANSPORT INTO MITOCHONDRIA OF CYTOPLASMICALLY TRANSLATED PROTEINS

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INTRODUCTION

In a preceding paper the kinetics of synthesis and transport of cytoplasmically synthesized proteins into the mitochondrion in intact cells of Neurospora crassa have been described (1). These in vivo studies have shown the existence of extramitochondrial pools of proteins which are transported into the mitochondria. These studies however give only limited information on the transfer mechanism, as it is experimentally difficult to separate the processes of synthesis and transport in vivo. We describe here an in vitro system which allows discrimination between the synthesis of mitochondrial proteins on cytoplasmic ribosomes and their subsequent transport into the mitochondria.

RESULTS

1. In vitro synthesis of mitochondrial proteins

Neurospora cells were grown in the presence of ^{35}S -sulphate, after 14 hrs growth the cells were given a chase of unlabelled sulphate for one hr. The cells were then washed and filtered dry. They were ground with sand in incubation mixture as described (2), supplemented with an ATP regenerating system and the homogenate centrifuged twice at 3000xg for 5 min. The cell free supernatant thus obtained was used for in vitro protein synthesis.

The time course of incorporation of ^3H -leucine into total homogenate is shown in Fig. 1A. Incorporation starts without any lag and reaches a maximum level within 10-20 min. The detectable ^3H -radioactivity in protein is approximately 5×10^6 counts \times min $^{-1}$ per ml of incubation mixture, corresponding to 0.5 g of cells (wet weight). These levels of incorporation enable us to detect the small amounts of mitochondrial proteins synthesized, while the double labelling of the proteins makes it possible to discriminate between newly synthesized and preexisting proteins.

The effect of carbonyl-cyanide-m-chlorophenylhydrazone (CCCP) on ^3H -leucine incorporation is also shown. The effect of CCCP is especially interesting in view of its inhibitory action on protein transport in vivo (1). The initial rate is unaffected by CCCP at levels which inhibit transport (cf. Fig. 4). The final

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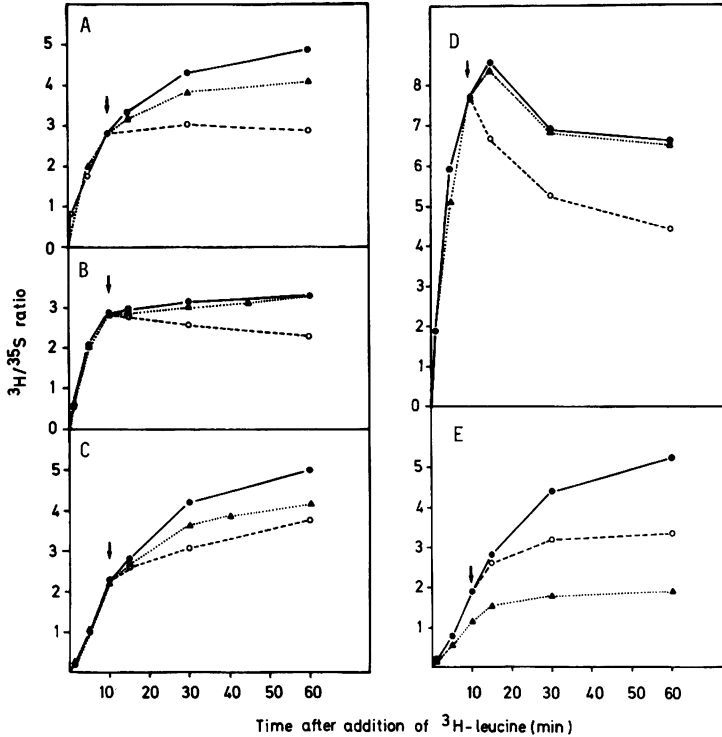


Fig. 1. Incorporation of ^3H -leucine into an ^{35}S -prelabelled cell free homogenate. Samples were withdrawn at the times indicated and fractionated. Labelling was as follows: a) continuous exposure to leucine —●—●—; b) 10 min ^3H -leucine + chase --o---o--; c) continuous ^3H -leucine + CCCP (25 μM)▲..... A, homogenate; B, cytoribosomes; C, cytosol; D, microsomes; E, mitochondria.

incorporation level is depressed by 10-15%, however. Cycloheximide inhibits incorporation by approx. 95%, the remainder of the incorporation represents mitochondrial translation.

2. Kinetics of labelling of subcellular fractions

A chase of cold leucine given at 10 min immediately halts any further incorporation of ^3H -leucine into total homogenate. The *in vitro* system was sampled at time intervals for separation into subcellular fractions as shown in Fig. 1, B-E.

The $^3\text{H}/^{35}\text{S}$ -ratio in the microsomal fraction rises rapidly reaching a maximum at 10 min. This maximum is followed by a gradual decline. Cytoribosomes also show a rapid increase in the $^3\text{H}/^{35}\text{S}$ -ratio which reaches a plateau within 10-15 min.

The addition of a chase causes a decrease in the ratio. The increase in the

$^3\text{H}/^{35}\text{S}$ -ratio of the cytosol is relatively slow and is not checked by the addition of the chase.

The mitochondrial labelling pattern shows a slow increase in the $^3\text{H}/^{35}\text{S}$ -ratio which is sustained over the experimental period. When a chase is given a parallel slow increase is maintained, showing no levelling off.

These results suggest that in the in vitro system synthesis of mitochondrial proteins and their subsequent transport into the mitochondria takes place.

The effect of CCCP on the progress of the $^3\text{H}/^{35}\text{S}$ -ratios in the cell fractions is included in Fig. 1B-E. The addition of CCCP has no significant effect on the cell fractions except the mitochondria, where it reduces the increase in the $^3\text{H}/^{35}\text{S}$ -ratio. The inhibition of mitochondrial labelling by CCCP is not complete. This can be attributed to mitochondrial translation which was found to continue in the presence of CCCP at the concentration level used (unpublished data). The in vitro system does not depend on endogeneous phosphorylation since an ATP regenerating system was added. Therefore it is reasonable to assume that CCCP inhibits transport of proteins into isolated mitochondria.

3. Transport of matrix proteins into the mitochondria

In vitro synthesis of defined mitochondrial proteins was shown by precipitation of mitochondrial matrix proteins with a specific antibody. We present in Fig. 2 electropherograms of the immunoprecipitates. We compared the profiles obtained from in vitro incorporation with those obtained from mitochondria isolated from pulse labelled whole cells (Fig. 3). There is good agreement between the two profiles indicating that the in vitro and in vivo synthesized proteins are the same. Similar results were obtained for the CAT-binding protein (3) and for cytochrome c (4) (see below).

To demonstrate transfer of matrix protein the following experiment was performed. A cell free system was prepared from ^{35}S prelabelled cells. Two parallel samples were set up. One was pulsed for 10 min with ^3H -leucine. The other was pulsed for 10 min and then chased for 30 min with cold leucine. Mitochondria and cytosol were prepared from both samples and matrix proteins immunoprecipitated. The $^3\text{H}/^{35}\text{S}$ -ratios of the immunoprecipitates are presented in Table 1.

TABLE 1

$^3\text{H}/^{35}\text{S}$ -ratios in immunoprecipitates of matrix proteins obtained from cytosol and mitochondria of a cell free system exposed to a pulse of ^3H -leucine and to a pulse plus chase

	10 min pulse	10 min pulse plus 30 min chase
Cytosol	1.55	1.34
Mitochondria	.08	.72

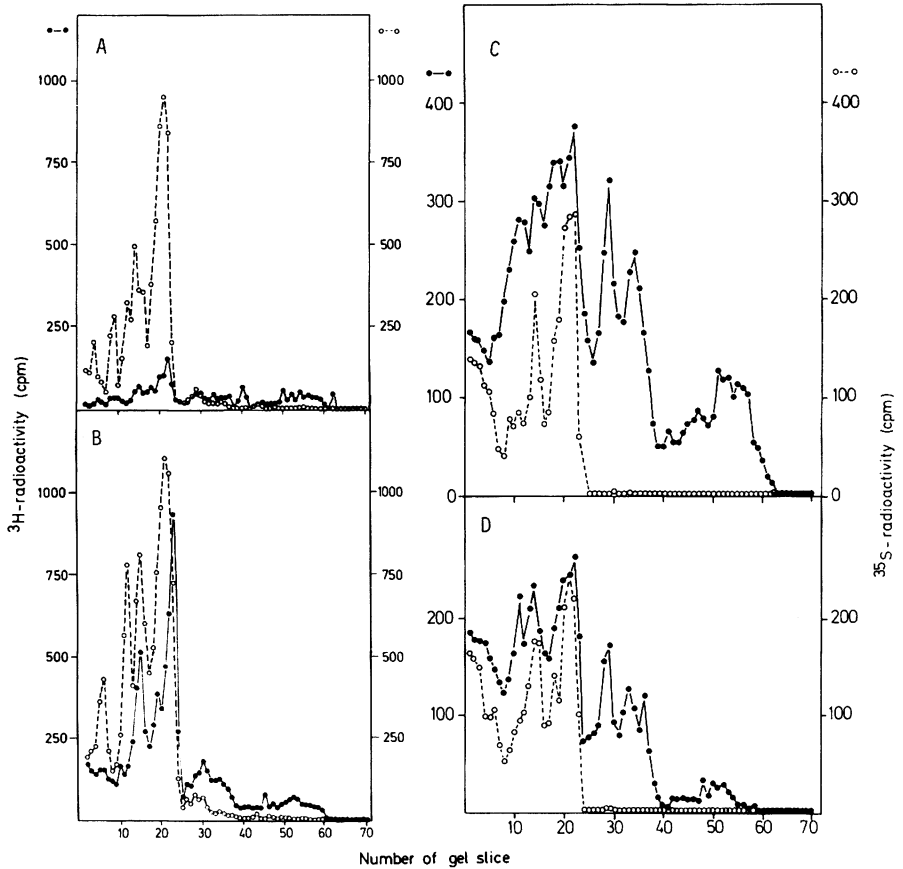


Fig. 2. SDS gel electrophoresis of immunoprecipitated matrix proteins from mitochondria (A,B) and cytosol (C,D). Fractions were isolated from the cell free homogenate after 10 min exposure to ^3H -leucine (A,C) and following a 30 min chase (B,D).

In the mitochondria the ratio increased ninefold during the chase indicating that import of matrix proteins into the mitochondria took place during the chase. simultaneously, the ratio in the cytosol decreased, suggesting export of newly synthesized matrix proteins. It is significant that the $^3\text{H}/^{35}\text{S}$ -ratio in the total cytosol increased appreciably during the chase whereas the immunoprecipitated matrix from the same fraction decreased (cf. Fig. 1).

SDS gel electrophoretic analysis of the immunoprecipitates is shown in Fig. 2. The ^{35}S -profiles confirm the occurrence of matrix proteins in the cytosol fraction,

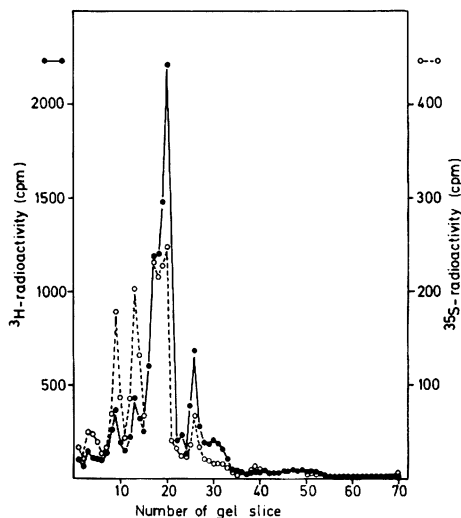


Fig. 3. SDS gel electrophoresis of matrix proteins from mitochondria isolated from pulse labelled cells. Cells were grown on ^{35}S -sulphate and pulsed with ^3H -leucine for 720 sec at 9°C .

obviously due to leakage during cell fractionation. The protein peaks are similar in all four samples. The ^3H -profiles clearly show that matrix proteins formed during the pulse period are accumulated in the mitochondria during the chase period (Fig. 2A,B). The ^3H gel profiles from the cytosol indicate that also in this fraction newly formed matrix proteins are found (Fig. 2C,D).

4. Transport of cytochrome c and inhibition by CCCP

To demonstrate transport of one specific protein into the mitochondria the following experiment was carried out. Homogenates were incubated with ^3H -leucine in the presence and absence of CCCP for 40 min. Mitochondria were then isolated and cytochrome c precipitated with a specific antibody. The immunoprecipitates were analysed by SDS gel electrophoresis (Fig. 4). The distribution of ^{35}S in the gels shows that there is specific precipitation of cytochrome c (mol. weight 12,300 (4)). The control sample shows a prominent ^3H -peak demonstrating synthesis and import of newly synthesized cytochrome c (or the apo-molecule) in the in vitro system. In the mitochondria from the CCCP treated samples the appearance of the ^3H -peak is strongly inhibited. CCCP therefore appears to prevent the import of newly synthesized cytochrome c (or the apo-molecule) into the mitochondria.

CONCLUSIONS

The results presented here demonstrate the synthesis of defined mitochondrial proteins on cytoplasmic ribosomes in the in vitro system. They further show that in this cell free system the transport system is functional. The labelling

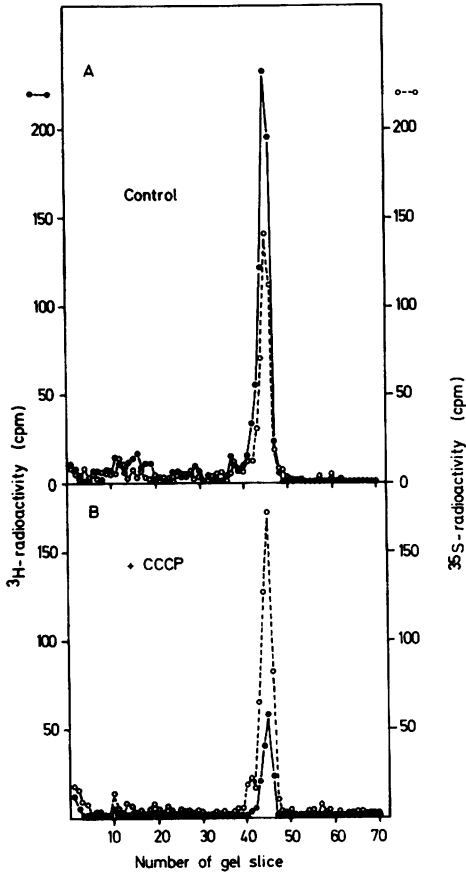


Fig. 4. SDS gel electrophoresis of immunoprecipitated cytochrome c of isolated mitochondria from cell free homogenate, exposed to ^3H -leucine for 40 min in the absence and presence of CCCP ($25\ \mu\text{M}$).

kinetics and the immunoprecipitation data demonstrate the existence of extramitochondrial pools of mitochondrial proteins, which are synthesized rapidly and exported relatively slowly into the mitochondria. The response of the system to CCCP makes it possible to inhibit transport while allowing synthesis of mitochondrial proteins to proceed unimpeded.

The need for a system to study synthesis and transport of cytoplasmically synthesized mitochondrial proteins has been expressed in a recent review (5). The system described here may partly meet this need. At present we are employing this system in an attempt to elucidate the molecular basis of the transport process.

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