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Th. Bücher
W. Neupert
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STUDIES ON THE SYNTHESIS OF MITOCHONDRIAL PROTEINS IN THE CYTOPLASM AND ON THEIR TRANSPORT INTO THE MITOCHONDRION

G. Hallermayer and W. Neupert

Institut für Physiologische Chemie, Physikalische Biochemie und Zellbiologie
der Universität München
Germany

INTRODUCTION

It is well established that the majority of the mitochondrial proteins are synthesized outside the mitochondrion on cytoplasmic ribosomes. However, the mechanisms by which these proteins are transported and integrated into their functional locations remain unknown (for review see (1,2)). This gap in knowledge stems mainly from the experimental difficulties experienced in trying to detect and identify the trace amounts of proteins on their path to the mitochondrion. Furthermore, serious problems arise from artefacts produced by isolation of cellular components, viz. leakage of proteins from cell organelles, unspecific adsorption and proteolytic degradation.

In this report we present experimental data obtained from Neurospora cells by (a) kinetic dual labelling studies and (b) immunological location of mitochondrial proteins in subcellular fractions.

RESULTS

1. Pulse- and Pulse-Chase-labelling of the proteins of Neurospora cell fractions

Neurospora cells were grown in the presence of ^{35}S-sulphate to obtain homogeneous labelling of total cellular protein. After 16 hrs growth (exponential phase) the cells were cooled to 9 ^{o}C and maintained at this temperature for 2 hrs. A pulse of ^{3}H-leucine was given. Aliquots of the cultures were removed at the times indicated in Fig. 1. Cells were harvested and fractionated by homogenisation and differential centrifugation, and the ^{3}H/^{35}S-ratio determined in the various cell fractions. In addition to the pulse labelling a portion of the culture was given a chase of unlabelled leucine after 90 sec exposure to ^{3}H-leucine and the ^{3}H/^{35}S-ratio in the cell fractions was determined at the time intervals shown (Fig. 1). All fractions were characterized by electron microscopy and enzymatic analysis.

In the cell homogenate the ^{3}H/^{35}S-ratio increases linearly reaching a constant level after about 360-720 sec (when all of the added leucine is incorporated into cellular proteins). Leucine uptake is complete in about 60 sec under our experimental conditions. The effectiveness of the leucine chase can be seen by the immediate check, it imposes on the increase of the ^{3}H/^{35}S-ratio (Fig. 1). The cytoribosomes show a faster increase in the ^{3}H/^{35}S-ratio than the total homogenate;

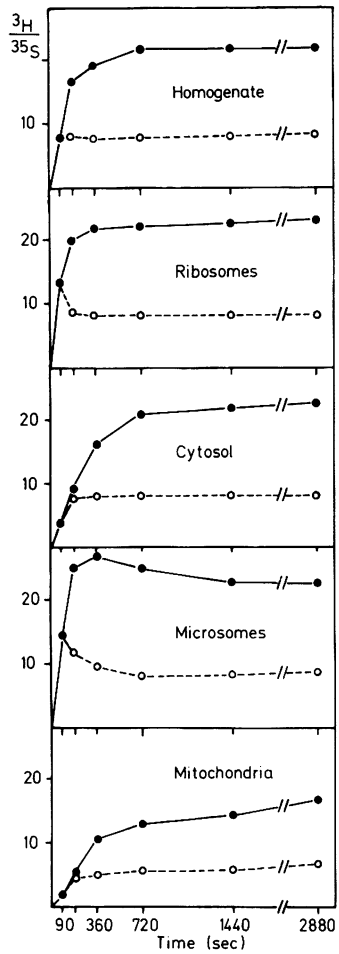

Fig. 1. Kinetics of the incorporation of ^3H-leucine into TCA-insoluble protein of total homogenate and subfractions of Neurospora crassa.
Cells were a) pulse labelled with ^3H-leucine -●——●-;
b) pulse labelled and chased with leucine after 90 sec -o----o-.
Specific ^3H-radioactivities of mitochondrial fractions are expressed by the $^3H/^{35}S$-ratio.

the addition of the chase causes a rapid decrease indicating a rapid replacement of radioactively labelled nascent polypeptide chains by unlabelled ones.

Labelling of the cytosolic proteins lags behind that of the ribosomes indicating that the ^3H-leucine has to pass through the pool of nascent chains on ribosomes, before it appears in the cytosol. During the first 90 sec after the chase, the $^3H/^{35}S$-ratio in the cytosol increases considerably and then stays constant, reflecting the rapid release of nascent chains from the ribosomes.

The microsomal fraction shows a unique labelling pattern, the $^3H/^{35}S$-ratio rises very rapidly to a maximum, before incorporation into whole cells is complete. This maximum is followed by a slow decline to a final level equal to that of the whole homogenate. A similar decrease is also seen after the chase. It should be noted that the decrease is slower than that due to the release of nascent chains from the ribosomes. This type of pattern would suggest that proteins are either synthesized or imported by the microsomal fraction and also exported.

The mitochondrial labelling is characterized by a pronounced lag with respect to both the total homogenate and the cytosolic proteins. The mitochondria have the slowest rate of increase in $^3H/^{35}S$-ratio. After 360 sec when the ^3H-incorporation of whole cells is almost completed, the ratio of the mitochondria is only about 1/2 of the final level of all other cell fractions. A plateau is not reached within 2880 sec. A similar slow increase is observed, when after 90 sec pulse labelling of proteins is stopped by a chase. Such a labelling pattern is consistent with the existence in the cell of an extramitochondrial pool of mitochondrial proteins. The time needed for synthesis appears to be small compared to the average time the mitochondrial

proteins remain in the extramitochondrial pool.

2. Kinetics of labelling of individual protein fractions of the mitochondria

Immunoprecipitation was used to follow the labelling of individual mitochondrial proteins. The proteins selected were cytochrome c (3), the carboxyatractyloside-binding protein (CAT-protein) (4), mitochondrial ribosomal proteins and whole matrix protein. Cells were exposed to ^{3}H-leucine as in Fig. 1. The kinetics of appearance of these ^{3}H-labelled proteins in the mitochondria are shown in Fig. 2 and Fig. 3. They differ considerably from each other, indicating that the pools suggested from Fig. 1 are of different size, depending on the protein in question. Furthermore, the increase of the $^{3}H/^{35}S$-ratio after the chase in all mitochondrial proteins studied substantiates the conclusion, that extramitochondrial pools do exist.

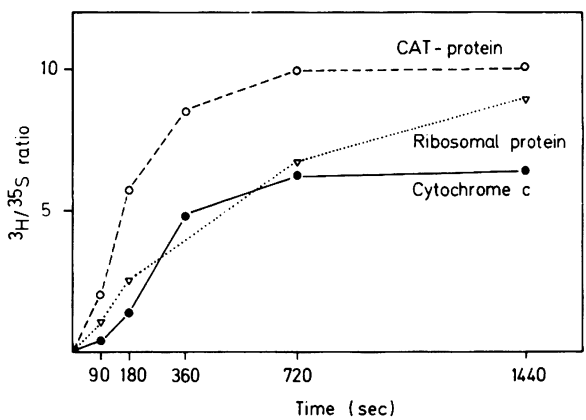

Fig. 2. Kinetics of the appearance of individual ^{3}H-labelled proteins in the mitochondria from cells which were exposed to ^{3}H-leucine at zero time. CAT-binding protein, mitochondrial ribosomal protein and cytochrome c were immunoprecipitated from mitochondria at times indicated.

3. Assessment of extramitochondrial pools of mitochondrial proteins

The extramitochondrial pool of matrix protein was studied by monitoring the kinetics of appearance of ^{3}H-leucine labelled matrix in the various cellular fractions. Matrix protein was immunoprecipitated from cytosolic, microsomal and mitochondrial fractions. The label appeared quite rapidly in the cytosol and after about 360 sec decreased slowly. The mitochondria on the other hand showed the characteristic delayed labelling, reflecting a continuous accumulation (Fig.3). The radioactivities shown in Fig. 3 are related for each fraction to 0.1 g of Neurospora cells (wet weight). It thus appears that there is a quantitative relationship between the disappearance of label from the cytosol and the concomitant increase in the mitochondria. Furthermore, the sum of the radioactivities in cytosol and mitochondria follows the kinetics of total cell homogenate minus cytoplasmic ribosomes. In accordance with this finding the microsomal fraction shows

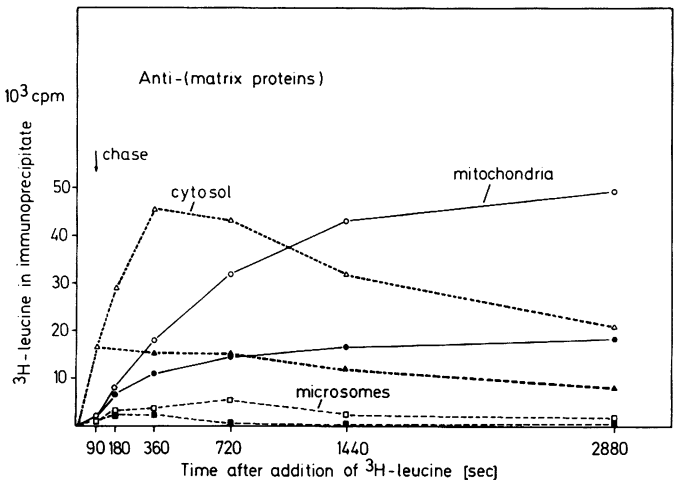

Fig. 3. The changes of ^{3}H-leucine radioactivity in mitochondrial matrix protein in subcellular fractions. (a) pulse label (open symbols); (b) after 90 sec pulse of ^{3}H-leucine a chase of unlabelled leucine was given (solid symbols).

a relatively low labelling.

These data would suggest that there exists a cytosolic pool of mitochondrial matrix protein and that proteins from this pool are transported into the mitochondrion. However, it must be considered that the cytosol may not be the real location of this pool, since fractionation may result in artefactual redistribution of proteins.

4. Inhibition of translocation by carbonyl-cyanide-m-chlorophenylhydrazone (CCCP)

When a culture was pulse-labelled as described in Fig. 1 and CCCP was added together with the chase, it was found that the increase in the ^{3}H/^{35}S-ratio in the mitochondria was greatly reduced. This suggests a block in the transport of proteins into the mitochondrion. To substantiate this conclusion, the CAT-binding protein was precipitated from mitochondria of cells labelled in the following ways: a) pulse for 180 sec; b) pulse 180 sec and chase 600 sec; c) pulse 180 sec and chase plus CCCP for 600 sec. The ^{3}H/^{35}S-ratios measured in the immunoprecipitates were 7.0, 10.1 and 6.7, respectively. Electrophoresis of the immunoprecipitates reveals a single peak with an apparent molecular weight of 32,000, corresponding to the CAT-binding protein (Fig. 4). The higher ratio in the peak of the chased sample compared to the control suggests import of newly formed CAT-protein during the chase period.

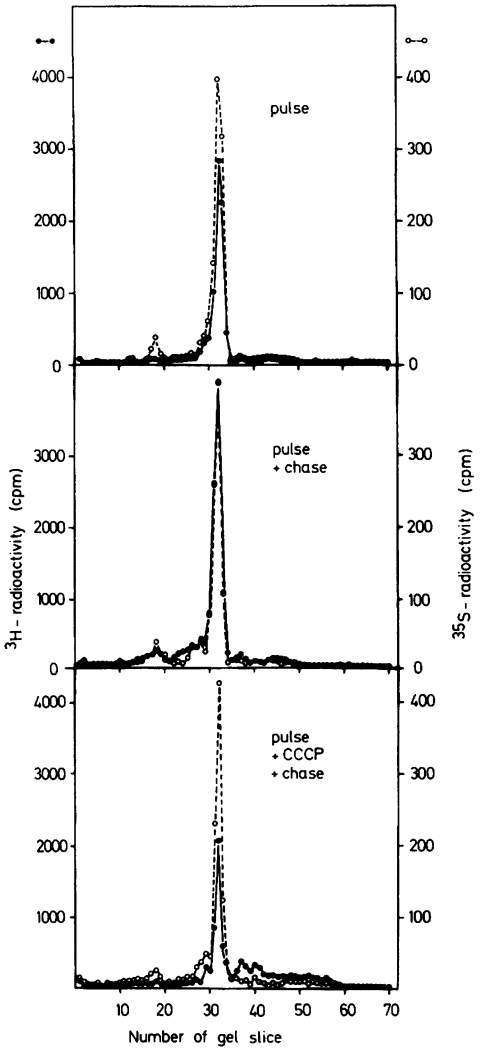

Fig. 4. SDS gel electrophoresis of immunoprecipitated CAT-binding protein from mitochondria of cells homogeneously labelled with ^{35}S.

a) 180 sec pulse of ^{3}H-leucine
b) 180 sec pulse followed by chase for 10 min
c) 180 sec pulse followed by simultaneous addition of CCCP and chase

In the CCCP treated sample ^{3}H-labelled low molecular weight material is also observed. Furthermore, the ^{3}H/^{35}S-ratio in the peak is relatively low in comparison to the ratio in the immunoprecipitate. The appearance of these low molecular weight components coupled with the low ^{3}H/^{35}S-ratio of the 32,000 MW peak suggests that inhibition of transport by CCCP is accompanied by degradation of newly synthesized protein. We are tempted to suggest that the newly synthesized protein is particularly labile when transport is inhibited, resulting in degradation to lower MW products. We have discussed a similar phenomenon concerning mitochondrially synthesized protein components on the basis of quite different experimental approaches (5).

DISCUSSION

The data presented show that extramitochondrial pools of newly synthesized mitochondrial proteins do exist. They further indicate that the sizes of these pools are different for different mitochondrial proteins. Newly formed mitochondrial matrix proteins were found to be localized predominantly in the

cytosol fraction. This finding must be interpreted with caution, since redistribution of proteins may cause serious artefacts. Preferential susceptibility to proteolysis of proteins which are on the path to their final location in the mitochondrion is also a possible source of error. The latter is especially suggested by results obtained with the CAT-binding protein.

Recently a mechanism for the transport of proteins into the mitochondrion was suggested (6). According to this proposal, mitochondrial proteins are inserted into the mitochondrion directly by ribosomes attached to the outer mitochondrial membrane at sites where outer and inner membranes are fused. Such a mechanism would have the following characteristics: a) the kinetics of the labelling of mitochondrial proteins should parallel that of cytosolic proteins; b) the labelling of different mitochondrial proteins should also show parallel kinetic patterns (with the exception of proteins which are modified after transport with respect to their antigenic properties, e.g. cytochrome c); c) the $^{3}H/^{35}S$-ratio of mitochondrial proteins detected outside the mitochondria should not exceed that of the intramitochondrial proteins.

The experimental system here described has none of the above characteristics and we find it difficult to reconcile our findings with the direct insertion hypothesis as a general mechanism.

In an accompanying paper we describe the transport process in a cell free system, in which translocation of in vitro synthesized proteins can be followed and in which transport is blocked by CCCP as in vivo, but protein synthesis is not inhibited (7).

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