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IN VITRO INCORPORATION OF (^{35}S)-METHIONINE IN MITOCHONDRIAL PROTEINS OF DROSOPHILA MELANOGASTER

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SUMMARY

In order to study the interrelation between nuclear genome and mitochondria during biogenesis of mitochondrial proteins, mitochondria of D. melanogaster were isolated from whole insects.

The fraction obtained showed respiratory control by ADP in the presence of pyruvate-malate substrates of up to 6-7.

Conditions of in vitro uptake of (^{35}S) methionine in polypeptides were studied. The energy necessary for protein synthesis is supplied by endogenous ATP. Bacterial and fungal contamination was severely repressed. Incorporation rates of 50-100 picomoles of (^{35}S) methionine mg protein^{-1} hour^{-1} could be measured under these conditions.

Electrophoresis in the presence of SDS of proteins of the internal membrane showed, after 50 min of incubation, a significantly labelled band of proteins at 22 Kd. Other bands at 80, 52 and 40 Kd were labelled to a lesser degree.

INTRODUCTION

A great deal of research has been carried out into the biogenesis of mitochondrial proteins in saccharomyces cerevisiae (1,2) Neurospora crassa (3), and in rat liver (4,5).

Isolated mitochondria have been shown to be capable of synthesising polypeptides, among which some have been identified (6) : subunits of cytochrome c oxydase (7), ATPase (8,9) or QH_2 cytochrome c reductase (10).

Tris	: Tris (hydroxy methyl) aminoethane
EGTA	: Ethylene glycol bis (β-aminoethyl ether)-N,N'-tetraacetic acid
TCA	: Trichloroacetic acid
BSA	: Bovineserum albumin
SDS	: Sodium dodecyl sulfate
POPOP	: 1,4 bis-2-(4 methyl-5 phenyl oxazolyl) benzene
PPO	: 2-5-diphenyl oxazole

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Mitochondrial DNA was isolated from D. melanogaster and studied (11). Electrophoretic detection could be carried out on one group of mitochondrial poly A mRNA (12) ; up until now no work has been published on the biogenesis of mitoribosomal proteins in this insect.

The results presented here show that (^{35}S) -methionine can be incorporated in vitro into certain proteins in the internal membrane of these mitochondria.

MATERIALS AND METHODS

- Isolation of mitochondria

This technique was inspired by that used by (13).

D. melanogaster is a wild strain obtained from flies caught in western France in 1972 (14) ; 2-3 g of flies previously put to sleep by the cold, were ground gently in a mortar containing 5 ml of the isolation medium (0.25 M sucrose, 10 mM Tris, 1 mM EGTA, 1 % bovine serum albumin pH 7.4). The resulting mixture was then homogenized in a Potter Elvehjen fitted with a teflon-pestle, then filtered through three layers of gauze and centrifuges at 300 x g for 5 min. The supernatant was then centrifuged at 3 000 x g for 10 min. The pellet was recovered with the isolation medium without BSA, and centrifuged at 7 000 x g for 5 min. 15 to 20 mg mitochondrial proteins were collected in an isolation buffer without bovine serum albumin. The mitochondria had respiration control of 6-7 in the presence of pyruvate and malate.

- Incorporation of (^{35}S) -methionine in mitochondria

The mitochondria (1 mg/ml) were incubated at 30°C in a medium containing 0.2 M sucrose, 10 mM Tris, 1 mM EGTA, 20 mM KCl, 10 mM KH_2PO_4, 5 mM $MgCl_2$, pH 7.4, 50 µg/ml cycloheximide, 50 µg/ml azaserine, 0.1 mg/ml sulfanilamide, 2 mM ADP, 5 mM pyruvate, 5 mM malate, 0.1 mM of all the amino acids, (^{35}S) -methionine 10^5 dpm/nmoles. 0.5 ml of the suspension were removed every 10 min. The reaction was stopped by the addition of 100 µl of the isolation medium containing 50 mM cold methionine.7 min. later, mitochondria were centrifuged (3 000 g - 2 min.), the pellet was resuspended in 0.5 ml of incubation medium containing cold methionine 50 mM. The mitochondrial proteins were then precipitated by 5 % TCA and recovered after another centrifugation. After washing with 5 % TCA, the pellet was dissolved in 100 µl formic acid and then analysed in liquid scintillation (toluene, POPOP/PPO system).

- Fractionation of mitochondria

The different mitochondrial fractions were obtained according to (15). The mitochondria were incubated for 15 min. at 0°C in the presence of digitonin (0.15 mg/mg protein). The reaction was stopped by the addition of 2 volumes isolation medium without bovine serum albumin. The suspension was centrifuged at 10 000 x g for 10 min. A pellet of mitoplasts was obtained and was resuspended and incubated for 15 min. at 0°C with lubrol WX (0.12 mg/mg protein). The reaction was stopped by the addition of 2 volumes isolation medium without BSA. The suspension was recentrifuged at 100 000 x g for 60 min., which allowed the internal membrane and the matrix fraction to be obtained.

The first supernatant obtained was centrifuged at 100 000 x g for 60 min. which allowed the external membrane and the inter-membrane space fraction to be obtained.

The cytochrome c oxydase and malate dehydrogenase activity were measured according to (16) and (17).

- Electrophoresis of the different mitochondrial protein fractions

For the technique used see (18). 1 % mercaptoethanol and 2 % SDS were added to 150 to 200 µg of each protein fraction and placed on top of the gels ; polyacrylamide gel electrophoresis was carried out for 7 hours. The apparent molecular weights were determined using standard proteins. The densitometric scans were obtained using a Vernon densitometer ØVI. The gels were cut into slices of 2 mm. Each slice was dissolved in 150 µl of H_2O_2 before the radioactive count.

RESULTS AND DISCUSSION

In order to measure the biogenesis of mitochondrial proteins in vitro, it is advisable to restrict as much as possible any bacterial or fungal contamination at the time of incubation.

In this study it was not possible to work in sterile conditions using mitochondria prepared from whole flies. The following measures were therefore taken :

a) - The kinetics of incorporation were limited to 50 min.

b) - Inhibitors of the biogenesis of nucleotides (azaserine, sulfanilamide) which could inhibit the growth of microorganisms without affecting a possible mitochondrial synthesis or which could inhibit fungal growth (cycloheximide), were introduced into the incubation medium : under these conditions, bacteriological examination showed that the number of microorganisms (mainly Gram$^-$) did not increase significantly during incubation (4.3×10^3 bacteria ml^{-1} at t = 0 ; 4.7×10^3 bacteria ml^{-1} after 50 min incubation in the presence of 50 µg/ml^{-1} cycloheximide, 50 µg ml^{-1} azaserine and 100 µg ml^{-1} sulfonilamide ; 2.5×10^4 bacteria ml^{-1} after 50 min incubation in the presence of 50 µg ml^{-1} cycloheximide alone).

Also, the incorporation of (^{35}S)-methionine was studied when the ATP source was intramitochondrial (respiratory control 6-7) in the presence of substrate (pyruvate, malate) ADP and phosphate.

- Incorporation of (^{35}S)methionine in mitochondria

Figure 1 shows that D. melanogaster mitochondria incorporate 80 to 100 pmoles (^{35}S)-méthionine per mg protein per hour. This value is close to

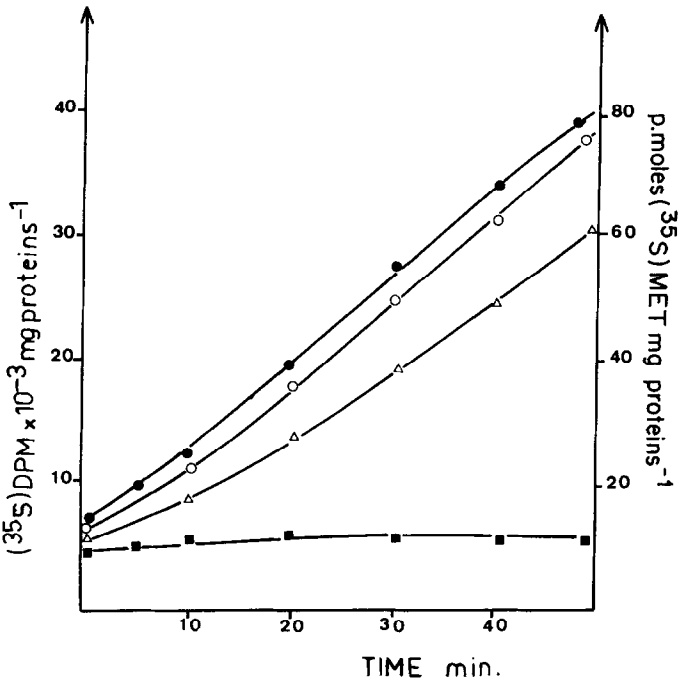

FIGURE 1 : Incorporation of (^{35}S) -methionin in mitochondria
Mitochondria 1 mg/ml were incubated under conditions described in materials and methods.
- without addition
- with cycloheximide 50 µg/ml
- with cycloheximide 50 µg/ml, sulfanilamide 0.1 mg/ml azaserine 50 µg/ml
- with cycloheximide 50 µg/ml, sulfanilamide 0.1 mg/ml azaserine 50 µg/ml, chloramphénicol 1 mg/ml.

that obtained with other mitochondria (19). Cycloheximide was observed to not perceivably modify this incorporation. However the azaserine and sulfanilamide decreased it slightly. Chloramphenicol, known to be a strong inhibitor of the biogenesis of bacterial and mitochondrial proteins (20, 21), inhibited it completely.

- Mitochondrial fractionation

Digitonin and lubrol allowed the separation of membranes and mitochondrial fractions. The internal membrane proteins represented about 60 % total mitochondrial proteins. The matrix proteins represented about 15 %.

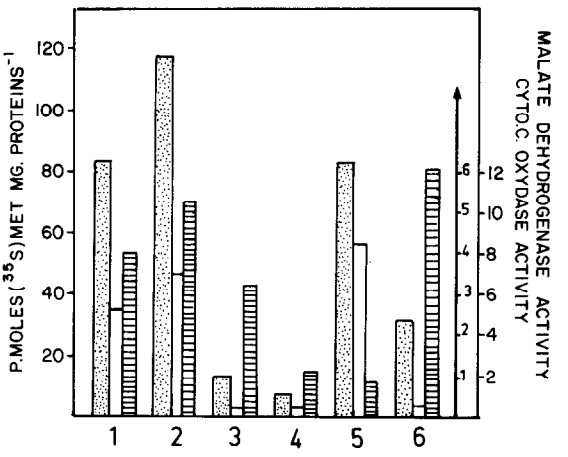

FIGURE 2 : Incorporation of (^{35}S)-methionin and determination of cytochrome c. oxydase and malate dehydrogenase activity in different mitochondrial fractions.
For details see materials and methods.
pmoles (^{35}S)-methionin mg proteins^{-1}
cytochrome c oxydase activity : µmoles cyto c reducted min^{-1} mg protein^{-1}
malate dehydrogenase : nmoles oxaloacetate reducted min^{-1} mg protein^{-1}
1 : whole mitochondria
2 : mitoplast
3 : external membrane
4 : intermembrane space
5 : inner membrane
6 : matrix

The radioactivity, cytochrome c oxydase and malic dehydrogenase activities were measured for each fraction. The results are shown in figure 2. High radioactivity was recorded in the fraction corresponding to the internal membrane (fig. 2 fraction 5). This fraction also showed maximum cytochrome c oxydase activity.

A not negligeable quantity of radioactivity was recorded in the fraction corresponding to the mitochondrial matrix. This incorporation could result from the soluble proteins in the matrix. The malic dehydrogenase activity was found above all in the matrix fraction ; this activity also exists in the external membrane and could result from contamination by a cytoplasmic form of this enzyme.

- Electrophoresis of the different mitochondrial fractions ; incorporation of (^{35}S)-methionine in the internal membrane

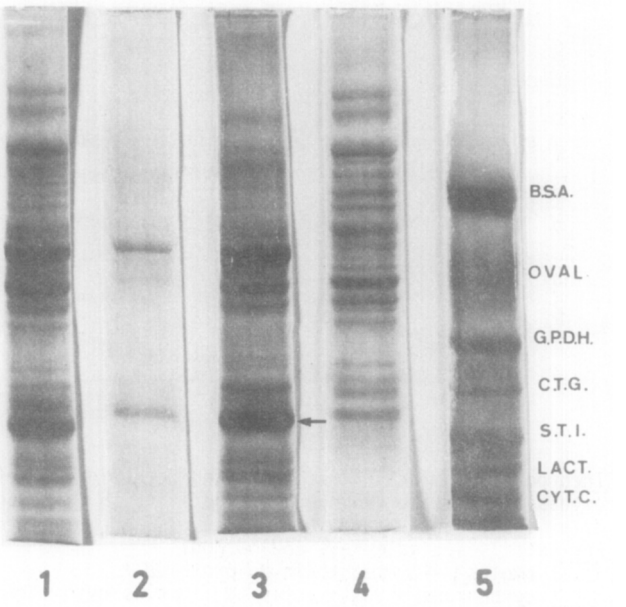

FIGURE 3 : Electrophoresis of different mitochondrial fractions
For detail see materials and methods
1 : whole mitochondria
2 : external membrane
3 : inner membrane
4 : matrix
5 : standard proteins
BSA : bovine serum albumin ; oval : ovalbumin ; GPDH : glyceraldehyde-3-phosphate dehydrogenase ; CTG : chymotrypsinogen A ; STI : soybean trypsin inhibitor ; lact : lactoglobulin ; cyt. c : cytochrome c.
Arrow shows the position of 22 Kd protein band.

Figure 3 shows the electrophoresis of the whole mitochondria, the external membrane, the internal membrane and the matrix fraction. Also the electrophoresis of standard proteins (fraction 5) is given which allows an approximate determination of the molecular weight.

The electrophoretic technique used allowed the resolution of 20-30 bands in the case of the internal membrane.

The measurement of radioactivity of gels by electrophoresis of the internal membrane (fig.4) shows that band IV of P.M. 22 Kd is significantly marked by (^{35}S)-methionine (arrow, electrophoresis 3, fig.3). This band represents about 10 % of the internal membrane proteins. This experiment was carried out 4 times.

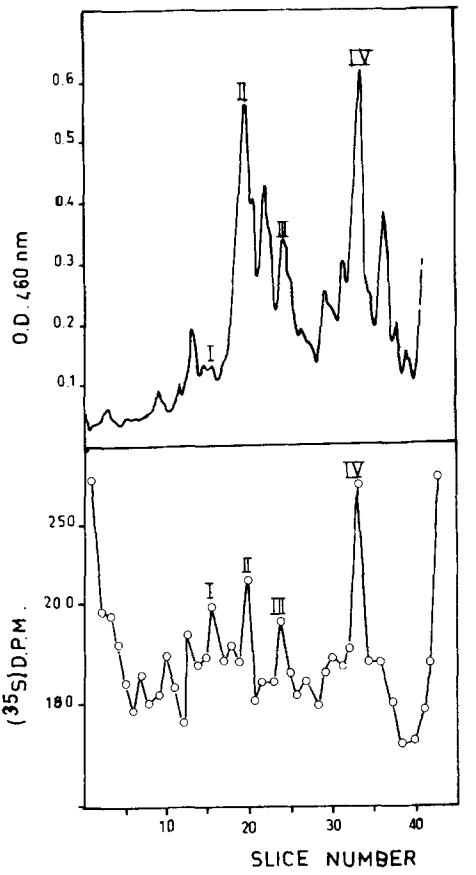

FIGURE 4 : Densitometric traces, and radioactive analysis of SDS gel electrophoresis of inner membrane
For detail, see materials and methods.

Bands I, II and III corresponding to P.M. of 80 Kd, 52 Kd and 42 Kd were less marked but appeared in the various experiments.

CONCLUSION

1 - Drosophila melanogaster mitochondria were isolated from whole flies and incubated under the presence of all the amino acids including (^{35}S)-methionine, in conditions of maximum intramitochondrial ATP synthesis and maximum restriction of any contamination by micro-organisms or fungi.

2 - These mitochondria were fractionated using digitonin and lubrol in order to obtain the various membranes and fractions. The internal membrane fraction was very significantly marked.

3 - The incorporated radioactivity was recorded above all in a protein band of P.M 22 Kd in the inner membrane ; work is being carried out to identify this band.

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