PROTEIN SYNTHESIS BY YEAST MITOCHONDRIA IN VIVO. QUANTITATIVE ESTIMATE OF MITOCHONDRIALLY GOVERNED SYNTHESIS OF MITOCHONDRIAL PROTEINS

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SUMMARY

Mitochondria of Saccharomyces cerevisiae selectively were labelled in vivo by incubating cells with radioactive leucine and cycloheximide. In yeast cells grown in galactose, 8-9% of the proteins of whole mitochondria and about 15% of those of mitochondrial membranes are calculated to be synthesized by the cycloheximide-resistant system of protein synthesis.

INTRODUCTION

After inhibition of cytoplasmatic ribosomal protein synthesis by cycloheximide, SEBALD et al. (1, 2) demonstrated in vivo persistence of mitochondrial protein synthesis in Locusta migratoria and Neurospora crassa. Protein synthesis of isolated mitochondria in vitro, however, is left unaffected by cycloheximide (3, 4). This experimentally well established result leads to the conclusion that in vivo residual synthesis of mitochondrial proteins, not inhibited by this antibiotic, is not governed by mitochondrial DNA. Cycloheximide thus allows a selective labelling of mitochondria in vivo, an approach avoiding the uncertainties of incorporation experiments with isolated mitochondria. This paper deals with experiments to use cycloheximide for studies with Saccharomyces cerevisiae. Experimental conditions have been elaborated which are assumed to allow quantitative measurements of the contributions of both the intramitochondrial and extramitochondrial protein synthesizing system to mitochondrial protein.

MATERIALS and METHODS

The haploid yeast strain Saccharomyces cerevisiae A 1327 A, ad_2, leu_1, a, originated from the Seattle Stock Culture, was a gift from Dr. Zimmermann, Forstbotan. Institut der Universität Freiburg. Yeast cells were grown at 30°C under rapid aeration in synthetic medium described by WICKERHAM (5) supplemented with 1% yeast extract and 2% galactose. Early stationary phase cells were isolated by centrifugation (5 min., 2000 x g, 25°C) washed in Wickerham synthetic medium and suspended to 30 mg wet weight/ml in the same medium supplemented with 1% galactose. The cells were aerated for 10 min. at 30°C. Cycloheximide (Sigma Chemical Company) and ^{14}C-leucine (The Radiochemical Centre Amersham) were added as indicated. Labelling was terminated by the addition of a hundredfold excess of unlabelled leucine. Tests for bacterial contamination were performed by plating 0,1 ml of the incubation mixture of Wickerham medium solidified by 1,5% agar and supplemented with 10 mg cycloheximide, 10 g yeast extract and 20 g galactose per litre.

Yeast cells were homogenized by use of a MERKENSCHLAGER Homogenizer (Braun, Melsungen) in a medium composed of 0,25 M mannitol, 0,02 M Tris, 10^{-3} M EDTA, final pH 7,4 following the method described by TUPPY et al. (6). For isolation of mitochondria the homogenate was successively centrifuged in the following way: 10 min at 800 x g, the resulting supernatant 20 min at 9,000 x g, the resuspended pellet 15 min 1,100 x g, the resulting supernatant 20 min at 7,500 x g. The postmitochondrial supernatant (9,000 x g) was centrifuged for 30 minutes at 30,000 x g. The resulting supernatant contained microsomes and cell sap and wad designated the 30,000 x g supernatant. In some experiments mitochondria were further purified by sucrose gradient centrifugation following the method described by TUPPY et al. (6).

Protein was determined by the biuret method (BEISENHERZ et al. (7). The preparation of insoluble mitochondrial protein from the mitochondrial pellet and the 30,000 x g supernatant and the determination of the specific radioactivity have been described. Succinate-cytochrome c reductase was estimated according to ARRIGONI et al. (10) and malate dehydrogenase according to BÜCHER et al. (11).

RESULTS

In the presence of about 50 µg cycloheximide per ml incubation medium the radioactivity incorporated into protein of the 30,000 x g supernatant is less than 1% of the control, whereas the radioactivity incorporated into mitochondrial protein amounts to about 7% of the control without cycloheximide (fig. 1). A further increase in cycloheximide concentration up to 1000 µg per ml is only of minor effect. Figure 2 shows that the inhibition of ^{14}C-leucine incorporation nearly reaches its maximum after a preincubation time of 1 min with cycloheximide. An increase

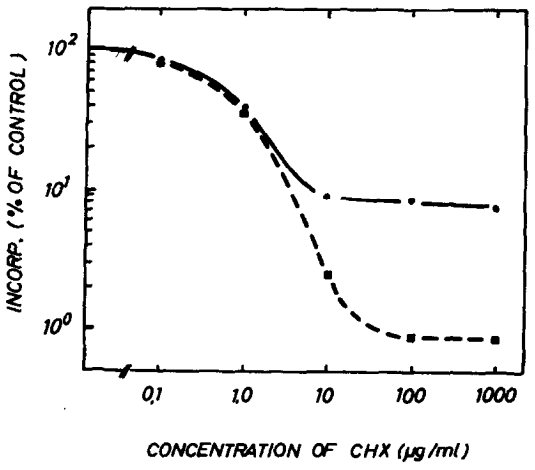

Fig. 1

In vivo incorporation of ^{14}C-leucine (5nCi/ml) into mitochondrial protein (● ——— ●) and into 3o,ooo x g supernatant protein (■ – – – – ■) for different cycloheximide concentrations. The values represent percentage of the control without cycloheximide. 1oo% correspond to about 2,ooo cpm/mg protein for mitochondrial fractions and to about 3ooo cpm/mg protein for the supernatant. Cycloheximide (CHX) was added 5 min prior to a 2o min labelling period.

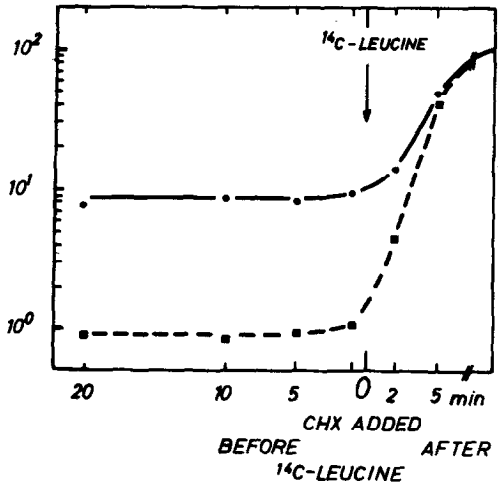

Fig. 2

In vivo incorporation of ^{14}C-leucine (5nCi/ml) into mitochondrial (● ——— ●) and supernatant proteins (■ – – – – ■) for different times of cycloheximide addition. The values represent percentage of the control without CHX. The labelling period was 2o min.

in preincubation time up to 2o min does not reduce the cycloheximide-resistant protein synthesis in mitochondria. By density gradient centrifugation, crude mitochondrial material (fig. 3) can be separated into two major bands, one (I) containing soluble (malate dehydrogenase) and insoluble (succinate-cytochrome c reductase) mitochondrial enzymes and the other (II) containing only insoluble, membrane-bound mitochondrial enzymes. Band I (density 1,18 - 1,19 g · cm^{-3}) is assumed to contain whole mitochondria, band II (density 1,14 - 1,15 g · cm^{-3}) to be com-

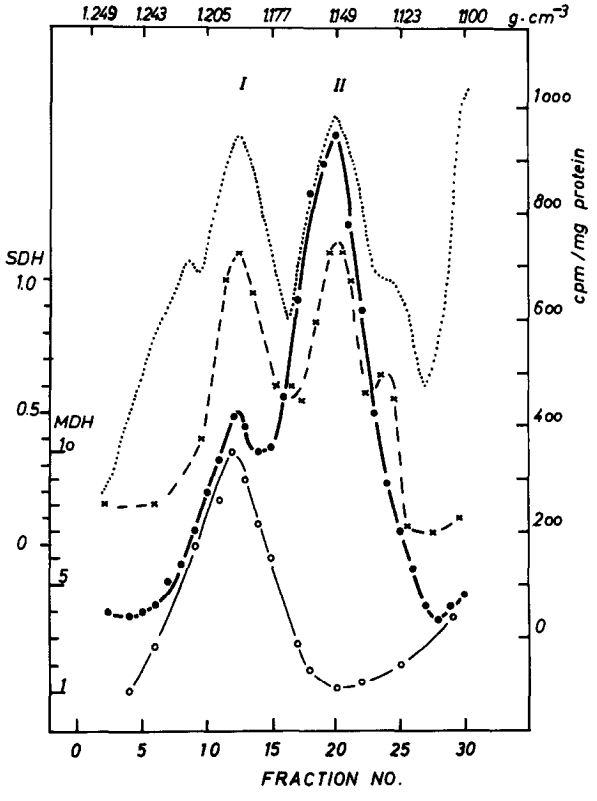

Fig. 3

Distribution of in vivo incorporation radioactivity (● ——— ●), succinate-cytochrome c reductase, ΔE 55o/min/mg, (x ------- x), malate dehydrogenase, units/min/mg (o ——— o) and extinction at 42o mμ (.....) after sedimentation of crude mitochondrial preparations in a sucrose gradient. Yeast cells of this preparation were incubated for 1o min with ^{14}C-leucine (2o nCi/ml) under CHX-inhibition (1oo μg/ml). The fractions of the density 1,18 - 1,19 g · cm^{-3} and 1,14 - 1.15 · cm^{-3} are assumed to contain whole mitochondria and mitochondrial fragments respectively (12).

posed of mitochondrial fragments, mainly mitochondrial membranes. After labelling the cells in the presence of cycloheximide, the specific radioactivity in band II is nearly twenty times higher than in non-mitochondrial fractions and about 5o% higher than band I. In the control without cycloheximide, there is only a slight increase in the specific activity of mitochondrial fractions in comparison to non-mitochondrial material.

The time-dependent incorporation of ^{14}C-leucine is shown in figure 4. Without cycloheximide the incorporation rate into mitochondrial and supernatant protein remains almost constant during the first 1o minutes and thereafter ceases rather rapidly due to exhaustion of the precursor. In the presence of cycloheximide, however, the incorporation rate into mitochondrial proteins remains constant for at least 25 minutes.

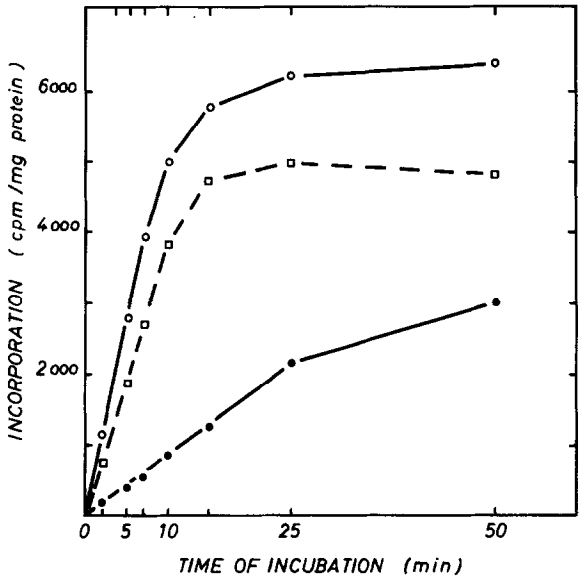

Fig. 4

Time course of in vivo incorporation of ^{14}C-leucine. Mitochondrial fragments were collected after sucrose gradient centrifugation. CHX (1oo μg/ml) was added 5 min prior to ^{14}C-leucine (1o nCi/ml).

o ------- o Mitochondrial protein without CHX
● ------- ● Mitochondrial protein with CHX
□ ------- □ Supernatant protein without CHX

These experiments were carried out with a leucine auxotrophic strain in aminoacid-free incubation medium with ^{14}C-leucine added for labelling. Dilution of the pool of radioactive leucine by synthesis of unlabelled leucine within the cell or by transport from outside therefore is eliminated. Since the incorporation rates of ^{14}C-leucine are constant at least during the initial labelling period (fig. 4), it is concluded that the turnover of cellular proteins or other factors do not influence the ratio of labelled to unlabelled leucine in the cell. The decrease of incorporation into mitochondrial proteins after prolonged cycloheximide inhibition is assumed to be due to secondary effects. Accordingly, it seems possible to compare the incorporation rates of ^{14}C-leucine in experiments with and without cycloheximide inhibition. Figure 4 and table 1 show that the cycloheximide-resistant incorporation of radioactivity into mitochondrial fragments or insoluble proteins during the first 1o minutes of labelling amounts to about 15% of the control. Both protein preparations represent mitochondrial membranes. From these experiments specific activity of whole mitochondria is calculated to be about 8 to 9 % of the control without cycloheximide. Soluble proteins of mitochondria show only slight radioactivity after labelling in the presence of cycloheximide.

Table 1

Influence of cycloheximide on the *in vivo* incorporation of ^{14}C-leucine into mitochondrial subfractions. Labelling of cells is identical with that in fig. 4. Mitochondria were isolated by sucrose gradient centrifugation.

protein fraction	cpm/mg protein	% of the control
3o,ooo x g supernatant	1o5	o,7
whole	14oo	8,5
mitochondria insoluble	28oo	15
soluble	125	1

DISCUSSION

The experiments were performed with a yeast strain defective in the synthesis of leucine, an amino acid which, for labelling of the cells, is added to the incubation medium otherwise free of leucine. Dilution of the cellular pool of radioactive leucine by transport of unlabelled leucine from outside the cell, or by synthesis within the cell, thus is excluded under the conditions applied. Accordingly, the time-dependent incorporation rate is expected to be constant for at least the initial phase of labelling. Since this has been proved in the experiments described, the cycloheximide-resistant synthesis of mitochondrial proteins directly can be correlated with the synthesis of these proteins in the control. The ratio of the incorporation rate with cycloheximide to the rate without the inhibitor is assumed to represent that part of mitochondrial proteins which is coded by the mitochondrial DNA and therefore synthesized by the mitochondrial system of protein synthesis. With view to the strain used and the growth conditions (early stationary phase cells grown in galactose), it is calculated that about 8 to 9% of whole mitochondria and 15% of mitochondrial membranes are synthesized by the mitochondrial protein synthesizing system. These values may slightly increase after further purification of mitochondrial preparations. It also must be mentioned that the cultures, due to the characteristics of the strain used for incubation, contained about 2o% respiratory-deficient cells. With a more indirect method, SEBALD et al. (13) for Neurospora crassa estimated the rate of mitochondrial protein synthesis to be in the same range.

For a cytoplasmic "petite" mutant of Saccharomyces cerevisiae (genotype $P\rho^-$) it was not possible to prove protein synthesis in mitochondria, although in genic "petite" mutants (genotype $p\rho^+$) incorporation of labelled amino acids by the mitochondrial protein synthesizing system could be demonstrated. This is in agreement with the results recently published by SCHATZ et al. (14).

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