THE INFLUENCE OF MITOCHONDRIA ON RIBOSOMES. CYCLOHEXIMIDE RESISTANCE OF RIBOSOMES FROM PETITE MUTANTS OF SACCHAROMYCES CEREVISIAE

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Summary.

The influence of cycloheximide on amino acid incorporation into protein of standard strain ρ + and cytoplasmic mutant ρ - of S.cerevisiae was determined in vivo and in vitro. In vivo cycloheximide at the concentration which inhibits protein synthesis in ρ + strain by over 60% has litle or no effect in mutant ρ - strain. In vitro cycloheximide in the range of 0.05 to 0.3 ug/ml of incubation medium inhibits polyphenylalanine synthesis in ρ + strain by 50% and in ρ - strain by less than 10%. Similar resistance to this antibiotic are shown in standard strain grown in anaerobic conditions. It has been found that the resistance to cycloheximide is associated with changes in cytoplasmic ribosomes and may depend on the integrity of mitochondrial system.

It has been found that mitochondria, besides their function of supplying energy for the cell, have also influence on different biochemical reactions which can not be directly connected with respiratory chain and oxidative phosphorylation. Induction of several enzymes of sugar metabolism is abolished in respiratory deficient „petite" ρ - strains of Saccharomyces cerevisiae (1, 2), and depends on the mitochondrial integrity which is a prerequisite for these enzyme induction (3). Moreover the growth of ρ - strains is always slower than that of standard strains of S.cerevisiae even under conditions of complete repression of mitochondrial system, i.e. when the energy is supplied only by alcoholic fermentation.

The present report provides information about the resistance of petite mutant ρ - of S.cerevisiae to cycloheximide, a potent inhibitor of protein synthesis in certain yeast, plant and mammalian cells, but not in bacteria, (4) and in mitochondrial protein biosynthetic

system (6). Protein biosynthesis catalysed by cell-free extracts of S. fragilis is unaffected by cycloheximide, while similar preparation of S. cerevisiae, is sensitive to the antibiotic (5, 8). The data presented in this study indicate that the resistance and the sensitivity to cycloheximide of cytoplasmic ribosomes isolated from standard ρ + and mutant ρ - strains of S.cerevisiae depend also on the integrity of the mitochondrial system.

Materials and Methods

Strains and growth conditions. The strains tested were: a diploid standard cycloheximide sensitive strain EE5 ρ + from the collection of P. Slonimski, Centre de Genetique Moleculaire, Gif sur Yvette, France and the corresponding ρ - mutants induced by treatment of standard strain with ethidium bromide (9), or of spontaneous origin. In the aerobic conditions the cells were grown at 30°C in minimal GO liquid medium containing 1% of glucose, in shaken Erlenmeyer flascs, starting from a stationary phase preculture by 10 fold dilution in fresh medium and harvested during late exponential growth.

In the anaerobic conditions liquid medium consisting of the following components was used (in 1500 ml): glucose 150 g, yeast extract 105 g, KH_2PO_4 7,5 g, Tween 20 0,45 g, Tween 80 1,9 g, and ergosterol 18 mg. The cells were grown for 18 h at 30°C under nitrogen in shaken Erlenmeyer flasks closed with hydraulic valves.

Labelling the cells in vivo. This is described in the legend to Figure 1. Up to this point all operations were performed under sterile conditions.

Preparation of cell free extracts. All operations were carried out at 0 - 2°C. The harvested cells were washed twice with cold buffer A (0,05 M Tris pH 7.6, 0,005 M $Mg/CH_3COO/_2$, 0,01 M KCL, 0,01 M 2-Mercaptoethanol and 0,0005 M Spermidine) and then disrupted and centrifuged according to Bretthauer et al. (10). Ribosomes were sedimented from

postmitochondrial supernatant by the method of Wettstein, et al. (12). Ribosomal pellets were suspended in buffer B (0,05 M Tris pH 7.6, 0,005 M $Mg/CH_3COO/_2$, 0,01 M KCL and 0,0005 M Spermidine) and diluted to the final concentration of 20 mg/ml. 1 mg of ribosomes was assumed to give an OD_{260} of 9,3 (8). To obtain 105 000 g supernatant fluid (enzymic fraction) free of ribosomes, the postmitochondrial extract was centrifuged at 105 000 g for 90 min. and the upper third of the centrifugal tubes was collected.

Total protein was determined by the method of Lowry et al. (7). Ribosomes and supernatant were used immediately for further investigation.

Assay for aminoacid incorporation. This was done according to Mans and Novelli (11). Radioactivity was assayed in the Liquid Scintillation Spectrometer Tricarb, Packard Model 3320.

Results

Effect of cycloheximide on the amino acid incorporation in vivo. Inhibition of the incorporation of radioactive leucine by various concentrations of cycloheximide is illustrated in Figure 1. Incorporation of ^{14}C-leucine by ρ + strain was inhibited by 40% at a concentration of 0.03 μg per 1 ml of incubation medium and by 70% at a concentration of 0.3 μg/ml. In contrast, the ρ - mutant of this strain seemed to be resistant to the effect of cycloheximide both at a concentration of 0.03 and 0.3 μg/ml. Above this concentration progressive inhibition of the ^{14}C-leucine incorporation become evident, but the differences in the sensitivity between ρ + and ρ - strains can be still observed even at a concentration 10 fold greater. At a concentration of cycloheximide 10 μg/ml the ρ + strain is inhibited by 97%, while the ρ - only by 75%.

Changes in polyribosomes. Sucrose gradients of cytoplasmic extracts prepared from cultures of ρ + and ρ - strains incubated with 1 μg of cycloheximide per ml of incubation medium showed that in the ρ + strain approximately 60% of ribosomes remained as polysomes, while in the mutant at

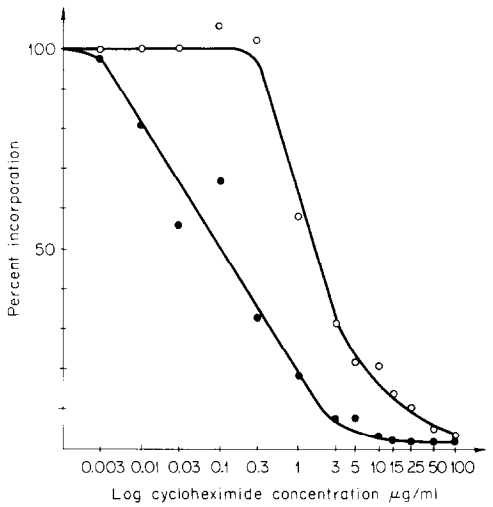

Figure 1

Effect of cycloheximide on L-(^{14}C)leucine incorporation by intact cells of standard strain ρ + (●——●) and „petite" mutant ρ - (o——o). Cells (about 10^7) were suspended in 1 ml of 0.1 M Phosphate buffer pH 6.5 containing 1% of glucose and incubated for 30 min at 30° with 1 µCi of L-(^{14}C) leucine (spec. act. 311 mCi/mmol, Ammersham, England) diluted with ^{12}C leucine to 10 mCi/mmol and the indicated concentration of cycloheximide. The reaction was terminated by the addition of 1 ml cold 10% trichloroacetic acid (TCA) which contained ^{12}C L-leucine at a concentration of 2×10^{-4}M. Precipitates were collected by centrifugation, dissolved in 1 ml of 1M NaOH which contained the same concentration ^{12}C L-leucine, kept at 37° for 20 min and reprecipitated with 5% TCA. After collection on fiber glass filters precipitates were washed three times on the filters with 5% TCA, and dried. Radioactivity was determined in a Tri Carb Packard Spectrometer using 0.4% solution of BBOT in toluene. Presented data are the mean value of five different experiments.

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least 90% were converted to monoribosomes (Figure 2). In yeast the decay of polysomes in cytoplasmic extracts is fast, it begins after one minute of incubation at 30°C and is esentially complete by five minutes (14), unless continued translation of mRNA into polypeptides is disturbed by cycloheximide. In such case the movement of ribosomes relative to mRNA and the breakdown of polysomes should be prevented. As it can be seen in Fig. 2 the decay of polysomes in mutant strain was not disturbed by cycloheximide. Thus it seems that the resistance of mutant strain to this antibiotic is associated with the change in translation system.

Effect of cycloheximide on the amino acid incorporation in vitro. As seen in Table 1 cycloheximide in the range of 0.05 to 0.3 µg/ml of incubation

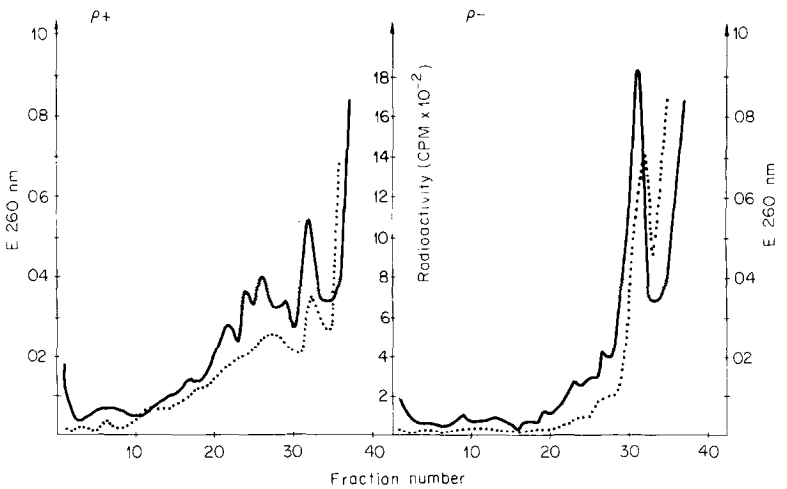

Figure 2

Polysome patterns from cells of standard strain ρ + and mutant ρ - in the presence of cycloheximide.
5 g of freshly harvested cells from each strain were suspended in 50 ml of 0.1M Phosphate buffer pH 6.5, containing 1% glucose and incubated for 5 min at 30° with 10 μCi of L-(^{14}C)leucine. Then 1 μg/ml of cycloheximide was added and the cells were incubated for additional 25 min at 30°. Postmitochondrial extracts were incubated for 5 min at 30° in the presence of 1 μg/ml of cycloheximide and analysed in 10 - 60% v/w sucrose gradients. A 0.4 - 0.5 ml sample of postmitochondrial supernatant (20 OD_{260}) was layered on the top of 14 ml of sucrose gradient containing 0.01 M Tris-HCl pH 7.6, 0.05 M $Mg(CH_3COO)_2$, 0.01 M KCl and 0.0005 M Spermidine, and centrifuged at 60 000 g at 5° for 4 h. To determine the distribution of ribosomes, 40 - 45 fractions, 17 drops each, were collected from the bottom of the gradient. Samples were adjusted with water to a volume of 1 ml and extinction was read in a Unicam SP 500 spectrophotometer. Radioactivity was measured in each fraction as described earlier (13). —— adsorbance at 260 nm, --- radioactivity in counts per min.

medium showed progressive inhibition of polyphenylalanine synthesis directed by poly(U) in the system isolated from cytoplasm of standard ρ + strain and had almost no effect in the system isolated from cytoplasm of mutant strain ρ -. Above this range progressive inhibition of polyphenylalanine synthesis become evident in both systems. In similar experiments chloramphenicol at the concentration of 0.1 to 1 μg/ml showed only very weak inhibition, and equal in the systems isolated from ρ + and ρ - strains. This observation excluded the possible influence of mitochondrial ribosomes in the studied cell-free protein biosynthetic system.

Effect of cycloheximide on mixed cell-free biosynthetic systems. In order

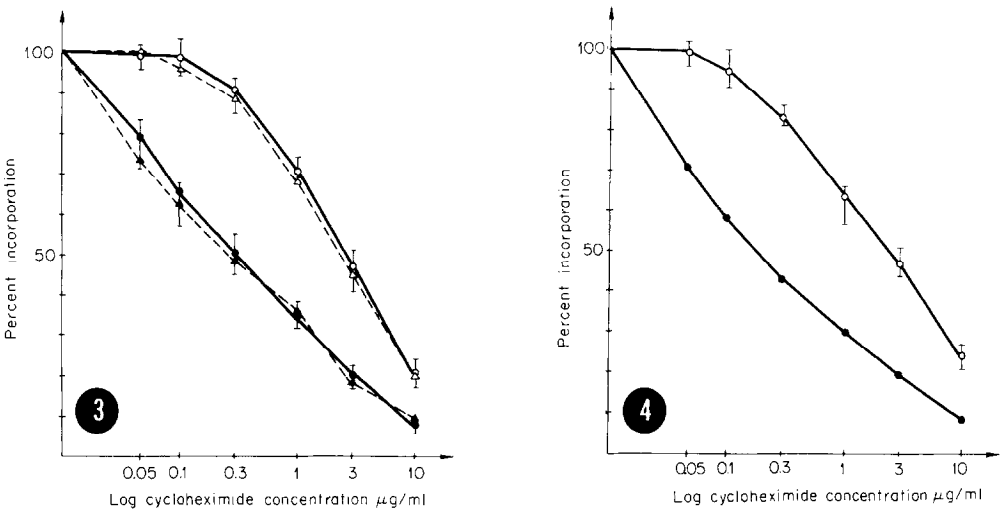

Figure 3

The response of the initial rate of phynylalanine incorporation to increasing cycloheximide concentration in cell free system composed of: (1) ribosomes from mutant ρ - strain and supernatant from standard ρ + strain (o——o), (2) reciprocal system of (1) (●——●). The other curves represent the control homologous cell free systems from mutant (Δ----Δ) and standard (▲----▲) strains.

Figure 4

Effect of the repression of mitochondrial system on the resistance to cycloheximide of ribosomes from standard strain ρ +. Cell free system was isolated from standard strain ρ + which was grown in:
(1) anaerobic conditions with high concentration of glucose (o——o),
(2) normal, aerobic conditions (●——●).

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to study if the resistance to cycloheximide of ρ - mutant is connected with ribosomes or with 105 000 g enzymic fraction, an experiment was performed in which ribosomes from one strain were combined with enzymic fraction from the other. As it can be seen from Figure 3 combination of ribosomes from mutant strain and supernatant from standard strain was as resistant to cycloheximide as the homologous system from mutant ρ -. The corresponding combined system from ρ + ribosomes and ρ - supernatant was completely sensitive to the effect of the antibiotic. These data indicate that the resistance is associated only with ribosomes of mutant strain.

Effect of cycloheximide on an anaerobically grown standard strain. As

Table 1

Inhibitor	Concentration	Incorporation CPM/ 0.1 mg of ribosomes		Inhibition %	
		Wild type ρ +	Mutant ρ -	ρ +	ρ -
NONE	-	7 705	9 840	-	-
Cycloheximide	0.05 µg/ml	5 624	9 835	27	0
"	0.1 "	4 623	9 320	40	6
"	0.3 "	3 626	8 550	53	13
"	1.0 "	2 701	6 690	65	32
"	3.0 "	1 403	4 430	82	56
"	10.0 "	911	1 950	89	80
Cloramphenicol	0.1 µg/ml	7 680	9 650	1	2
"	0.5 "	7 240	9 125	6	8
"	1.0 "	6 912	8 532	10	13

Comparison of the effect of antibiotics on poly(U) directed polyphenylalanine synthesis in cell free system isolated from standard strain ρ + and cytoplasmic mutant ρ - of S.cerevisiae.The incubation medium contained the following components (in µmoles per ml, unless otherwise specified) 30 Tris-HCl pH 7.6 6 $Mg(CH_3COO)_2$, 7 KCl, 0.4 Spermidine, 0.5 2-Mercaptoethanol, 6 Phospho(enol)pyruvate 25 µg Pyruvate kinase, 1 ATP, 0.25 GTP, 0.5 µCi L-(^{14}C) Phenylalanine (spec.act. 468 mCi/mmol, Amersham, England), 100 µg of Polyurydylic acid (Poly(U)), 100 µg of yeast tRNA.Ribosomes (0.8 mg) were added prior to the indicated quantity of the antibiotic. 0.7 mg of supernatant protein was added last.Incubation was carried out at 30° for 45 min in a total volume of 0.5 ml. The reaction was stopped by placing a 0.1 ml portion of the incubation mixture on Whatman 3MM filter paper discs which was then dried in a stream of hot air for 20 sec. and placed in cold 10% trichloroacetic acid solution containing 0.1 M L-phenylalanine and left for 12 h prior to further treatment (11). The data shown are the mean values of five experiments.

seen in Figure 4 cell free system isolated from the cells of ρ + strain became resistant to cycloheximide in the condition of full repression of mitochondria, i.e. under nitrogen and at high concentration of glucose in the growth medium. The resistance was similar to that observed in cell free system isolated from ρ - mutant (Table 1 and Figure 3).

Discussion

The data presented here strongly suggest that the resistance of ri-

bosomes of the studied strains of S.cerevisiae to cycloheximide depends on the presence of active mitochondria in the cell. The resistance of ρ - strain to cycloheximide was mentioned earlier by Schatz and Saltzgaber (15). Our experiments in vivo confirm their observation and show that the resistance is the most pronounced at low concentration range of the antibiotic. Experiments described here indicate that the absence of active mitochondria caused by mutation of mitochondrial DNA in ρ - strain, or by repression through growing conditions resulted in similar increase of the resistance of cytoplasmic ribosomes to cycloheximide. It is known from earlier reports that the resistance of S.fragilis to cycloheximide is associated with 60 S ribosomal subunits (8, 16). Studies on the influence of this antibiotic on other eucariotic systems show that at different concentration ranges cycloheximide inhibits initiation, translocation and peptide release (18,19,20,21), i.e. reactions which require GTP (17).

The absence of mitochondria or at least their active forms can affect each of these reactions. This may have a direct influence on the composition or conformation of ribosomal proteins responsible for cycloheximide binding affinity, or may have an indirect effect through the changes in energy metabolism of the cell.

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