PROTEIN SYNTHESIS BY YEAST PROMITOCHONDRIA IN VIVO *

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SUMMARY

Promitochondria of anaerobically-grown Saccharomyces cerevisiae were selectively labeled in vivo by incubating the cells with radioactive leucine and cycloheximide under nitrogen. Promitochondrial labeling was sensitive to chloramphenicol. It was not detectable in cytoplasmic "petite" mutants.

We have reported earlier that anaerobically-grown S. cerevisiae cells contain mitochondria-like organelles (1-4). These "promitochondria" lack a functional respiratory chain but still possess mitochondrial DNA as well as oligomycin-sensitive mitochondrial ATPase (F_1). Promitochondria of cytoplasmic "petite" mutants exhibit oligomycin-insensitive F_1 and, thus, differ from the corresponding organelles of the wild-type strains. Since the cytoplasmic "petite" mutation specifically affects the mitochondrial genetic system (5) we proposed that at least part of this system was still operative in the anaerobically-grown wild-type cells (2).

The experiments described here substantiate this suggestion. It is shown that promitochondria in wild-type yeast cells can incorporate amino acids into protein; promitochondria in cytoplasmic "petite" mutants cannot. Significantly, our approach avoids the uncertainties of incorporation experiments with isolated mitochondria and eliminates virtually all phenotypic differences between wild-type and respiration-deficient strains.

METHODS

Cell strains and growth conditions: The wild-type S. cerevisiae strains D 273-10B ($\alpha P\varsigma^+$,haploid) and DT XII ($P\varsigma^+$,diploid) as well as the corresponding "petite" mutants D 273-10B-1 and DT XIIa were grown anaerobically

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in the presence of Tween 80 and ergosterol as described earlier (2) except that the concentration of glucose in the growth medium was lowered to 0.3 %. For aerobic growth, Tween 80 and ergosterol were omitted and the concentration of glucose was 0.8 %. Parallel experiments with the two wild-type strains, or with the two "petite" mutants, always gave essentially the same results.

Labeling of (pro)mitochondria in vivo: The stationary yeast cultures were chilled to 0° and poisoned with cycloheximide (final concentration 25 µg/ml). The cells were isolated by centrifugation in the cold (5 min at 1500 x g), washed three times with ice-cold 40 mM phosphate buffer pH 7.4 - 0.05 % bovine serum albumin - 25 µg/ml cycloheximide and suspended to 50 mg wet weight/ml in 40 mM phosphate buffer pH 7.4 - 40 µg/ml cycloheximide. Three hundred ml of this suspension were mixed with 120 ml of 1 M glucose, 120 ml of 0.2 M phosphate buffer pH 7.4, 45 ml of cycloheximide solution (500 µg/ml) and 15 ml of water and shaken for 10 min at 28° under a stream of nitrogen. 4,5-^{3}H-L-leucine (0.6 mCi; 5 Ci/mmole) was then added anaerobically and the incubation continued for 30 min. Up to this point, all operations were performed under sterile conditions. Labeling was terminated with 600 ml of 0.2 M unlabeled leucine pH 7.4 and incubating for additional 10 min.

Fractionation of the labeled cells: All steps were performed in the cold. The labeled cells were isolated by centrifugation and washed three times with homogenization medium (0.25 M mannitol - 20 mM Tris SO_4 pH 7.4 - 20 mM unlabeled leucine - 2 mM EDTA - 25 µg/ml cycloheximide). After disruption of the cells (2) in this medium, large fragments were removed by two consecutive centrifugations for 10 min at 2000 x g. The supernatant from the second centrifugation (termed "homogenate") was divided into two aliquots. One aliquot was centrifuged for 90 min at 50,000 rpm in a Spinco No. 50 rotor to obtain the "soluble proteins". The second aliquot was centrifuged for 30 min at 17,500 rpm in a Spinco No. 30 rotor. The resulting pellet of "promitochondria" was washed once with homogenization medium by recentrifugation and purified further by 90 min centrifugation in a 20 - 70 % sucrose gradient (2). The aerobically-grown cells were labeled and fractionated under exactly the same conditions.

Analytical procedures: Cell growth was monitored by direct cell counts or turbidity measurements at 550 mµ. Protein, F_1-ATPase and NADH oxidase were assayed as described (2). For the determination of protein-bound radioactivity, the samples were precipitated with an equal volume of 10 % trichloroacetic acid, washed twice with 5 % trichloroacetic acid, and heated in 5 % trichloroacetic acid for 15 min at 90°. The residue was washed twice with ethanol:ether (3:1), dried in vacuo, dissolved in concentrated formic acid, and counted in a liquid scintillation spectrometer.

RESULTS

It is well established that, in intact aerobic yeast cells, cycloheximide inhibits protein synthesis by cytoplasmic ribosomes whereas mitochondrial protein synthesis is unaffected (5). Therefore, if yeast cells are incubated with a radioactive amino acid in the presence of cycloheximide, only the products of the mitochondrial system should become labeled. Control experiments with aerobically-grown yeast cells confirmed this prediction (Table I).

Table I.

Selective labeling of yeast mitochondria in vivo by ^{3}H-leucine

Fraction	cpm/mg protein	% of total incorporation
homogenate	1120	(100)
soluble proteins	204	10
mitochondria	7950	89

The experiment was carried out with aerobically-grown cells of the wild-type strain D 273-10B. The concentration of mitochondria in the homogenate was determined by measuring the specific activities of NADH oxidase and F_1-ATPase in the homogenate and in the purified mitochondria (2).

Table II.

Selective labeling of promitochondria in vivo by ^{3}H-leucine

Fraction	cpm/mg protein	% of total incorporation
homogenate	633	(100)
soluble proteins	76	5.6
promitochondria	15,850	102

The experiment was carried out with anaerobically-grown cells of the wild-type strain DT XII. The concentration of promitochondria in the homogenates was determined by ATPase-measurements (2).

The results with anaerobically-grown yeast cells were closely similar (Table II). Indeed, virtually all of the label incorporated into the cellular proteins proved to be associated with the promitochondrial fraction. The specific radioactivity

of this fraction was more than 200 times higher than that of the soluble proteins. In contrast, when the cells were labeled and chased in the absence of cycloheximide, the specific radioactivity of the isolated promitochondria was only about twice that of the soluble proteins.

Since the concentration of cycloheximide employed in these experiments inhibited cellular protein synthesis over 99 %, the data of Tables I and II may reflect labeling by contaminating bacteria or spurious side-reactions. However, the following considerations make these possibilities extremely unlikely:

a. All steps preceding the chase with unlabeled leucine were carried out under sterile conditions.
b. Isopycnic banding of the labeled promitochondria in a sucrose gradient showed a close correlation between radioactivity and mitochondrial ATPase activity.
c. Labeling of the promitochondria was approximately 90 % inhibited by 4 mg/ml of chloramphenicol, a specific inhibitor of mitochondrial protein synthesis (5).

It is thus reasonable to conclude that the cycloheximide-resistant labeling of promitochondria in vivo is mediated by the protein synthesizing system of these organelles.

Most significantly, analogous experiments with two different cytoplasmic "petite" mutants failed to show any comparable labeling of the promitochondrial fraction.

Table III.

Lack of selective promitochondrial labeling in a cytoplasmic "petite" mutant

Fraction	control cpm/mg protein	plus 4 mg/ml chloramphenicol cpm/mg protein
homogenate	174	174
soluble proteins	38	39
promitochondria	120	138

The experiment was identical to that specified in Table II except that anaerobically-grown cells of the cytoplasmic "petite" mutant DT XIIa were used and that one aliquot of the cells was labeled in the presence of cycloheximide and 4 mg/ml chloramphenicol. Only the specific radioactivities are given since the impaired binding of mitochondrial ATPase to the mutant promitochondria (2) made it impossible to measure the concentration of these particles in the homogenates.

The radioactivity incorporated into these cells was rather diffusely distributed between the various subcellular fractions (Table III) and was partly associated with cytoplasmic ribosomes (not shown). The specific radioactivity of the mutant promitochondria was only 0.8 % of that observed with promitochondria of the wild-type (cf. Table II). This small residual labeling was completely insensitive to 4 mg/ml chloramphenicol (Table III). Thus, the labeling pattern of the mutants suggests that these cells lack promitochondrial protein synthesis and that their cytoplasmic system shows a minor "cycloheximide-leak" that is less evident in experiments with the wild-type strains.

DISCUSSION

The present report outlines a promising experimental system for studying mitochondrial protein synthesis within living yeast cells. A similar procedure has been independently developed by Sebald, Schwab and Bücher for Neurospora crassa cells (6). Unlike experiments with isolated mitochondria, the procedure described here is simple and not subject to uncertainties of energy supply, proper incubation media, and isolation artefacts. It also permits extremely efficient labeling of the (pro)mitochondrial proteins (10,000 - 20,000 cpm per mg) and may thus facilitate the identification of (pro)mitochondrial gene products. Another novel feature of our experiments is the maintenance of anaerobiosis, both during growth and labeling of the cells. This method effectively minimizes phenotypic differences between wild-type and respiration-deficient yeast strains and, hence, uncovers differences at the level of the genetic system. It should be emphasized, however, that the present labeling procedure is limited to yeast strains that are highly sensitive to cycloheximide.

The results described here show that promitochondria of wild-type yeast cells exhibit a chloramphenicol-sensitive protein synthesizing system. This conclusion is supported by the recent finding that promitochondria isolated from galactose-grown yeast cells incorporate amino acids into protein (7). The nature of the promitochondrial product(s) is obviously of great interest. Since respiratory adaptation is inhibited by chloramphenicol (5), unadapted promitochondria may not synthesize all of the proteins that are elaborated by promitochondria undergoing adaptation to oxygen. Indeed, the observations reported in the accompanying paper (8) raise the possibility that specific cistrons of promitochondrial DNA are repressed in the absence of oxygen.

The labeling pattern observed with the two cytoplasmic "petite" mutants

shows that these strains have lost a mitochondrial protein synthesizing system. In this context it is significant that one of the two mutant strains used here seems to lack mitochondrial ribosomal RNA (9). Our results fully confirm the recent findings of Kužela and Grečná (10) that isolated mitochondria from aerobically-grown cytoplasmic "petite" mutants are unable to incorporate amino acids into protein. The present in vivo technique establishes now conclusively that this defect of the mutant mitochondria is not a consequence of their respiration-deficiency or their increased lability.

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1) Schatz, G. Biochim. Biophys. Acta 96:342 (1965).
2) Criddle, R.S. and Schatz, G. Biochemistry 8:322 (1969).
3) Paltauf, F. and Schatz, G. Biochemistry 8:335 (1969).
4) Plattner, H. and Schatz, G. Biochemistry 8:339 (1969).
5) Roodyn, D.B. and Wilkie, D. The Biogenesis of Mitochondria, Methuen, London (1968).
6) Sebald, W., Schwab, A.J. and Bücher, Th. FEBS Letters 4:243 (1969).
7) Davey,P.J., Yu, R. and Linnane, A.W. Biochem. Biophys. Res. Commun. 36:30 (1969).
8) Rouslin, W. and Schatz, G. accompanying paper
9) Wintersberger, E. Z. physiol. Chemie 348:1701 (1967).
10) Kužela, S. and Grečná, E. Experientia 25:776 (1969).