ISOLATION AND CHARACTERIZATION OF RIBOSOMES FROM YEAST MITOCHONDRIA

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The existence of small granules in mitochondria from different sources has recently been reported by several groups of workers [1–9]. The cytochemical identification of these mitochondrial granules with ribosomes (mitoribosomes) has been achieved independently by André and Marinozzi [2] and by Swift [3]. Ribosomes isolated from rat liver mitochondria [10, 11] and from *Neurospora crassa* mitochondria [12, 13] differ from cytoplasmic ribosomes (cytoribosomes) by their size, their sedimentation coefficient and their susceptibility to dissociation into subunits. In this report we describe the isolation of ribosomes from *Candida utilis* mitochondria, their sedimentation pattern in a sucrose gradient and their ability to function in protein synthesis. Polysome-like structures were demonstrated in thin sections of intact mitochondria by electron microscopy.

Experimental

Candida utilis (Strain CBS 1516) was grown for 16–18 hr at 25° under forced aeration in fermentor jars containing 10 l of a medium made of 1% yeast extract, 2% peptone and 1.5% glucose. Mitochondrial fractions were obtained as described by Mattoon and Sherman [14] with minor modifications. Fragments of mitochondrial pellets were prepared for electron microscopy by fixation for 30 min with 1% glutaraldehyde in 0.1 M phosphate, pH 7.2, and then with 2% osmium tetroxide in 0.1 M phosphate, pH 7.2. After dehydration by ethanol, the particles were embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate. As shown in fig. 1, a satisfactory degree of purity of the mitochondrial preparations was achieved. Free ribosomes were obtained by disruption of mitochondria with Na desoxycholate (DOC) and were purified by centrifugation in a sucrose gradient. In routine preparations, about 60 mg of yeast mitochondria were suspended in 7.5 ml of 0.01 M $MgCl_2$, 0.01 M Tris-HCl buffer, pH 7.6. Then 7.5 ml of 1% DOC in 1 mM Tris-HCl, pH 7.6, were added to this suspension (weight ratio of DOC to mitochondrial protein equal to 1.25). After standing for 30 min at 4°, the preparation was centrifuged at 20 000 rpm for 20 min (Spinco SW 50 rotor). The resulting supernatant fluid was centrifuged at 48 000 rpm for 60 min (SW 50 rotor). The ribosomal pellet recovered at this stage was washed three times with a medium made of 5 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.6, and 50 mM KCl (MTK medium), and it was finally suspended in about 0.5 ml of the same medium. The cloudy suspension was clarified by centrifugation at 2000 g for 15 min. An aliquot (0.2 ml) of the supernatant fluid was layered on a linear 5–20% sucrose gradient containing 5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 7.6, and the tubes were centrifuged for 60 min at 40000 rpm (SW 50 rotor). After centrifugation the bottoms of the tubes were punctured

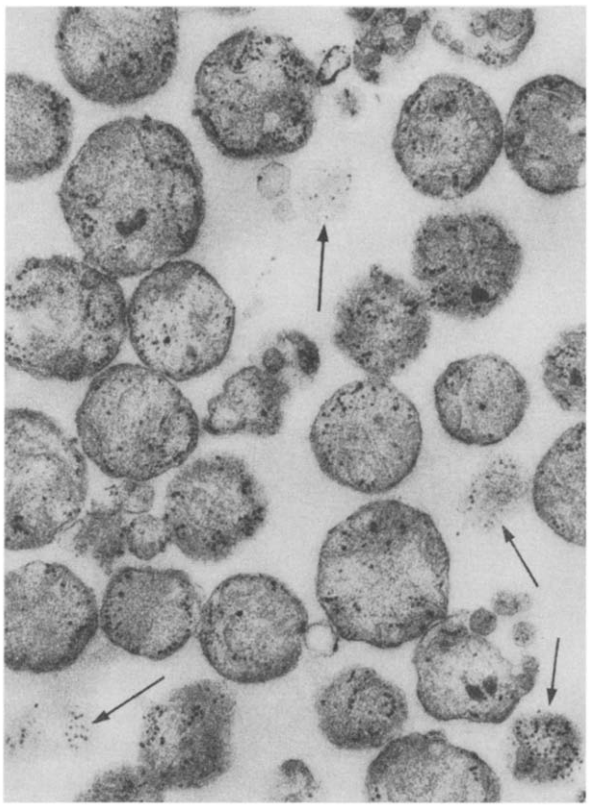

Fig. 1. Electron micrograph of a thin section of a mitochondrial pellet showing the almost complete absence of cytoplasmic contamination. The arrows point to tangential sections of mitochondria, not be be confused with contamination. X 24000.

and the contents pumped through a flow cell. The absorbance at 234 mμ was recorded automatically using an Uvicord type 4701 connected to a Varian recorded G 14A-1. Five drop fractions were collected and diluted to 3 ml for measurement of absorbancy at different wavelengths and for determination of radioactivity by scintillation counting.

A microsome fraction was obtained by high speed contrifugation (100 000 g for 60 min at 4°) of the mitochondria-free supernatant of the yeast homogenate. To prepare the cytoribosomes, the microsome fraction was treated with DOC, essentially as described for the preparation of mitoribosomes, except that the weight ratio of DOC to protein was 4.8. Cytoribosomes were washed and suspended in the MTK medium and they were separated on a linear 5–20% sucrose gradient containing 5 mM $MgCl_2$, 50 mM KCl and 10 mM Tris-HCl, pH 7.6.

Uniformly labelled L-[^{14}C] leucine (129 mC/mmole) was obtained from the C.E.A. (Saclay). The [^{14}C]-leucine incorporation was assayed essentially as described by Lamb et al. [15]. Mitochondria (about 60 mg) were incubated aerobically at 25°C for 10 min with 100 ml of a medium containing, per ml: 40 μmoles of Tris-HCl, pH 7.4, 5 μmoles of KH_2PO_4, 100 μmoles of KCl, 8 μmoles of $MgCl_2$, 100 μmoles of sorbitol, 0.1 μmole of EDTA, 1 μmole of ATP, 5 μmoles of phosphoenolpyruvate, 25 μg of pyruvate kinase, 25 μg of oligomycin, 0.4 μC of [^{14}C] leucine. The incorporation was ended by centrifugation at 25 000 *g* for 15 min at 2°C. The mitochondrial pellet was washed with 0.65 M mannitol and used for the preparation of mitoribosomes as described above.

To minimize bacterial contamination during the [^{14}C] leucine incorporation experiments, the incubation medium was filtered through a 0.22 μm Millipore membrane; glassware used for incubation was sterilized by autoclaving; furthermore, mitochondria were used immediately after isolation. Assays for total viable bacteria were made at the end of each incubation by plating o.2 ml samples of the medium on nutrient agar in four different dilutions and incubating for three days at 25°C. Usually less than 5×10^3 bacteria per ml of the incubation medium were counted.

Results and discussion

Thin sections of mitochondrial pellets show few distortions of the mitochondrial structure; mitochondria are neither extensively swollen nor contracted (fig. 1). Mitoribosomes are remarkably numerous. Most of them appear attached along the matricial side of the inner membrane and of the cristae (fig. 2), in conformity with preceding descriptions [2, 3]. Tangential sections of these membranes show that the mitoribosomes are commonly lined up in curved rows, rosettes and spirals (fig. 3), very similar to the polysomes of rough endoplasmic reticulum. Rows of 7–8 particles are frequent; higher numbers, up to 12, are less frequent. The diameter of the mitoribosomes averages 180 Å. This dimension is greater than most of those reported in the literature.

The biochemical properties of these mitoribosomes will now be described. In fig. 4, a sucrose gradient

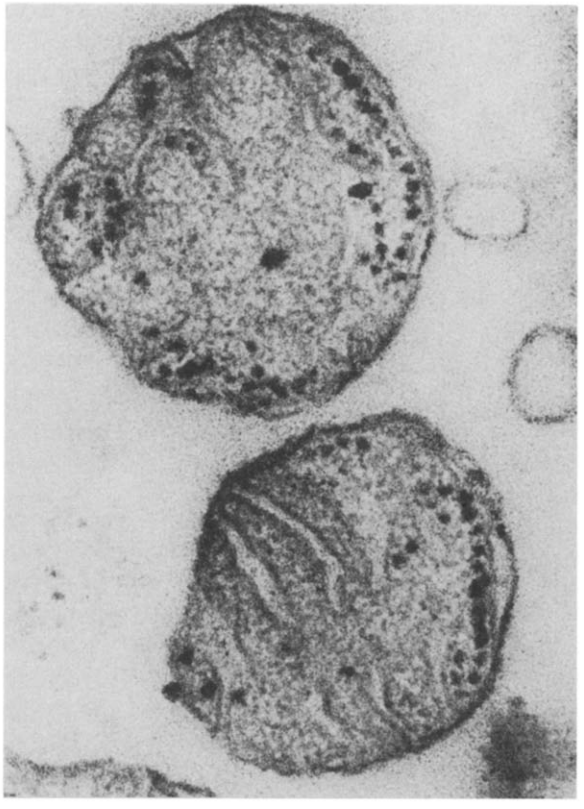

Fig. 2. Thin section of mitochondria showing a reasonably well preserved mitochondrial structure. A number of mitoribosomes are alined against the inner side of the inner membrane. X 90000.

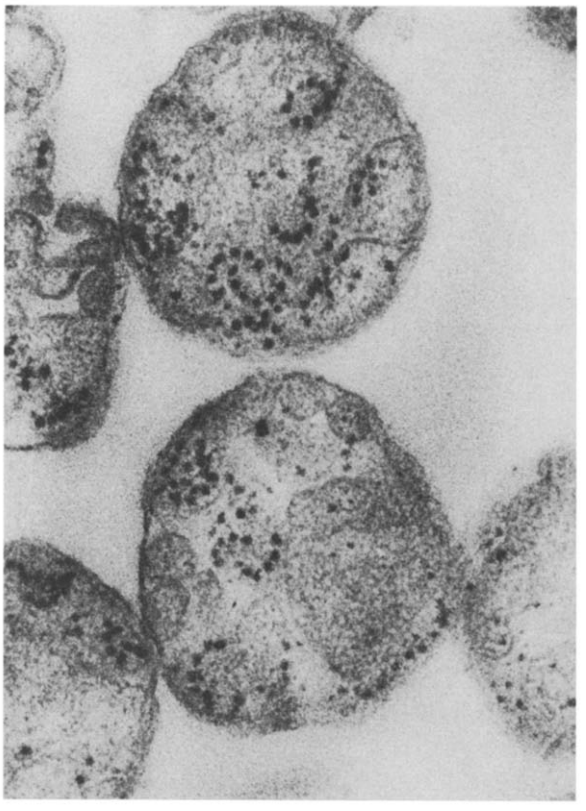

Fig. 3. Mitochondria in which several cristae are seen in grazing section. Most mitoribosomes are lined up in curved rows, spirals, etc., reminiscent of polysomes. X 60000.

profile of a crude preparation of mitoribosomes is compared with a profile of cytoribosomes isolated from the same strain of *Candida utilis*. Cytoribosomes show a typical peak of 80*S* ribosomes (this 80*S* peak was calibrated in a separate experiment with 80*S* cytoribosomes isolated from rat liver). The yeast cytoribosome profile shows other peaks with sedimentation coefficients of 36*S*, 60*S*, 120*S* and 154*S*. In contrast with the cytoribosome profile, the mitoribosome profile is characterized by a limited number of peaks. The assignment of an approximate value of 52–54*S* to the main peak of mitoribosomes is based on a comparison with the 80*S* cytoribosomes. In addition to the 52–54*S* peak, a marked shoulder characterized by a sedimentation coefficient of 77–80*S* and a minor one with a sedimentation coefficient of 32–34*S* are observed. It is noteworthy that the sedimentation patterns of mitochondrial extracts are essentially the same, whether the mitochondria have been washed only twice or five times with mannitol prior to their treatment with DOC. This result is in contrast with a recent report [16] pointing to the reversible binding of cytoribosomes to mouse liver mitochondria.

The sedimentation profiles differ according to the $MgCl_2$ concentration in the sucrose gradient. For instance, when $MgCl_2$ is present at a concentration of 20 mM (instead of 5 mM) the sedimentation coefficients are lowered from 77–80*S* to about 70–72*S* and from 52–54*S* to about 50*S*. When 5 mM EDTA replaces $MgCl_2$ in the MTK medium, the 77–80*S* peak almost completely disappears and the area cor-

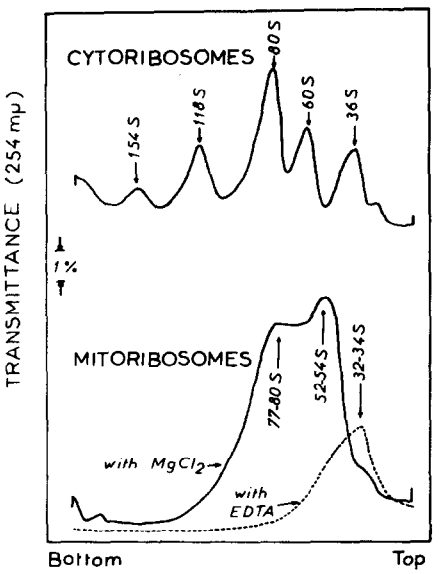

Fig. 4. Sedimentation profile of mitoribosomes and cytoribosomes from *Candida utilis*. Centrifugation was for 60 min at 40000 rpm in the Spinco SW 50 rotor at 4°. The tracing obtained with mitoribosomes treated with $MgCl_2$ (MTK medium) (see Experimental) has been superimposed on the tracing obtained with mitoribosomes treated with EDTA.

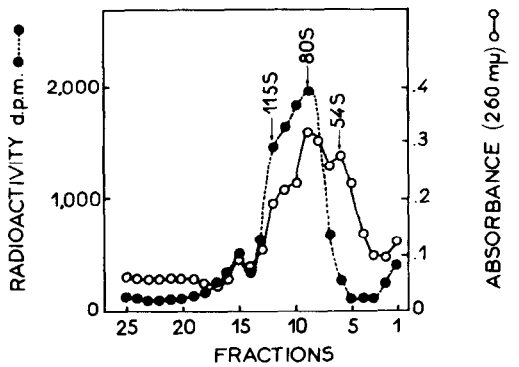

Fig. 5. Sucrose gradient analysis of the ribosome fraction isolated from *Candida utilis* mitochondria which were incubated with [^{14}C] leucine as described under Experimental. Centrifugation was for 60 min at 40000 rpm in the Spinco SW 50 rotor at 4°.

responding to the 52–54*S* peak decreases with a parallel increase in the 32–34*S* peak (fig. 4). This suggests that the 32–34*S* particles and possibly the 52–54*S* particles are subunits resulting from EDTA-induced dissociation of the 77–80*S* mitoribosomes.

The UV spectra of the three main fractions of mitoribosomes (77–80*S*, 52–54*S* and 32–34*S*) were characterized by a maximum absorbancy at 260 mμ. The ratios of absorbancy $A_{260}:A_{280}$ for the material sedimenting at 77–80*S*, 52–54*S* and at 32–34*S* were in the range of 1.8 to 1.9. The ratio $A_{260}:A_{235}$ was about 1.1 for the 32–34*S* peak and in the range of 1.5 to 1.7 for the 52–54*S* and 77–80*S* peaks.

In experiments on the incorporation of [^{14}C]-leucine, mitochondria were incubated in the presence of both [^{14}C] leucine and an ATP regenerating system as described under Experimental. Ribosomes isolated from [^{14}C] leucine-labelled mitochondria were analyzed in a sucrose gradient. A major peak of radioactivity was observed at 77–80*S* with a shoulder at 115*S* (fig. 5). In some experiments, a minor shoulder at 52–54*S* was detected. Chloramphenicol at 1.6 mM totally inhibits the [^{14}C] leucine incorporation; on the other hand, less than 20% inhibition was obtained by addition of 0.5 mM cycloheximide, indicating that the incorporation is not due to contamination by cytoribosomes [15].

The preferential association of radioactivity with the 77–80*S* component of mitoribosomes from *Candida utilis* is reminiscent of data reported by Tashiro and Siekewitz [17] concerning cytoribosomes isolated from guinea-pig liver. In their experiment, after dissociation of hepatic polysomes into monosomes, most of the radioactivity was recovered with the monomer particles; further dissociation of the monomer particles into subunits resulted in the release of radioactivity as soluble material. These features suggest that in our experiments the active unit and most probably the monomer form of ribosome in mitochondria from *Candida utilis* have a sedimentation coefficient of 77–80*S*.

Rifkin et al. [12] and Küntzel and Noll [13] have calculated a sedimentation coefficient of 81*S* and of 73*S* respectively for the monomer form of ribosomes isolated from mitochondria of *Neurospora crassa*. O'Brien and Kalf [11] have, however, concluded that in rat liver mitochondria the 55*S* particle represents the monomeric form of mitoribosomes. This conclusion was partly based on the fact that, after incorporation of [^{14}C] leucine in liver mitochondria, the most labelled particles are the 55*S* particles. This discrepancy may be paralleled with the difference in size seen by

electron microscopy of intact mitochondria. Particles in thin sections measure 120–150 Å for the mitochondira of liver and a variety of other organs of higher vertebrates and higher plants [2, 3] and 160 Å for *Neurospora crassa* mitochondria [12]. We report here a size of 180 Å for yeast. It may well be that mitoribosomes from fungi differ in size and biochemical properties from those of higher organisms. Another point of interest is the fact not previously noted that, in our material, mitoribosomes are alined in polysomes resembling those of rough endoplasmic reticulum. This is another feature of the protein synthesis machinery that the cytoplasm and the mitochondria have in common.

Acknowledgements

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