CHARACTERIZATION OF MITOCHONDRIAL AND CYTOPLASMIC RIBOSOMES FROM *PARAMECIUM AURELIA*

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ABSTRACT

The ribosomes extracted from the mitochondria of the ciliate, *Paramecium aurelia*, have been shown to sediment at 80S in sucrose gradients. The cytoplasmic ribosomes also sediment at 80S but can be distinguished from their mitochondrial counterparts by a number of criteria. Lowering of the Mg$^{++}$ concentration, addition of EDTA, or high KCl concentrations results in the dissociation of the cytoplasmic ribosomes into 60S and 40S subunits, whereas the mitochondrial ribosomes dissociate into a single sedimentation class at 55S. Furthermore, the relative sensitivity of the two types of ribosome to dissociating conditions can be distinguished. Electron microscopy of negatively stained 80S particles from both sources has also shown that the two types can be differentiated. The cytoplasmic particles show dimensions of 270 × 220 Å whereas the mitochondrial particles are larger (330 × 240 Å). In addition, there are several distinctive morphological features. The incorporation of $^{14}$C]leucine into nascent polypeptides associated with both mitochondrial and cytoplasmic ribosomes has been shown: the incorporation into cytoplasmic 80S particles is resistant to erythromycin and chloramphenicol but sensitive to cycloheximide, whereas incorporation into the mitochondrial particles is sensitive to erythromycin and chloramphenicol but resistant to cycloheximide.

Antibiotic-resistant mutants have been isolated in *Paramecium* (1, 2) and in yeast (3, 4), and these mutations have been shown to be determined by the mitochondrial genetic system (5-8). The site of alteration causing the resistant phenotype has been shown to be the mitochondrial ribosome in both yeast (9) and *Paramecium* (10). Preliminary studies in our laboratory (5) suggested that an alteration in a mitochondrial ribosomal protein is associated with the antibiotic resistance. This work was undertaken with crude mitochondrial ribosome preparations, but before further investigations could be undertaken these ribosomes required further purification and characterization. As the mitochondrial ribosomes sediment with the same S-value as the cytoplasmic ribosomes (10), it is necessary to use criteria other than S-value to distinguish between them. Furthermore, it has been suggested (11) that high sedimentation values for mitochondrial ribosomes could arise as a result of preparation artifacts, and so further investigation is required to determine whether the mitochondrial ribosomes are truly 80S.

Mitochondrial ribosomes have been isolated from various classes of organisms, and it is becoming increasingly evident that they do not form a homogeneous group in terms of sedimentation coefficient (12). To date, three main sedimentation classes have been observed: the 55S–60S ribosomes of mammalian and amphibian cells (13–15), the 72S–74S ribosomes of the Ascomycetes (16, 17), and the 80S ribosomes of the plants (18).
Isolation of Mitochondria

The clear pellet was stored at -20°C until required.

MATERIALS AND METHODS

Stocks and Culture Conditions

Stocks 513 and 513-E8-48 of Paramecium aurelia were used in this study; 513-E8-48 was isolated from stock 513 as a spontaneous mutant resistant to 250 μg/ml erythromycin. Paramecium was cultured on a grass infusion inoculated with Klebsiella aerogenes (23). 20-liter cultures were concentrated by continuous flow centrifugation, followed by centrifugation in an oil testing rotor. The clear, straw-colored supernate was then layered on 20-ml gradients of 15-30% sucrose in TMK and centrifuged at 100,000 g for 90 min in an 8 x 25-ml angle rotor; the tubes were pierced and the effluent was monitored at 254 nm. Sedimentation coefficients were calculated assuming a linear relationship between the S value and the distance of the peak position from the top of the gradient. Bacterial 50S subunits were used as standards for this measurement.

Electron Microscopy

Negative staining was carried out on both fixed and unfixed ribosomes and their subunits. A small amount of ribosome suspension was placed on a carbon film mounted on a 400-mesh copper grid. The grid was stained with 1% uranyl acetate for 15-30 s and the excess fluid removed. After drying, the grids were examined in an AEI 6B microscope and photographed at a magnification of 60,000 (calibrated with a grating replica). For measurement of the ribosome dimensions, 100 particles were measured at a magnification of 300,000.

Incorporation of [3H]Leucine

Mitochondria were prepared as described above and incubated with 0.5 μCi/ml [3H]leucine (300 mCi/mmol, Radiochemical Center, Amersham) in Medium B as described by Roodyn et al. (26) at a concentration of 10 mg/ml. Incubation was carried out in 10-ml flasks which were shaken at 30°C for 12 min, the reaction being terminated by the addition of excess [3H]leucine and by cooling the mixture in ice. In experiments in which inhibitors were used, the reaction mixture was incubated in ice for 5 min with the inhibitor, followed by 3 min at 30°C before the addition of [3H]leucine. The mitochondria were sedimented by layering the reaction mixture on 20 ml of Medium B minus amino acids and centrifuging at 10,000 g for 10 min. The mitochondrial pellet was lysed and mitochondrial ribosomes were prepared on
FIGURE 1 Sedimentation profiles of (a) mitochondrial and (b) cytoplasmic ribosome particles in sucrose gradients under different ionic conditions. The ribosomes were prepared (and sucrose gradients [15–30%] prepared) and fractionated as described in the text. The ribosomes were resuspended in 1.0 ml of 50 mM Tris-HCl (pH 7.4) buffer containing 10 mM KCl and the Mg$^{2+}$ concentrations indicated in the figure.

Sucrose gradients as described above. The fractions from such gradients were then analyzed for radioactivity by adding them to 10 ml of scintillation fluid and 2 ml of NE520 solubilizer (Nuclear Enterprises Inc., San Carlos, Calif.) and counting in a scintillation counter. Control experiments in which TCA precipitation of gradient fractions was carried out showed little or no difference with respect to the gradients analyzed by the method described.

Incorporation into nascent polypeptides on cytoplasmic ribosomes was carried out by incubating postmitochondrial supernates (prepared as described for the preparation of cytoplasmic ribosomes) with [${}^{14}$C]leucine (0.5 μCi/ml) for 5 min at 30°C. The incubation mixture contained 40 mM NaCl, 100 mM KCl, 10 mM MgSO$_4$, 6 mM mercaptoethanol, 30 μg/ml pyruvate kinase, 0.2 mM GTP, 1 mM ATP, 5 mM phosphoenolpyruvate, 10 mM Tris-HCl, pH 7.4, and 50 μg/ml of an amino acid mixture. After incubation, 0.6 μmol/ml [${}^{14}$C]leucine was added and the reaction mixture was chilled and centrifuged at 110,000 g for 90 min in a 10 × 10-ml angle rotor. The resulting ribosomal pellet was resuspended in TMK and layered on 20-ml gradients of 15–30% sucrose in TMK and centrifuged for 4 h at 100,000 g in a 3 × 23-ml swing out rotor. The gradients were fractionated and the radioactivity was estimated as described above.

RESULTS

Sedimentation Characteristics of Mitochondrial and Cytoplasmic Ribosomes

Both mitochondrial and cytoplasmic ribosomes were analyzed under a variety of ionic conditions
with a view to characterizing the monosomes and subunits in terms of both the sedimentation coefficient and the conditions under which dissociation occurred. The sedimentation patterns obtained with both mitochondrial and cytoplasmic ribosomes in various magnesium ion and EDTA concentrations are shown in Fig. 1.

Both mitochondrial and cytoplasmic ribosomes sedimented at 80S in 10 mM Mg$^{2+}$ with small amounts of dissociation products (61S and 39S for cytoplasmic and 55S for mitochondrial). When the Mg$^{2+}$ concentration was lowered to 2 mM, the cytoplasmic ribosomes were partially converted to 70S particles while the mitochondrial ribosomes sedimented at 80S with increased amounts of the 55S particle. When the Mg$^{2+}$ concentration was further lowered to 0.1 mM, the cytoplasmic ribosomes were completely converted to 70S particles while the mitochondrial ribosomes were largely converted to a 55S particle with some material at 64S. In the presence of 0.1 mM EDTA, 0 mM Mg$^{2+}$, the cytoplasmic ribosomes were converted to their two subunits sedimenting at 61S and 39S, and the mitochondrial ribosomes were converted to 55S particles.

From these data, it is clear that although the monosomes of both cytoplasmic and mitochondrial ribosomes have the same sedimentation in 10 mM Mg$^{2+}$, their behavior when the Mg$^{2+}$ concentration is lowered is different in terms of both the sedimentation properties of the particles formed and the conditions under which dissociation occurs. Complete conversion to the subunit forms of both mitochondrial and cytoplasmic particles occurs at 0.1 mM Mg$^{2+}$ if the KCl concentration is raised to 100 mM.

The absorption at 235, 260, and 280 nm of the 80S mitochondrial ribosome fractions was measured. After the first sucrose gradient, the ratios $E_{260}/280 = 1.59-1.62$ and $E_{260}/235 = 0.91-1.1$, while after a second sucrose gradient these ratios were increased to 1.8-1.85 and 1.3-1.4, respectively. Although these ratios are not so high as in some other systems (11), they are sufficiently high to suggest that contamination by membrane fragments is unlikely.

**Electron Microscopy of Cytoplasmic and Mitochondrial Ribosomes**

Examination of negatively stained cytoplasmic ribosomes in the electron microscope shows them to be similar to those isolated from other organisms (27). They appear to be a relatively uniform population of particles measuring 270 × 220 Å. A series of selected profiles and interpretive drawings is shown in Fig. 2. Some of the particles observed are divided into two subunits, the smaller being flattened and the larger being more triangular in shape (Fig. 2b). There is often an electron-dense spot between the two subunits (s) (Fig. 2a). Other rarer profiles show a kidney-shaped or "waisted" particle (Fig. 2c).

Negatively stained preparations of mitochondrial ribosomes are considerably larger than their cytoplasmic counterparts, being ~330 × 240 Å. The most common profile observed has an electron-dense spot close to the center (Fig. 3a). Further profiles (Fig. 3b) show that the ribosome is divided into roughly equal parts by an electron-dense cleft (c). A rarer set of kidney-shaped profiles also shows the particle divided into two parts by a cleft (Fig. 3c).

Profiles of mitochondrial ribosomes have several characteristic features. A small lobe (l) is frequently seen attached to one of the subunits (Fig. 3a and b). The subunit not carrying the lobe often shows an electron-dense notch (n) as seen in Fig. 3a. The kidney-shaped profile (Fig. 3c) is clearly different from the analogous cytoplasmic ribosomal profile (Fig. 2c).

General views of negatively stained cytoplasmic (Fig. 4a) and mitochondrial (Fig. 4b) ribosomal particles show many of the features described earlier. In particular, the morphological differences between the two types of ribosomes are evident. The 55S peak from sucrose gradients was also examined by negative staining; these preparations contained a very heterogeneous population of profiles and it was not possible to distinguish the morphological features seen in the 80S mitochondrial ribosomes.
A series of selected profiles of negatively stained cytoplasmic ribosomes (80S) and interpretive drawings. Three main views are shown: (a) view with an electron-dense spot (s) located centrally; (b) view showing cleft (c) which apparently divides the ribosome into two subunits, the smaller flattened and the larger more triangular in shape; and (c) view of rarer kidney-shaped profile. \( \times 300,000 \).

A series of selected profiles of negatively stained mitochondrial ribosomes (80S). The more irregular outline and larger size can be observed. Three main profiles are shown: (a) the most common profile showing a large electron-dense spot associated with a cleft (c), which divides the particle into two parts, and a notch (n), which is associated with one of the subunits; (b) a further view showing the cleft (c) and also a characteristic lobe (l) attached to one of the subunits; (c) a set of kidney-shaped profiles. \( \times 300,000 \).
Figure 4 General views of cytoplasmic and mitochondrial ribosomal particles. × 90,000. (a) cytoplasmic ribosomes; (b) mitochondrial ribosomes (80S), showing the greater size and more varied morphology.
Amino Acid Incorporation into Ribosomes

It has been shown in many cases that incorporation of \([^{14}C]\)leucine into mitochondria is sensitive to inhibition by erythromycin and chloramphenicol but resistant to cycloheximide (for review, see reference 13). Therefore, sensitivity or resistance to these inhibitors provides a useful way of distinguishing between cytoplasmic and mitochondrial protein synthesis. Experiments in which these criteria were used, have been undertaken to distinguish further the 80S mitochondrial ribosomes, by examining the effect of these inhibitors on incorporation into nascent polypeptide chains attached to the ribosome.

Incorporation of \([^{14}C]\)leucine into nascent polypeptide chains on the mitochondrial ribosomes is shown in Table I A. If the mitochondria were also incubated with chloramphenicol or erythromycin the incorporation was extensively inhibited, whereas when cycloheximide was added to the incorporation system little inhibition was observed. In studies of this type, the presence of bacteria in the incubation mixture could contribute to the incorporation and would also be sensitive to chloramphenicol and erythromycin. Although this is unlikely, as the incorporation occurs in the 80S peak but not in the bacterial 70S peak, an experiment was undertaken to eliminate this possibility. Mitochondria were prepared from the erythromycin-resistant stock 513 E\(^{84}\) and incubated with \([^{14}C]\)leucine in the presence and absence of erythromycin; the incorporation into the 80S ribosome peak was measured with both the sensitive parent stock and the mutant as shown in Table I A. It is clear that at a concentration of 100 \(\mu g/ml\) of erythromycin, incorporation into sensitive Paramecium mitochondrial ribosomes was inhibited by 58.5\%, whereas incorporation into resistant mitochondrial ribosomes was barely inhibited. Furthermore, it is clear from the data presented that the ribosomes isolated from the mitochondria of sensitive cells are sensitive to chloramphenicol and erythromycin but resistant to cycloheximide. In contrast, protein synthesis on cytoplasmic ribosomes is sensitive to cycloheximide and resistant to chloramphenicol as shown by the incorporation experiments with a mitochondria-free supernate (Table I B). Thus it can be concluded that the mitochondrial 80S ribosomes are distinct, in terms of their sensitivity to inhibitors, from cytoplasmic ribosomes.

### Table I

<table>
<thead>
<tr>
<th>A. Mitochondrial Protein Synthesis</th>
<th>[(^{14}C)Leucine] incorporated cpm/E(_{260})</th>
<th>Inhibition</th>
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<tbody>
<tr>
<td>Mitochondria</td>
<td>Inhibitor</td>
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<tr>
<td>513s</td>
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<td>1025</td>
</tr>
<tr>
<td>E(^{84})</td>
<td>ERY 0.1 mg/ml</td>
<td>425</td>
</tr>
<tr>
<td></td>
<td>CAP 0.1 mg/ml</td>
<td>412</td>
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<tr>
<td></td>
<td>CHI 0.1 mg/ml</td>
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</tr>
<tr>
<td>Ea-48</td>
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<tr>
<td></td>
<td>ERY 0.1 mg/ml</td>
<td>984</td>
</tr>
<tr>
<td></td>
<td>CAP 0.1 mg/ml</td>
<td>393</td>
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B. Cytoplasmic Protein Synthesis

<table>
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<tr>
<th>Inhibitor</th>
<th>Concentration</th>
<th>[(^{14}C)Leucine] incorporated cpm/E(_{260})</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
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<td>766</td>
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<tr>
<td>CAP</td>
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<td>747</td>
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</tr>
<tr>
<td>CHI</td>
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<td>338</td>
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</tr>
<tr>
<td>CHI</td>
<td>250</td>
<td>252</td>
<td>67.0</td>
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ERY, erythromycin; CAP, chloramphenicol; CHI, cycloheximide. Mitochondria (A) or mitochondria-free supernate (B) were incubated with \([^{14}C]\)leucine as described in Materials and Methods. The \([^{14}C]\)leucine incorporated into the 80S ribosome peak was measured by counting the gradient fractions containing this ribosome peak. The results are expressed as the total cpm in the peak per E\(_{260}\) unit.

### DISCUSSION

It has been demonstrated in this paper that the ribosomal particles obtained from either the mitochondria or the cytoplasm of Paramecium sediment at 78S–80S in sucrose gradients. The mitochondrial ribosomes can be differentiated from their cytoplasmic counterparts in a number of ways: firstly by the sedimentation coefficients of the dissociation products in low concentrations of Mg\(^{++}\) or EDTA, secondly by the conditions under which this dissociation occurs, thirdly by the size and morphology of the particles, and fourthly by the differential effect of protein synthesis inhibitors (chloramphenicol, erythromycin, and cycloheximide) on the incorporation of \([^{14}C]\)leucine into nascent polypeptides attached to the 80S particles. The sedimentation values of mitochondrial ribosomes in other organisms (except Tetrahymena) are considerably lower than 80S, and the possibility remains that this high value is not the true one. Three main explanations have been considered for a high sedimentation value, namely,
the association of membrane fragments with the ribosomes, the dimerization of a smaller "true" monosome, and the dimerization of one of the subunits of such a monosome. These possibilities have been examined by electron microscopy and measurement of the ratios of absorbance at 280, 260, and 235 nm.

The sedimentation properties of Paramecium mitochondrial ribosomal particles are very similar to those reported for Tetrahymena (19). In the latter study the ionic conditions used were slightly different from those used in this study, and hence a direct comparison is difficult. At 2 mM Mg"⁺ the Paramecium 80S particle partially dissociates into a 55S particle, while in Tetrahymena at 1 mM Mg"⁺ no such dissociation is observed. At 0.1 mM Mg"⁺ almost complete dissociation into 55S particles occurs in Paramecium although some material sediments at 64S, while in Tetrahymena a 70S peak is observed at this Mg"⁺ concentration. These differences are slight and could arise from either the different ionic conditions used or the different preparative methods. Again, the cytoplasmic ribosomes from Paramecium show slight differences in dissociation properties compared to cytoplasmic ribosomes from Tetrahymena (19) or to those obtained from Paramecium by others (28). At 0.1 mM Mg"⁺ the Paramecium cytoplasmic ribosomes are converted to a 70S particle, while those of Tetrahymena dissociate into subunits, although the latter results were obtained in the presence of 100 mM KCl, under which conditions Paramecium ribosomes dissociate into subunits. The results obtained in this paper do not agree with those of Reisner et al. (28), who studied the effect of various EDTA concentrations on the sedimentation pattern of cytoplasmic ribosomes in the presence of Mg"⁺. The differences in ionic conditions used could well account for the differences between their results and those presented here.

The high sedimentation value of Paramecium mitochondrial ribosomes could arise as a result of the attachment of membrane fragments to a smaller true ribosome, but this is rendered unlikely by the high 260/280 and 260/235 absorption ratios obtained as well as by the lack of such fragments on the ribosomes observed in the electron microscope. The recent observation (21) that under appropriate conditions, Neurospora mitochondrial ribosomes sediment as 80S rather than 73S as previously reported, suggests that mitochondrial ribosomes of the 80S class are not limited to protozoa but may be more widely occurring than has previously been supposed. The possibility that the 80S mitochondrial ribosomes represent dimers of a smaller monosome is extremely unlikely in view of the specific morphology of the two subunits observed in negatively stained preparations. Furthermore, the heterogeneity of profiles seen in the preparations of 55S particles suggest that these could not be the monosomes. It could still be argued that the spreading technique disperses any dimers, but the size of these ribosomes in the electron microscope, coupled with the lower molecular weight of rRNA (unpublished observations), are compatible with a similar sedimentation value for the cytoplasmic ribosomes. It has been observed in yeast (17) that 80S particles can be produced by dimerization of one of the ribosomal subunits; in view of the asymmetric morphology of the 80S particles reported here, this is considered unlikely. Furthermore, this would necessitate the 55S particles being the true monosomes, but these particles showed no clefs or other divisions as seen in the 55S monosomes of rat liver (14) and so are presumed not to be monosomes but a mixture of both types of subunit, although two distinct types could not be identified as they have been in other organisms (17, 29). There is considerable heterogeneity in these preparations, and this is likely to be due in part to the presence of two types of subunit. It is clear from the negatively stained preparations of mitochondrial and cytoplasmic ribosomes that they are different in size, general morphology, and relative size of subunits. These observations are similar to those made in Tetrahymena (20) and in particular demonstrate certain unique structural features, namely the centrally located dense spot between the two approximately equal subunits and the lobe attached to one of the subunits. It has been reported (17, 29) that the mitochondrial ribosomes are smaller than their cytoplasmic counterparts, but our results show that they are larger in Paramecium. It is not clear whether these differences in size are produced by the isolation procedure or whether they exist in vivo.

In summary, it is clear that the cytoplasmic and mitochondrial 80S ribosomal particles are distinct and that the 80S particle is probably the true monosome for the mitochondrial ribosomes. The two putative subunits cannot be separated on sucrose gradients, although it would be necessary to analyse the rRNA from this peak in order to be certain that both subunits were present. It is clear
that despite some very minor differences, the mitochondrial ribosomes of Paramecium are strikingly similar to those of Tetrahymena, and this suggests that the protozoa have a unique type of mitochondrial ribosome. To establish this point more definitively, it would of course be necessary to look at other classes of protozoa; although Paramecium and Tetrahymena are very different in many respects, they are both ciliates. The characterization of the ribosomes presented here will be of value in further work on the biogenesis of the mitochondrial ribosomes in Paramecium by a combined genetic, immunological, and biochemical approach.

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