THE ISOLATION, PURIFICATION AND PARTIAL CHARACTERIZATION OF CYTOPLASMIC AND MITOCHONDRIAL RIBOSOMES FROM A WATER-MOLD *ACHLYA*

by

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ABSTRACT

Cytoplasmic and mitochondrial ribosomes from the genus Achlya, a water-mold of the order Saprolegniales, class Oomycetes were isolated, purified, and partially characterised. The sedimentation constants of both the cytoplasmic and mitochondrial ribosomes were found to be 82S, and their subunits 53S and 38S. The stability of the ribosomes with respect to changes in Mg^{++} concentration was also studied. Cytoplasmic ribosomes dissociated readily at 0.1 mM Mg^{++} while mitochondrial ribosomes dissociated into ribosomes only in the presence of EDTA and not by lowering the Mg^{++} concentration. Cytoplasmic ribosomal RNA was isolated and found to be 25S and 18S, using E.coli 23S and 16S ribosomal RNA as standards. Mitochondrial ribosomal RNA's were observed to be very labile, and even the best preparations suffered partial degradation. But experimental evidence suggested they may have 'S' values of 25 and 18. The extreme lability of mitochondrial ribosomal RNA suggested the existence of latent nucleolytic activity in the ribosomes. Preliminary evidence points to the possible existence of a 9S ribosome-bound RNA in the mitochondrial ribosome.

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LIST OF ABBREVIATIONS

RNA	-	Ribonucleic acid
rRNA	-	Ribosomal ribonucleic acid
tRNA	-	Transfer ribonucleic acid
DNA	-	Deoxyribonucleic acid
RNase	-	Ribonuclease
DTT	-	Dithiothreitol
EDTA-Na	-	(Ethylenedinitrilo)tetraacetate, Tetrasodium Salt
Tris	-	Tris (hydroxymethyl)aminomethane
Tris-HCl	-	Tris(hydroxymethyl)aminomethane adjusted to required pH with hydrochloric acid
SDS	-	Sodium dodecyl sulphate

INTRODUCTION

Fourteen years ago, Rendi (1959) studied the effect of chloramphenicol on protein synthesis using isolated subcellular fractions from rat liver. He discovered that chloramphenicol had no effect on the cytoplasmic protein synthesizing systems, but inhibited protein synthesis in the microsomal fraction containing mitochondria. The same phenomenon was observed independently by Mager (1960) with *Tetrahymena pyriformis*, rat and guinea pig livers. This suggested the presence of another protein-synthesizing system in eucaryotic cells apart from the cytoplasmic one.

In the years that followed, this fundamental observation was pursued with fervor. After years of hard work, a general scheme finally emerged. The presence of another protein-synthesizing system in mitochondria differing from the one in cytoplasm was established when a tRNA and aminoacyl-synthetase specific for mitochondria were isolated from *Neurospora crassa* (Barnett et al, 1967). In all eucaryotic organisms studied, the mitochondrial protein-synthesizing system is also inhibited by antibiotics that specifically interfere with bacterial protein synthesis at the ribosome level. For example, tetracycline, lincomycin, erythromycin,

spiramycin and others (Clark-Walker and Linnane, 1966; Lamb et al, 1968; Thomas and Wilkie, 1968; Linnane et al, 1968), but is insensitive to inhibitors of cell-sap protein synthesis that do not interfere with bacterial protein synthesis, like cycloheximide and emetin (Beattie et al, 1967; Perlman and Penman, 1970). Since all the antibiotics mentioned above act on ribosomes (Weisblum and Davies, 1968), the concensus was that mitochondria contain "bacterial-type" ribosomes (Huang et al, 1966).

This hypothesis was further strengthened by studies on the size and sedimentation constants of mitochondrial ribosomes and their respective rRNA's from various plants (Clark et al, 1964; Stutz and Noll, 1967; Rifkin et al, 1967; Kuntzel et al, 1967). Chloroplasts and mitochondria of plants contained polysomes consisting of 70S ribosomes, in contrast to the 80S ribosomes which make up the polysomes of the surrounding cytoplasm. They also showed that chloroplast, mitochondrial and bacterial ribosomes were indistinguishable with respect to the sedimentation behavior of the monomeric particle and the sedimentation coefficients (23S and 17S) of the RNA components corresponding to their 50S and 30S subunits. Neurospora crassa, an ascomycete, was one of the organisms used for the above studies.

Yeast, also a member of the ascomycetes, however, presented different results. Mitochondrial and cytoplasmic ribosomes from this organism have identical sedimentation coefficients (80S) and dissociate into 60S and 40S subunits (Morimoto and Halvorson, 1971). Although there exists this discrepancy in size between yeast mitochondrial ribosomes and bacterial ribosomes, yet peptide chain elongation factors and ribosomes between the two of them are interchangeable and results in active protein synthesis, whereas the cytoplasmic factors cannot be interchanged with ribosomes from mitochondria or bacteria (Richter and Lipmann, 1970). Similar results on interchangeability between mitochondrial and cytoplasmic factors and ribosomes were also obtained from Neurospora crassa (Kuntzel, 1969).

The initiation process in protein synthesis is also strikingly similar between mitochondria and bacteria. Formylmethionyl-tRNA has been found in mitochondria from yeast, Neurospora, rat liver and HeLa cells (Smith and Marcker, 1969; Epler et al, 1970); transformylase activity has been detected in Neurospora mitochondria (Epler et al, 1970). Mitochondrial ribosomes from Neurospora are able to recognise, bind and translocate E.coli formylmethionyl-tRNA in response to the codon AUG, while cytoplasmic ribosomes produced negligible translocation (Sala and Kuntzel, 1970).

A ribosome-bound, low molecular weight RNA, distinct from tRNA, was first discovered in E.coli by Rosset and Monier in 1963. This RNA, which has a sedimentation coefficient of 5S, is now considered to be a universal component of ribosomes. By reconstitution experiments, it has been found that 5S RNA plays an essential structural role in the assembly of active 50S ribosomal subunit (Erdmann et al,1971). In its absence, some functionally important proteins fail to join the reconstituting particle to form an active subunit. It has also been shown that all procaryotic 5S RNA's tested were active in reconstituted 50S subunits, whilst eucaryotic 5S RNA's were not (Wrede and Erdmann, 1973). However, the eucaryotic 5S RNA's used were from cytoplasmic ribosomes, not mitochondrial ribosomes. Isolation of 5S RNA's from mitochondria of Neurospora (Lizardi and Luck, 1971) and other organisms (Borst and Grivell, 1971) has been unsuccessful so far. The data obtained to date show some interesting and striking similarities between mitochondrial ribosomes of ascomycetes and bacterial ribosomes. It would be most interesting, therefore, to see whether an organism from Oomycetes, a group that has been suggested to have evolved independently from most of the rest of the fungi (Bartnicki-Garcia, 1970;

Lovett and Haselbey, 1971; LeJohn, 1972), would complement or contradict the results that have been accumulated so far. It is the purpose of this thesis to act as a first step in the elucidation of these problems.

MATERIALS AND METHODS:

I. Organism

The fungus used is a water-mold, Achlya sp. (1969)*, from Dr. J.S. Lovett, Purdue University.

II. Media, Buffers and Chemicals

a) G_2Y - 5 g glucose, 0.5 g yeast extract in one litre of distilled water.

b) PYG - 2 g peptone, 2 g yeast extract, 3 g glucose in one litre of distilled water.

c) AMT - 100 mM ammonium chloride, 10 mM magnesium chloride, 10 mM Tris-HCl, pH 7.5.

d) Electrophoresis Buffer (0.2% SDS-TEB) - 2.0 g sodium dodecyl sulphate, 10.8 g Tris, 0.93 g Na-EDTA, 5.5 g boric acid in 1 litre of distilled water.

e) Acrylamide - Baker grade, J.T. Baker Chem. Co.

f) Bisacrylamide (N', N'-methylene-bis-acrylamide) - Canal Industrial Corp.

g) Agarose - Biorad Lab, electrophoresis grade.

h) TEMED (tetramethylethylene diamine) - Biorad Lab, electrophoresis grade.

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* Designation given by our laboratory.

i) Sucrose - Baker analysed reagent, J.T. Baker Chem. Co. All sucrose solutions used were autoclaved for 5 min at 10 psi to destroy any contaminating ribonucleases.

III. Growth of Organism

Stock cultures were kept on slants on Cantino's PYG agar (Cantino and Lovett, 1960). Whenever needed, a few mycelial threads were transferred into 20 ml of G_2Y medium dispensed in standard size plastic petri plates, and then incubated at 22 C for 48 hr for complete growth and sporulation. Four to six cell mats were then transferred aseptically into a flask containing 100 ml of distilled water, shaken vigorously for about 1 min, the cell mat removed by filtering through sterile cotton-gauze, and the spore suspension used as inoculum for carboys. Ten Petri plates of cultures were used for each 7-litre PYG medium. The spores were allowed to germinate and grow under forced aeration for 16-18 hr (mid-exponential growth phase), the mycelial threads were then harvested by filtration on Whatman No. 1 filter paper, and washed with

0.44 M sucrose-AMT buffer. About 40 g of cells may be obtained from a single 7-l culture.

IV. Extraction of Ribosomes

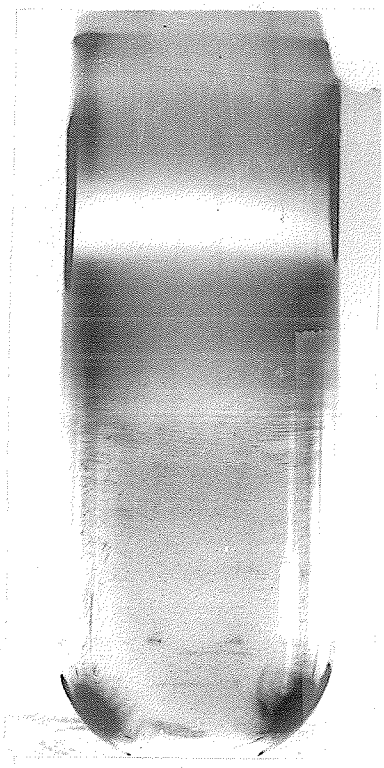
From this point onwards, all glasswares used was acid-washed with 6N hydrochloric acid, then rinsed with deionised millipored water, and heated at 500 C for three hours before use. Disposable plastic gloves were also worn during preparation of materials. Stable buffers and chemicals were autoclaved before use. The precautions taken were to minimise ribonuclease contamination and reduce heavy metal contamination and disruption of RNA.

The freshly collected cell mat was broken up into small pieces, put into a pre-cooled Waring blender, and 4 vol of cold 0.44M sucrose-AMT buffer added. Cells were then homogenized at high speed with 30 sec bursts, 5 times, with 30 sec cooling intervals in between bursts. The homogenate was then filtered through 4 double layers of cotton gauze, and the extract was centrifuged at 2 C at 5,900 X g for 10 min in a Sorvall RC2-B centrifuge to remove cell debris. The supernatant was then

centrifuged at 27,000 X g for 20 min to sediment mitochondria. The post-mitochondrial supernatant was saved for isolation of cytoplasmic ribosomes. The crude mitochondrial pellet was then suspended in 0.44M sucrose-AMT (1 ml per 20 gm wet weight of cells). Two millilitre portions of this suspension was layered on top of 60 ml linear (40% - 60% w/v) sucrose-AMT gradients with 10 ml of 60% sucrose as a cushion. Centrifugation was carried out with a Beckman SW25.2 rotor for 2½ hr at 106,000 X g at 4 C in a Spinco ultracentrifuge. A yellowish mitochondrial band was formed about half way down the gradient and this collected by aspiration from the top using a Pasteur pipette (Fig. 1). To prepare mitochondrial ribosomes, the mitochondrial fraction was diluted with 1½ vol of AMT buffer, containing 5% (v/v) Triton X-100 and mixed thoroughly to lyse the mitochondria. Treatment with Triton X-100 does not lyse any possible contaminating bacteria (Parenti and Margulies, 1967). The lysate was then subjected to centrifugation at 65,000 X g for 20 min, using a Beckman Type 60 Ti rotor, the pellet discarded, and supernatant again centrifuged at 100,000 X g for 1 hr. The translucent pellet (mitochondrial ribosomes) was dissolved in a very small volume of

Figure 1

PURIFICATION OF MITOCHONDRIAL FRACTION BY ISOPYCNIC CENTRIFUGATION IN LINEAR SUCROSE GRADIENT. PROCEDURES AS DESCRIBED IN MATERIALS AND METHODS.


MITOCHONDRIAL
BAND

AMT buffer and kept at -70 C until used.

To prepare cytoplasmic ribosomes, the post-mitochondrial supernatant was centrifuged in a Sorvall RC2-B at 48,200 X g for 20 min, pellet discarded and the supernatant recentrifuged at 100,000 X g for 1 hr in a Spinco ultracentrifuge. The translucent yellow pellet was rinsed with AMT buffer and dissolved in same. After a clarifying spin at 48,200 X g for 20 min, the turbid supernatant containing the cytoplasmic ribosomes was stored at -70 C until used.

V. Biological Activity of Mitochondria

To ensure that the 'mitochondrial' preparations obtained from the sucrose gradients were indeed mitochondrial, their ability to oxidize metabolizable substrates such as glutamate, etc., was monitored with a Gilson's oxygraph. The reaction mixture consisted of 0.3 ml 0.1M Tris-acetate buffer pH 7.0, 0.5 ml distilled water, and 10-20 µl of mitochondrial preparation (depending on the concentration). After endogenous substrates were used up, then the following substrates were added in a total volume of 1 ml: 100 µl of each either 50 mM malate or 50 mM glutamate,

and 10 mM $CaCl_2$. The rate of oxygen uptake was then followed.

VI. Sedimentation Analysis

Determination of the sedimentation values (S) of the two types of ribosomes was achieved using a Beckman Model E analytical ultracentrifuge. Before the analysis, the two types of ribosomes (in AMT buffer) were dialysed against a buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT) for 8 hr at 5 C. To determine the sedimentation values of the subunits of the ribosomes, they were dialysed instead in another buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, 1 mM EDTA, 1 mM DTT. Samples were run at 130,000 X g at 20 C, and after attaining the pre-set speed, Schlieren photographs were taken at 4-min intervals. Approximately 50 O.D. units of ribosomal material (0.5 ml) were used in each run.

VII. Zone Sedimentation Analysis

The effect of Mg^{++} ion concentration on the dissociation and re-association of the subunits of the ribosomes were also studied in zone velocity

centrifugation in isokinetic sucrose gradients (Noll, 1967). Two O.D. units of ribosomes in 20 µl buffer was layered onto 5 ml sucrose density gradients (5 - 20% w/v) containing 10 mM Tris-Cl pH 7.5, 50 mM NH_4Cl and varying Mg^{++} concentrations or EDTA. Centrifugation was carried out with an SW 50.1 rotor in a Spinco L2B-65 ultracentrifuge at 125,000 X g for 1 hr. The absorption spectrum of the gradient was determined at A_{260} in a Gilford model 2400 recording spectrophotometer with a flow cell attachment. For preparation of subunits, 60-ml 5% - 20% linear sucrose gradients containing the same buffer but with 1 mM EDTA added were used. Centrifugation was for 11½ hr at 100,000 X g using an SW 25.2 rotor at 4 C. One and a half ml fractions were collected using an ISCO fraction collector Model 272, and analysed spectrophotometrically at A_{260}. Fractions that belong to the same peak were pooled, adjusted to 10 mM with Mg^{++}, and subunits precipitated by addition of 0.7 vol of -20 C absolute ethanol, and stored at -20 C overnight. The precipitate were then collected by centrifugation, redissolved in a buffer containing 10 mM Tris-HCl pH 7.5, 50 mM NH_4Cl, and 5 mM $MgCl_2$, dialysed against the same buffer with

DTT added in the cold for 4 hr. Samples not used immediately were stored at -70 C.

VIII. Extraction of rRNA

Cytoplasmic rRNA were extracted using the method of Lovett and Leaver (1969). But instead of using whole cell extracts, purified cytoplasmic ribosomes were used. This is to eliminate any possible contamination by mitochondrial rRNA. Ribosomal suspensions were added to 5 mls of cold PAS-TNS (6% p-aminosalicylic acid and 1% triisopropylnaphthalene sulfonic acid in 0.01 M Tris-HCl buffer at pH 7.5), 5 ml of buffer saturated redistilled phenol, and mixed rapidly for 10 secs with a vortex mixer. The phases were then separated by centrifugation at 770 X g for 5 min at 0 C, and the upper, clear, aqueous phase re-extracted two more times with phenol by the same procedure. The RNA was precipitated from the aqueous layer with 0.1 volume of 20% potassium acetate (pH 5.0) and 2 volumes of cold absolute ethanol at -15 C, and stored as precipitate at -20 C until used. When necessary, the precipitate was centrifuged at 2,000 X g for 5 min, washed, and 'redissolved' in electrophoresis buffer.

IX. Gel Electrophoresis

RNA or ribosome samples were analysed by method of Peacock and Dingman (1967, 1968). Acrylamide and bisacrylamide were recrystallized before use. The final gel concentration in all cases were 0.5% agarose and 2.5% polyacrylamide unless otherwise specified. Gels were cast in 10 cm X 0.6 cm plexiglass tubes. Between 0.5 and 1.0 O.D. units of either RNA or ribosomes dissolved in 45 µl electrophoresis buffer and 5 µl glycerol were applied to each gel, and electrophoresed at 1 mA/tube for 10 min, then at 5 mA/tube for 70 min at 8 C. At the end of the electrophoretic run, gels were removed and soaked in 1% acetic acid for 1 hr. Absorption profiles of the gels were then obtained by scanning them at 258 nm using either a Joyce-Loebl Chromoscan or a Shimadzu Model MPS-50L Multipurpose Recording Spectrophotometer.

RESULTS AND DISCUSSIONS

I. Purity of Mitochondrial Preparations

To eliminate significant contamination of mitochondrial preparations by cytoplasmic ribosomes we have isolated mitochondria by isopycnic centrifugation in sucrose gradients according to the method by Luck (1963). Preparations of mitochondria thus obtained are essentially pure and free from contamination. The biological activity of the 'mitochondrial' preparations were assessed by measuring their ability to oxidize either glutamate or malate in a Gilson's oxygraph.

II. Physical Properties of Cytoplasmic and Mitochondrial Ribosomes

To determine whether cytoplasmic and mitochondrial ribosomes are different, the sedimentation behavior of ribosome preparations from the two sources were examined using zone velocity centrifugation in isokinetic sucrose gradients (Noll, 1967). Figures 2 and 3 show the sedimentation patterns of mitochondrial and cytoplasmic ribosomes. Both preparations sedimented as strong monomeric peaks. The distance travelled by the monomeric peak down the gradient was the same