THE FUNCTIONAL CHARACTERIZATION OF RIBOSOMES FROM RAT LIVER MITOCHONDRIA

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Received January 20, 1970

SUMMARY. When isolated sterile rat liver mitochondria are incubated with ^{14}C-leucine and disrupted with detergent, radioactivity is associated predominantly with a particle sedimenting at 50-55S in sucrose gradients. Experiments using puromycin, cycloheximide and chloramphenicol suggest that this radioactivity represents peptide chains associated with mitoribosomes or a mitoribosomal subunit.

Mitochondria have been shown to synthesize protein in vitro (see review by Work, Coote and Ashwell, 1968) and the evidence so far indicates that this protein synthesis takes place on mitochondrial ribosomes which we shall refer to as 'mitoribosomes'. Ribosome-like particles have been demonstrated in mitochondria from many tissues in electron micrographs (Andre and Marinozzi, 1965) and these indicate that in mammalian cells especially there are very few mitoribosomes. In general these appear smaller than their cytoplasmic counterparts. Contamination with the latter may explain why attempts to determine the size of mitoribosomes in isolated mitochondria have so far led to discrepant results (O'Brien and Kalf, 1967a; b; Elaev, 1964; Georgatsos and Papsarantopoulou, 1968). In our own experiments we decided to detect mitoribosomes solely by pulse-labelling under conditions in which contaminating cytoribosomes are thought not to be labelled. Our results indicate that when isolated mitochondria are pulse-labelled with ^{14}C-leucine, label is associated with a particle released by neutral detergent and sedimenting at 50-55S.

The kinetics of labelling of this particle and the effect of

various drugs suggest that it is ribosomal in nature. As yet it is impossible to state definitely that this particle is the mitoribosomal monomer rather than a subunit and further experiments are in progress in an attempt to settle this question. Dawid (1969) has reported the labelling of a 55S particle in frog oocyte mitochondria after short term pulses.

METHODS

Sterile rat liver mitochondria were isolated either in:-

a) SEN - 0.3M sucrose, 2mM EDTA, 30mM nicotinamide

b) S-T - 0.3M sucrose, 10mM Tris-HCl, pH 7.6

c) S-TM(30)K(100) - 0.3M sucrose, 10mM Tris-HCl, pH 7.6, 30mM MgAc, 100mM KCl.

At least three washes of the mitochondrial pellet were routinely given.

Mitochondria were resuspended in Medium 1 used by Ashwell and Work (1968) at a concentration of about 5 mg. protein/ml. ^{14}C-leucine (311 mC/mMole) was obtained from the Radiochemical Centre, Amersham, Bucks.). Mitochondria were incubated at 30^{o}for the time indicated and any drugs were added as shown. After ^{14}C-incorporation had been stopped with excess ^{12}C-leucine the mitochondria were spun down, washed free of incubation medium, resuspended in S-TM(10)K buffer (0.2M sucrose, 10mM Tris-HCl, pH 7.6, 10mM MgAc, 10mM KCl) and lysed using either Brij 58 (Honeywill-Atlas Ltd.) at concentrations from 0.2 to 1.0^{o}/o, sodium deoxycholate (0.1^{o}/o) or Nonidet NP 40 (0.5^{o}/o) (Shell Chemicals). Usually, the whole mitochondrial lysate plus E. coli optical density marker (50S ribosomal subunit) was layered directly onto a 15-30^{o}/o sucrose gradient, buffered with TM(10)K and spun in either the Spinco SW 25 rotor or the Spinco 30 rotor. 1 ml. gradient fractions were collected using a syringe device. $O.D._{260}$ measurements were made using a Unicam SP 500 spectrophotometer and radioactivity of trichloroacetic acid precipitable material was determined using the Packard Tri-Carb

Scintillation Counter with ^{14}C efficiency of 80%.

RESULTS

Fig. 1 shows that when mitochondria are incubated with ^{14}C-leucine for 5 min. and disrupted, radioactivity is associated with a particle which sediments very close to the E. coli 50S ribosomal subunit. Treatment of labelled mitochondria with puromycin (100 μM) for 30 sec. releases all radioactivity from this peak. This suggests that the peak is due to incomplete peptide chains associating with the mitoribosomes and released by the action of puromycin (Nathans and Lipmann, 1961). The increased radioactivity at the top of the gradient probably represents released chains. A similar effect with puromycin has been

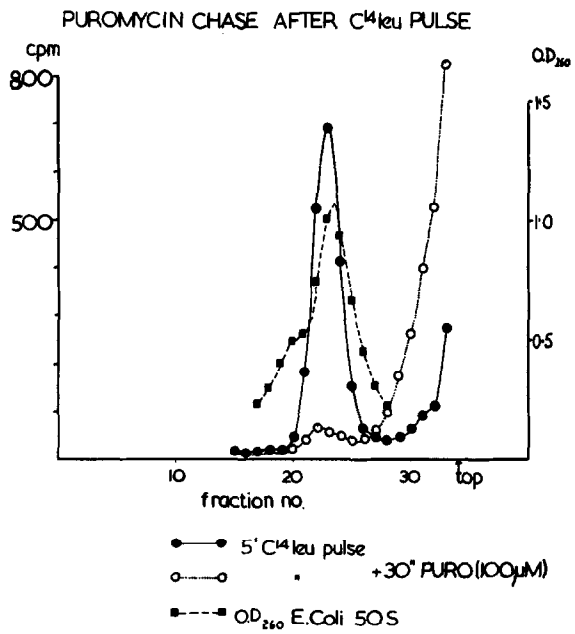

Fig. 1. Two identical batches of sterile rat liver mitochondria were incubated 5 min. at 30° at a concentration of 5 mg/ml in the presence of 0.7 μCi/ml ^{14}C-leucine. Puromycin (100 μM) was added to one batch for 30 sec. before both reactions were stopped. Mitochondrial lysates (0.2% Brij) were layered over 15-30% sucrose - TM(10)K gradients, together with 9 O.D.$_{260}$ units E. coli 50S subunits as optical density marker, and spun at 30,000 rpm in the Spinco 30 rotor for 100 min.

demonstrated by Neupert, Sebald, Schwab, Pfaller and Bücher (1969) using Neurospora crassa mitochondria.

Fig. 2 shows how the percentage of total counts associated with the peak decreases as the pulse is increased from 2 to 20 min. At the same time the percentage of counts in the gradient pellet increases. This would suggest that completed proteins are being transferred from the mitoribosomes to the mitochondrial membranes which are found in the gradient pellet. Even if mitochondria are labelled for 60 min. the 50-55S peak does not increase significantly in size and a puromycin chase will still remove all the counts. This result would support the view held by Kuntzel (1969) and Davey, Yu and Linnane (1969) that mitoribosomes do not make their own ribosomal proteins.

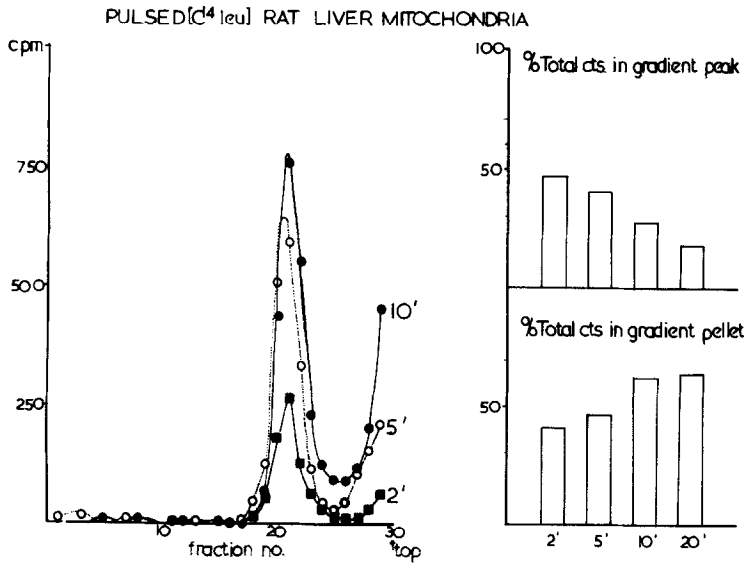

Fig. 2. Mitochondria were incubated at 30° for 2, 5, 10 or 20 min. in the presence of 0.7 μCi/ml ^{14}C-leucine. Mitochondrial lysates (0.2% Brij) were layered over 15-30% sucrose - TM(10)K gradients in Spinco 30 tubes and spun for 90 min. at 30,000 rpm. In each case the total radioactivity in the gradient pellet and peak was calculated.

Further evidence that the radioactive peak represents mitoribosomes is shown by the action of the antibiotics cycloheximide (CH) and chloramphenicol (CAP). CH is thought to inhibit selectively protein synthesis on cytoplasmic ribosomes whereas CAP selectively inhibits protein synthesis on bacterial ribosomes. Fig. 3 shows that if mitochondria are pulse-labelled in the presence of these antibiotics, then CAP will strongly inhibit the labelling of the peak whereas CH causes only slight inhibition.

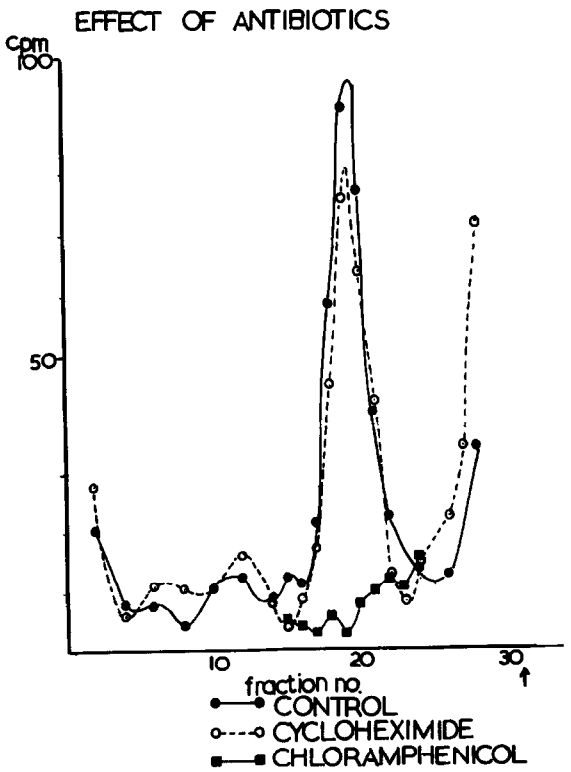

Fig. 3. Three identical batches of mitochondria were incubated at 30° in the presence of 0.3 μCi/ml ^{14}C-leucine for 5 min. One batch was pre-incubated for 10 min. with 100 μg/ml cycloheximide and one batch with 100 μg/ml chloramphenicol. Mitochondrial lysates (0.2°/o Brij) were layered over 15-30°/o sucrose - TM(10)K gradients in Spinco SW 25 tubes and spun for 16 hr. at 17,500 rpm.

Both Cannon (1968) and Weber and de Moss (1969) have shown that the presence of CAP will prevent the puromycin induced release of of peptides from bacterial ribosomes. The same effect can be demonstrated with rat liver mitoribosomes. If mitochondria are treated with CAP after a ^{14}C-leucine pulse, a subsequent puromycin chase is only 50% as effective as it would have been without CAP pre-treatment (see Fig. 4).

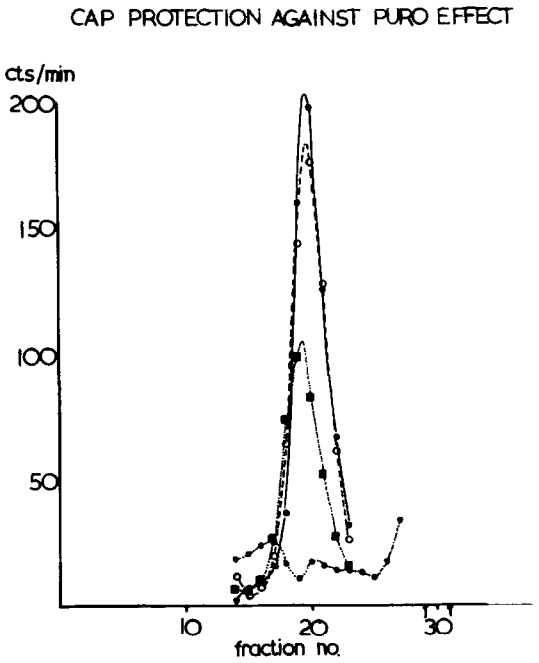

Fig. 4. Four identical batches of mitochondria were incubated at 30° with 0.3 μCi/ml ^{14}C-leucine. Each was pulsed for 5 min. and then CAP (500 μg/ml) and puromycin (100 μM) were added as shown. Lysis and gradient conditions as for Fig. 3.

●——● 5' ^{14}C-leu ; O- - - - - -O 5' ^{14}C-leu + 5' CAP

●·····● 5' ^{14}C-leu + 10' PURO; ■·····■ 5' ^{14}C-leu + 5' CAP + 10' PURO

Since our usual mitochondrial isolation medium contained EDTA it was considered necessary to prepare mitochondria in EDTA-free isolation media to show that the 50-55S peak was not due to a breakdown product of either mitoribosomes or cytoribosomes. Mitochondria prepared in S-T or

S-TM (30)K(100), pulsed and lysed in the usual manner showed the normal radioactive peak but removal of cytoribosomes was not so complete as in our standard method and cytoribosomes were quite often detected as an unlabelled optical density peak at 78S.

DISCUSSION

Our own results support those of O'Brien and Kalf and suggest that a 50-55S particle is present in rat liver mitochondria. The experiments using CH and CAP provide the best evidence that this particle is mitoribosomal in nature.

Neupert et al. (1969) have shown that a large proportion of the radioactivity from a 20-min. ^{14}C-leucine pulse is associated with the polysome region when lysed Neurospora mitochondria are analyzed on a sucrose gradient. Some of our experiments indicate a small percentage of counts in this region especially if the gradient pellet is re-lysed. In fact counts are also lost from the gradient pellet after puromycin treatment which suggests that some ribosomal particles are still in this pellet. Therefore it is quite possible that polysomes are present but these might be more tightly bound to membranes, and therefore not normally released by gentle detergent action. We have also been able to demonstrate substantial nuclease activity associated with the mitochondria and this could be another reason why few larger particles are detected.

As we have already mentioned, we are still not entirely convinced that the 50-55S particle represents the monomeric form of the ribosome rather than the large subunit of a 70S particle. Certainly all the evidence so far points to mitochondria having bacterial-like ribosomes and the mitoribosomes isolated from Neurospora (Kuntzel and Noll, 1967; Rifkin, Wood and Luck, 1967) and yeast (Vignais, Huet and Andre', 1969; Schmitt, 1969) have S-values between 70 and 80. However it is quite possible that mammalian mitoribosomes have a different role to play in

the functioning of the intact cell and therefore their size and form has correspondingly changed. This hypothesis is supported by a study of mitochondrial DNA (M-DNA) from different cells. M-DNA from cells of lower organisms have molecular weights considerably greater than 10^7 daltons which seems to be the characteristic MW of M-DNA from mammalian cells (see Borst and Kroon, 1969, for review).

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