SMALL SIZED RIBOSOMES FROM MITOCHONDRIA OF LOCUSTA MIGRATORIA

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1. Introduction

RNA species from locust flight muscle mitochondria with apparent molecular weights of about 0.5 and 0.2×10^6 have been reported [1–3]. In this paper data are presented which establish the existence of particles in locust muscle mitochondria having essential properties in common with ribosomes from which these RNA species may be derived. The sedimentation constant of these particles was estimated to be about 60 S.

This is in accordance with the finding of a type of small ribosome in mitochondria from various vertebrate tissues [4–7].

2. Materials and methods

Locust thorax muscles (1–8 days after imaginal moult) were homogenized as described previously [3]. For labelling experiments homogenisation was performed in SMT medium (0.3 M sucrose, 0.01 M $MgCl_2$, 0.01 M triethanolamine buffer pH 7.2) and mitochondria were isolated by differential centrifugation [3]. Mitochondria devoid of cytoplasmic ribosomes were isolated in SET medium (0.3 M sucrose, 0.05 M EDTA, 0.01 M triethanolamine buffer pH 7.2). After homogenisation, the 10 min 10,000 *g* pellet was washed once with SET. The resulting pellet was subjected to sucrose density gradient centrifugation and mitochondria were collected from the gradient as described [3]. Pulse labelling of isolated mitochondria with radioactive amino acids was performed for 7 min at 30°. The incubation medium contained 0.1 M KCl, 0.004 M KH_2PO_4, 0.002 M ATP, 0.003 M $MgCl_2$, 0.02 M triethanolamine buffer pH 6.85, 1.7 mg/ml of an amino acid mixture (other than leucine, isoleucine and phenylalanine) [8] and 5–20 mg mitochondrial protein per ml [9]. Incubation was started by addition of ^{3}H-L-leucine (390 mCi/mmole), ^{3}H-L-isoleucine (3.1 Ci/mmole), and ^{3}H-L-phenylalanine (8.7 Ci/mmole), 6 μCi/ml each, and stopped by cooling to 0°.

For preparation of ribosomes, mitochondrial pellets (approx. 100 mg protein) were lysed by suspension in 10–20 ml of 2.5% Triton X-100 in AMT medium (0.1 M NH_4Cl, 0.01 M $MgCl_2$, 0.03 M tris-buffer pH 7.6). After a clarifying spin for 30 min at 24,000 *g*, particles were sedimented by centrifugation for 120 min at 100,000 *g*. The brown pellet obtained was resuspended in AMT and placed on top of an isokinetic gradient made from 10.25% and 48% sucrose in AMT according to Noll [10]. In the labelling experiments lysis of mitochondrial pellets was performed in a volume of 0.4 ml and after a clarifying spin, the clear lysate was directly layered onto the density gradient. After 4 hr of centrifugation in the Spinco SW 41 rotor at 41,000 rpm 0.3 ml fractions were collected. All procedures were carried out at 0–4°. After diluting the fractions to 1 ml with AMT, the absorbancy at 260 and 280 nm was recorded. Radioactivity was measured in a Packard Tricarb counter using butyl-PBD-scintillator (6 g/l in toluene–2-metoxyethanol 3:2, v/v).

3. Results and discussion

Large amounts of cytoplasmic ribosomes adhere on mitochondria isolated from locust flight muscle in

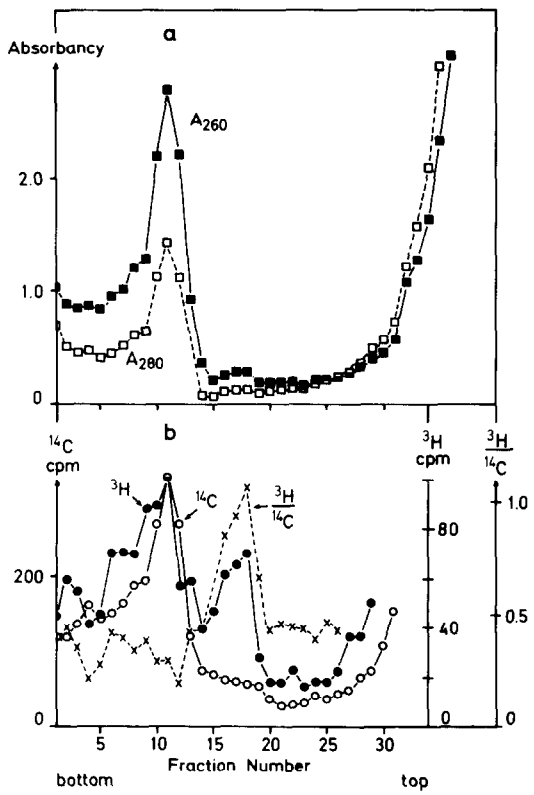

Fig. 1. Effect of cycloheximide on *in vivo* pulse labelling with radioactive amino acids of ribosomal particles associated with mitochondria. Five locusts at the first day after imaginal moult were injected with 2.5 μCi ^{14}C-L-isoleucine and 2.5 μCi ^{14}C-L-phenylalanine. Another 5 locusts were injected first with 80 μg cycloheximide, and after 10 min with 20 μCi ^{3}H-L-isoleucine and 20 μCi ^{3}H-L-phenylalanine. Both portions of insects were sacrificed 7 min after the application of the radioactive amino acids. The lysate of the 10 min 100,000 *g* sediment from the combined homogenates was placed on a sucrose density gradient.
■—■: absorbancy at 260 nm;
□—□: absorbancy at 280 nm;
○—○: ^{14}C-radioactivity;
●—●: ^{3}H-radioactivity;
x- - -x: ratio ^{3}H/^{14}C-radioactivity.

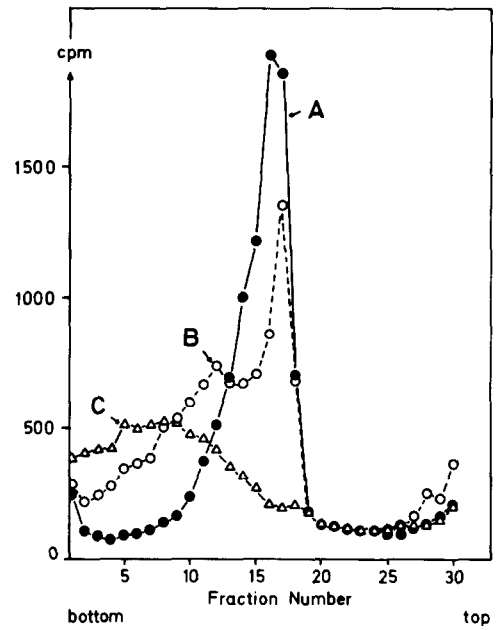

Fig. 2. Influence of concentration of mitochondria during lysis on the distribution of radioactivity in the sucrose density gradient after pulse labelling of isolated mitochondria with radioactive amino acids. After incubation with radioactive amino acids the mitochondrial suspension was divided into three unequal parts. Lysis was performed after centrifugation by adding identical amounts (0.4 ml each) of Triton–AMT to each pellet. The radioactivity of the curves is corrected for the different absolute amounts of mitochondrial protein placed on the gradient.
A) (●—●) 150 mg mitochondrial protein/ml Triton-AMT;
B) (○—○) 80 mg mitochondrial protein/ml Triton-AMT;
C) (△—△) 70 mg mitochondrial protein/ml Triton-AMT.

a medium with or without magnesium ions. Therefore, mitochondrial ribosomes have to be discriminated from a 20–30 fold excess of cytoplasmic ribosomes. In a first approach, conditions were established where mitochondria but not cytoplasmic ribosomes incorporate amino acids into their peptidyl-tRNA.

Selective labelling *in vivo* of nascent peptide chains on mitochondrial ribosomes was achieved by inhibiting cytoplasmic protein synthesis with cycloheximide [11]. This is shown in fig. 1. SMT medium was used for isolation of mitochondria. The distribution of the absorbance at 260 and 280 nm on the gradient shows a main peak at fraction 11 (approx. 80 S).

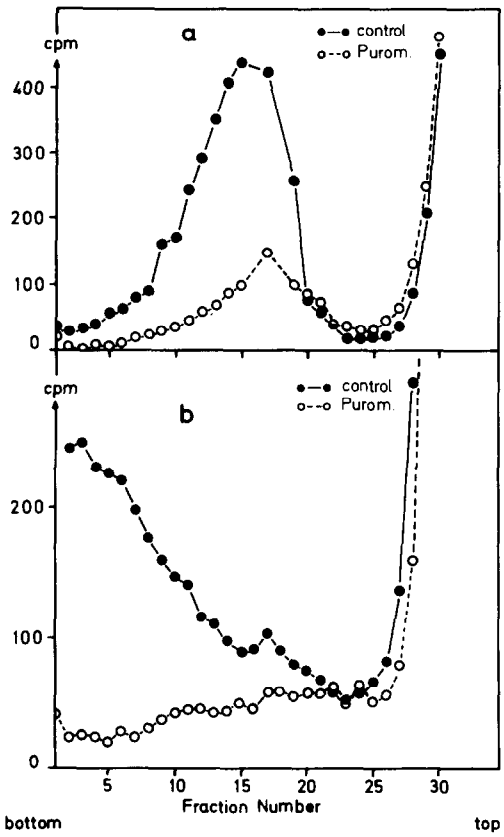

Fig. 3. Effect of puromycin on *in vitro* pulse labelling of mitochondrial ribosomal particles with radioactive amino acids. In two separate experiments (a and b), mitochondria were incubated with radioactive amino acids for 7 min, then divided into two equal portions. To one portion puromycin (final concentration 0.47 mM) was immediately added and both portions were allowed to stand for another 3 min at 30°. They were then cooled to 0° and after lysis placed on the density gradients. In (a) the concentration of mitochondrial protein during lysis was 110 mg/ml Triton–AMT, in (b) 60 mg/ml Triton–AMT.

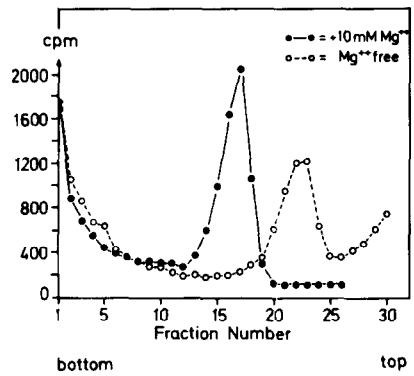

Fig. 4. Influence of magnesium ions in the sucrose density gradient on the distribution of radioactivity after pulse labelling *in vitro*. Mitochondria were incubated in one batch with radioactive amino acids. Lysis was performed at a concentration of 200 mg mitochondrial protein per ml Triton–AMT. After lysis one half was placed on a sucrose density gradient containing AMT; the other half was placed on an identical gradient except that the 0.01 M $MgCl_2$ was replaced by 0.0005 M EDTA.

Without cycloheximide the radioactive label (^{14}C-radioactivity) coincides with the absorbance. It serves as a control. The peaks at fraction 11 are attributed to cytoplasmic monoribosomes. In the presence of cycloheximide (^{3}H-radioactivity) the label in fraction 11 is greatly diminished (about 96% inhibition taking into account the 8-fold higher ^{3}H-radioactivity applied to the insects, see legend to fig. 1). A second peak of ^{3}H-amino acid label appears in fractions 16–18. This is also demonstrated by the ^{3}H/^{14}C-ratios and represents cycloheximide insensitive labelling. Using monomers and subunits of *Neurospora cytoplasmic* ribosomes (77 S, 60 S and 37 S, respectively [12]) for calibration of the gradient, fraction 17 corresponds to a sedimentation constant of 60 S.

For selective labelling *in vitro* isolated mitochondria (SMT medium) were incubated with radioactive amino acids. In contrast to the *in vivo* labelling in the absence of cycloheximide (^{14}C-radioactivity in fig. 1), the radioactivity in these experiments (fig. 2) does not coincide with the absorbance pattern (fig. 1). The distribution of the radioactivity on the gradient depends on the concentration of mitochondria during lysis. This observation is detailed below. At the highest concentration, one single peak at fractions 16–17 is seen (curve A in fig. 2). The specific radioactivity of the peak fraction on a protein basis surmounts the specific radioactivity of whole mitochondria by a factor of 20–50.

Preincubation of isolated mitochondria with chloramphenicol, a specific inhibitor of mitochondrial

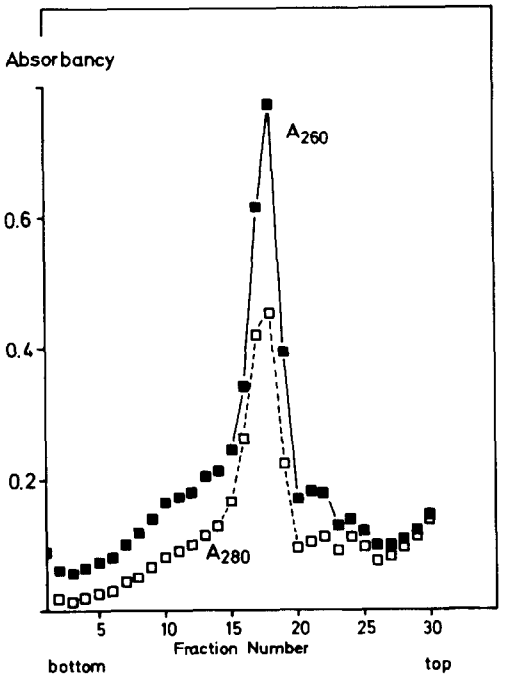

Fig. 5. Absorbancy profiles (260 and 280 nm) of a sucrose density gradient. Ribosomal particles were prepared from mitochondria freed of cytoplasmic ribosomes and added to the gradient.

protein synthesis, results in a 50% decrease of the radioactivity found on the gradient. This partial inhibition by chloramphenicol is in agreement with earlier results with isolated mitochondria [13, 14].

By adding puromycin after pulse labelling of isolated mitochondria [15], the radioactivity is greatly diminished (fig. 3a). The release by puromycin indicates that the radioactive amino acids are present in peptidyl-tRNA chains.

When the concentration of mitochondria during lysis is lowered, a second peak appears at fraction 12 (curve B in fig. 2). At the lowest concentration used, the peak at fraction 17 disappears almost completely and the radioactivity is distributed in a broad fashion over the lower 15 fractions (curve C in fig. 2). The radioactivity in these fractions is also sensitive to puromycin (see fig. 3b).

These observations suggest that the gradient centrifugation resolves particles of different sizes involved in mitochondrial polypeptide synthesis. It is concluded that the radioactivity peak in fractions 17–18 corresponds to a monomeric form of the mitochondrial ribosome, whereas under certain conditions, such as low concentration of mitochondria during lysis, polymeric forms are obtained. One possible explanation for the influence of protein concentration during lysis on the appearance of polymeric forms is the existence of an endogenous mitochondrial nuclease working more effectively at high concentrations. This hypothesis is supported by the observation that at low concentrations of mitochondria in the lysate, a shift of radioactivity from the lower fractions toward fraction 17 can be achieved by incubating at 37° [16].

The monosome character of the 60 S radioactivity peak is substantiated by the following observation. When a mitochondrial lysate high in protein concentration is centrifuged through a Mg^{2+}-free density gradient, an upward shift of the radioactive peak by 4–5 fractions is observed (see fig. 4). This suggests that dissociation of a 60 S particle into subunits occurred. The radioactivity may represent residual peptidyl-tRNA chains bound to a large subunit of about 45 S.

A further approach toward the problem of characterizing mitochondrial ribosomes was to remove the cytoplasmic ribosomes adhering on the mitochondria. This was achieved by homogenizing the flight muscles and isolating the mitochondria in a medium containing 0.05 M EDTA (see Materials and methods). After this treatment particles could be isolated which show a sharp absorbancy peak (260 and 280 nm) at fraction 18 and two further small peaks at fractions 21 and 24 in the sucrose density gradient (fig. 5). The properties of this nucleoprotein particle with a sedimentation constant of about 60 S are now under study. It is highly suggestive that this particle represents purified mitochondrial monoribosomes.

Acknowledgements

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References

[1] W.Kleinow and W.Neupert, Z. Physiol. Chem. 350 (1969) 1166.
[2] W.Kleinow, W.Sebald, W.Neupert and Th.Bücher, in: Autonomy and Biogenesis of Mitochondria and Chloroplasts, eds. N.K.Boardman, A.W.Linnane and R.M.Smillie (North-Holland, Amsterdam, 1971) in press.
[3] W.Kleinow and W.Neupert, Z. Physiol. Chem. 351 (1970) 1205.
[4] R.F.Swanson and J.B.Dawid, Proc. Natl. Acad. Sci. U.S. 66 (1970) 117.
[5] M.A.Ashwell and T.S.Work, Biochem. Biophys. Res. Commun. 39 (1970) 204.
[6] S.Perlman and S.Penman, Nature 227 (1970) 133.
[7] T.W.O'Brien, D.E.Lowe, A.F.Heishman, Federation Proc. 29 (1970) 913.
[8] V.Bronsert and W.Neupert, in: Regulation of Metabolic Processes in Mitochondria, eds. I.M.Tager, S.Papa, E. Quagliariello and E.C.Slater (Elsevier, Amsterdam, 1966) p. 426.
[9] W.Neupert, Thesis, München, 1968.
[10] H.Noll, Nature 215 (1967) 360.
[11] W.Sebald, Th.Hofstötter, D.Hacker, Th.Bücher, FEBS Letters 2 (1969) 177.
[12] H.Küntzel, J. Mol. Biol. 40 (1969) 315.
[13] E.Wintersberger, Biochem. Z. 341 (1965) 409.
[14] W.Sebald, Th.Bücher, B.Olbrich and F.Kaudewitz FEBS Letters 1 (1968) 235.
[15] W.Neupert, W.Sebald, A.J.Schwab, A.Pfaller, Th.Bücher European J. Biochem. 10 (1969) 585.
[16] W.Kleinow, unpublished results.