The Reversible Dissociation and Activity of Ribosomal Subunits of Liver and Skeletal Muscle from Rats

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Ribosomal subunits were prepared from rat liver and skeletal muscle after incubation with puromycin and treatment at low concentrations of Mg^{2+} . After isolation the resulting subunits could be recombined and the particles effected the synthesis of polyphenylalanine in the presence of polyuridylic acid. Hybrid particles formed from subunits of liver and muscle respectively were also active. The homogeneity of the isolated subunits was checked by polyacrylamide-gel-agarose electrophoresis. The method is shown to be a reliable and comparatively simple way of preparing active ribosomal subunits from skeletal muscle.

Martin and Wool (Martin & Wool, 1968, 1969; Martin, Rolleston, Low & Wool, 1969) were the first to demonstrate that ribosomes from skeletal muscle and liver could be dissociated into subunits and recombined to give particles that were active for the synthesis of polyphenylalanine in the presence of poly U. An essential feature of the method was the use of high concentrations (0.5M) of potassium chloride. An alternative method was later described by Lawford (1969) for the dissociation of liver ribosomes. In this method the ribosomes are first incubated in the presence of puromycin to remove nascent polypeptide chains, after which they can be dissociated without being subjected to high concentrations of potassium chloride.

In the present work the method of Lawford (1969) was applied to ribosomes from rat skeletal muscle and hybrid ribosomes were made with ribosomal subunits from liver. The homogeneity of the subunits was checked both by polyacrylamide-gel-agarose electrophoresis of the particles and of the extracted RNA (Loening, 1968; Dahlberg, Dingman & Peacock, 1969).

EXPERIMENTAL

Materials. Chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, U.K., and were of AnalaR grade wherever possible. L-[U- ^{14}C]Phenylalanine (specific radioactivity 475 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., puromycin dihydrochloride from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A., poly U from Miles Labora-

tories Inc., Kankakee, Ill., U.S.A., sodium tri-isopropyl-naphthalenesulphonate from Eastman Chemical, Kodak Ltd., Kirkby, Lancs., U.K., Sephadex G-25 from Pharmacia Fine Chemicals, Uppsala, Sweden and heparin (sodium salt) from Sigma Chemical Co., St Louis, Mo., U.S.A. Other chemicals were as specified by Campbell, Lowe & Serck-Hanssen (1967).

Animals. Wistar male or female rats of 150-250 g body wt. were used. The rats were starved overnight when subcellular preparations of liver were to be prepared. Non-starved animals were used for subcellular preparations from muscle.

Preparation of polyribosomes. Polyribosomes from liver were prepared as described by Wettstein, Staehelin & Noll (1963). They were suspended in medium A (50 mM-tris-KCl buffer, pH 7.8 at 25°C, 25 mM-KCl, 5 mM-MgSO₄). Polyribosomes of muscle were prepared as described by von der Decken & Omstedt (1970).

Preparation of cell sap from liver. The tissue was minced and homogenized in 2.5 vol. of medium B (50 mM-tris-HCl buffer, pH 7.8 at 25°C, 25 mM-KCl, 10 mM-MgSO₄, 0.15 M-sucrose). The 120 000g-min supernatant was centrifuged at 105 000g_{av.} for 60 min and the supernatant was siphoned off to provide the cell sap. The cell sap was passed through a column (150 mm × 14 mm) of Sephadex G-25 equilibrated with sucrose-free medium B. The column was eluted with the same medium.

Dissociation of polyribosomes into ribosomal subunits. The method with minor modifications was that of Lawford (1969). This involves the incubation of polyribosomes under conditions for protein synthesis by using untreated cell sap in the absence of ^{14}C -labelled amino acids but in the presence of 0.7 mM-puromycin. The ribosome concentration was 1.6 mg/ml as measured on the basis of $E_{1cm}^{1\%}$ 134 at 260 nm. Incubation was carried out in a volume of 10 ml in a 100 ml conical flask for 60 min. The flask was placed in an ice bath and the Mg^{2+} concentration was decreased to 2 mM and the K^+ concentration increased to 100 mM by the addition of 1.5 vol. of a mixture of 50 mM-tris-HCl buffer, pH 7.8 at 25°C, and 0.15 M-KCl. To

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separate the ribosomal subunits 4 ml of the diluted suspension was loaded on to each of six tubes containing 33 ml of a sucrose density gradient [15–35% (w/v) sucrose in 50 mM-tris-HCl buffer, pH 7.8 at 25°C, 0.15 M-KCl, 1 mM-MgSO₄] in the SW 27 rotor of the Beckman L2/65B ultracentrifuge. Centrifugation was for 8 h at 95 100 g_{av.}. The extinction at 260 nm was measured in a Gilford model 2000 recording spectrophotometer by pumping the gradients from the bottom through a flow cell at a constant flow rate of about 1 ml/min. The two subunit peaks were collected and pooled in chilled tubes and 0.5 vol. of medium A was added to each. The two subunits were pelleted by centrifugation for 3 h at 165 000 g_{av.}. Pellets were stored for up to 48 h in the deep freeze.

Amino acid incorporation in vitro. The concentrations of substances in the cell-free system in a final volume of 150 µl were: ATP, 1 mM; GTP, 0.25 mM; phosphoenolpyruvate, 10 mM; pyruvate kinase, 40 µg/ml; [¹⁴C]-phenylalanine (0.4 µCi/ml) 0.84 µM; MgSO₄, 5 mM; KCl, 75 mM; tris-HCl buffer pH 7.8, 50 mM. Also present were cell sap (0.4 mg of protein) and 5–35 µg of ribosomes or subunits. When indicated 80 µg/ml of poly U was added. Incubation was for 30 min at 37°C. Samples (100 µl) were transferred to filter-paper discs and the proteins were precipitated and extracted as described by Mans & Novelli (1961). The radioactivities of the filter-paper discs were counted in a Beckman model LS 200B liquid-scintillation spectrometer, as described by Ragnotti, Lawford & Campbell (1969), at about 65% efficiency.

Polyacrylamide-gel-agarose electrophoresis. Polyacrylamide-gel electrophoresis was in 2.4% acrylamide–0.06% agarose (Loening, 1968; see also Dahlberg *et al.* 1969) in 4 mm × 110 mm tubes for 3 h at 4 mA/tube (4°C). For electrophoresis of subunits the buffer described by Loening (1968) was used, the sodium dodecyl sulphate being omitted. The subunits were suspended in 10% (w/v) sucrose–10 mM-EDTA and an amount equivalent to E_{260} 0.15–0.4 was applied to the gels. After electrophoresis the gels were stained in a solution of 0.25% Coomassie Brilliant Blue–20% (w/v) salicylsulphonic acid–20%

(w/v) trichloroacetic acid. After 1 h at room temperature the gels were destained overnight in 10% (w/v) salicylsulphonic acid. The best colour development was then achieved by keeping the gels for at least 5 h in 10% (w/v) trichloroacetic acid in the dark. A parallel electrophoretic run was stained overnight in 0.2% Toluidine Blue in 40% (v/v) 2-ethoxyethanol and destained in 40% (v/v) 2-ethoxyethanol.

For identification of RNA the subunits were suspended in diluted electrophoresis buffer containing 10% sucrose and 2% sodium dodecyl sulphate. Electrophoresis conditions were as described for subunits but with 0.1% sodium tri-isopropyl-naphthalenesulphonate present in the gels and in the electrophoresis buffer. After electrophoresis the gels were stained in Toluidine Blue–2-ethoxyethanol and destained in 2-ethoxyethanol as described for the subunits.

The gels were scanned in a Joyce-Loebl Chromascan, the 5–045 filter being used.

Reassociation of ribosomal subunits. The subunit pellets were gently resuspended with a glass rod in medium A to give a concentration of 0.1–0.3 mg of ribosomes/ml for the small subunit and 0.2–0.7 mg/ml for the large subunit. The subunit suspensions were incubated either separately or together, under the conditions for protein synthesis.

In this paper the small and large particles obtained from ribosomes are referred to as 40S and 60S subunits.

RESULTS

Polyribosomes obtained from rat liver were dissociated into subunits after incubation with puromycin in the presence of an energy source and cell sap, followed by treatment with low concentrations of Mg²⁺ (Lawford, 1969). The same method was applied to ribosomes obtained from skeletal muscle. After separation in a sucrose density gradient the 40S and 60S subunits were collected

Table 1. *Incorporation of [¹⁴C]phenylalanine by ribosomal subunits from rat liver and muscle*

Incubation was in a final volume of 150 µl. The concentration of the substances was as follows: ATP, 1 mM; GTP, 0.25 mM; phosphoenolpyruvate, 10 mM; pyruvate kinase, 40 µg/ml; [¹⁴C]phenylalanine, 0.4 µCi/ml; MgSO₄, 5 mM; KCl, 75 mM; tris-HCl buffer, pH 7.8, 50 mM. When indicated 80 µg of poly U/ml was added. Cell sap (0.4 mg of protein) was present in all tubes. The type of subunit in each tube is as indicated. In each case an amount corresponding to E_{260} 0.075 of the 40S particle and 0.175 of the 60S particle was present. From the total c.p.m. has been subtracted those when ribosomes were omitted.

Source of subunits		Poly U	Total radioactivity (c.p.m.)
Liver	Muscle		
40S	—	+	56
60S	—	+	32
—	40S	+	91
—	60S	+	82
40S+60S	—	+	750
—	40S+60S	+	712
40S	60S	—	88
40S	60S	+	608
60S	40S	—	91
60S	40S	+	670

Fig. 1. Gel electrophoresis of ribosomal subunits. Subunit preparations were analysed by polyacrylamide-gel-agarose electrophoresis. After staining with Coomassie Brilliant Blue the gels were scanned and the proportional distribution was calculated (see Table 2). (a) 40S subunits from liver, an amount corresponding to E_{260} 0.28 being applied to the gel; (b) 60S subunits from liver, an amount corresponding to E_{260} 0.20 being applied to the gel; (c) 40S subunits from muscle, an amount corresponding to E_{260} 0.24 being applied to the gel; (d) 60S subunits from muscle, an amount corresponding to E_{260} 0.18 being applied to the gel.

Table 2. Homogeneity of subunits obtained from ribosomes of liver and muscle

The proportional distribution of the particles and the RNA present in the subunit preparations was calculated on the basis of the electrophoretic pattern obtained after polyacrylamide-gel-agarose electrophoresis as shown in Figs. 1 and 2. The calculations are based on three to six independent electrophoretic analyses. (a) 150g rats; (b) 250g rats.

Tissue	Subunits analysed	Particle composition (%)				RNA composition (%)			
		80S	60S	40S	Other	28S	18S	>18S	<18S
(a) Liver	40S	3.6	3.1	92.5	0.8	4.2	93.2	0.5	2.1
	60S	6.6	91.6	1.9	—	75.7	1.3	23.1	—
(b) Liver	40S	—	11.7	81.7	6.2	7.9	79.5	2.1	10.8
	60S	3.7	92.5	3.8	—	65.5	3.6	29.7	1.4
(a) Muscle	40S	6.2	12.4	81.2	—	5.5	68.9	—	25.7
	60S	6.8	90.0	3.2	—	53.0	8.2	38.9	—

as a pellet by centrifugation. The recombined subunits obtained from liver were active in incorporating phenylalanine into polypeptide in the presence of poly U as shown in Table 1. Table 1 also shows that similar results may be obtained when the subunits are derived from muscle.

In previous work, in which high concentrations of potassium chloride were used for the dissociation of ribosomes, active particles were formed from the combination of ribosomal subunits from liver and muscle (Martin & Wool, 1969). As shown in Table 1 hybrid particles could also be obtained when the

muscle ribosomes were dissociated by the method of Lawford (1969).

From the results in Table 1 it is apparent that either the 40S or 60S subunits obtained from liver or muscle have a low but significant incorporating activity. The homogeneity of the particles obtained after sucrose-density-gradient centrifugation was therefore tested by means of polyacrylamide-gel-agarose electrophoresis in 1mm-EDTA. The gels were stained for protein with Coomassie Brilliant Blue and the patterns obtained for the various particles are shown in Fig. 1. From the

Fig. 2. Gel electrophoresis of RNA obtained from ribosomal subunits. Subunit preparations were suspended in sodium dodecyl sulphate and analysed for RNA by polyacrylamide-gel-agarose electrophoresis. After staining with Toluidine Blue the gels were scanned and the proportional distribution was calculated (see Table 2). (a) RNA (11 μ g) of 40S subunits from liver; (b) RNA (13 μ g) of 60S subunits from liver; (c) RNA (9 μ g) of 40S subunits from muscle; (d) RNA (11 μ g) of 60S subunits from muscle.

areas under the peaks the homogeneity of the particles could be determined. The results are shown in Table 2. When the particles were stained for RNA with Toluidine Blue identical results were obtained. Unfortunately the 40S subunits tended to spread, making accurate quantitative evaluation more difficult.

As an alternative method for determining the homogeneity of the ribosomal subunits the RNA was released from the proteins in the presence of sodium dodecyl sulphate. The product was analysed by polyacrylamide-gel-agarose electrophoresis. The patterns for the various particles are shown in Fig. 2 and the proportional distribution of RNA is shown in Table 2. The amount of 28S RNA present in the 40S subunits and that of 18S RNA present in the 60S subunits is between 3 and 8% for both muscle and liver. In addition, four to six distinct bands were located between the 28S and 18S RNA when RNA preparations of the 60S subunit were analysed electrophoretically. These bands constantly appeared in the preparations of liver and muscle. It was noted that the RNA from the 40S subunits from muscle contained a considerable amount that moved ahead of the 18S RNA on electrophoresis. In liver the number of additional bands of RNA obtained appeared to increase with the age of the rats. Because these bands may have been derived from the action of

ribonuclease both polyribosomes and subunits from liver were prepared in the presence of heparin (sodium salt) (Palmiter, Christensen & Schimke, 1970). However, this treatment was without effect on the number of additional bands.

DISCUSSION

Although the original method described by Martin & Wool (1968) for the reversible dissociation of animal ribosomes marked a considerable advance, it did involve the treatment of the ribosomes with high concentrations of potassium chloride. Such conditions are known to be likely to cause the release of protein factors important in the formation of polypeptide chains. The method of Lawford (1969) avoids these conditions and it was decided to apply it to muscle. The results obtained show that it is very satisfactory in this respect, giving virtually complete dissociation of muscle ribosomes. The homogeneity of the subunits from muscle was as good as that from liver.

The activity of the reconstituted particles has only been assessed with respect to the synthesis of polyphenylalanine in the presence of poly U. We are aware that this is by no means a fully satisfactory assessment of the protein-synthesizing activity of the ribosomes. However, under these conditions the activity of the reconstituted ribo-

somes from muscle was very similar to that of those from liver. The small activity of the subunits incubated separately could be accounted for by contamination by the corresponding subunit.

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