Two Fractions Required for Cell-Free Protein Synthesis with Components from Rabbit Reticulocytes

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An extract was prepared from rabbit reticulocyte ribosomes after treatment with potassium chloride as described by Miller, Hamada, Yang, Cohen & Schweet (1967). This extract has been shown to convert monoribosomes into polyribosomes during protein synthesis *in vitro* (Cohen, 1968). The nature of this extract was studied in greater detail. Centrifugation of the extract through a sucrose density gradient separated the activity into a fast-sedimenting and a slow-sedimenting fraction. The two fractions were shown to have different functions in stimulating cell-free protein synthesis and their active components were shown to be protein or partly protein in nature. Each fraction was analysed by electrophoresis and in the analytical ultracentrifuge. It was concluded that the active component in the fast-sedimenting fraction had a sedimentation coefficient of 15.5s and that of the slow-sedimenting fraction 10.5s.

The treatment of rabbit reticulocyte ribosomes with high concentrations of potassium chloride removes a fraction that is required in cell-free protein synthesis (Miller, Hamada, Yang, Cohen & Schweet, 1967; Miller & Schweet, 1968; Bonanou, Cox, Higginson & Kanagalingam, 1968; Cohen, 1968). This fraction was shown to convert monoribosomes into polyribosomes during protein synthesis (Cohen, 1968) and the active component or components was shown to be protein and not messenger RNA (Miller *et al.* 1967; Miller & Schweet, 1968; Cohen, 1968).

This paper describes a more detailed analysis of this fraction. Centrifugation through a sucrose gradient showed that the activity separated into two fractions, both of which were shown to contain protein. The two fractions were then analysed by ultracentrifugation. The results show that one fraction consists principally of one component with a sedimentation coefficient of 15.5s. The other fraction contains a component with a coefficient of 10.5s as well as containing haemoglobin.

Results are also presented which suggest that the two fractions perform different functions, since they enhance each other in stimulating protein synthesis in cell-free systems with either potassium chloride-washed ribosomes or monoribosomes derived from polyribosomes.

MATERIALS AND METHODS

Trypsin (3× crystallized) and soya-bean trypsin inhibitor (crystalline) were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Ribonuclease A (bovine

pancreas; 5× crystallized) was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6.

The following were prepared as described previously (Cohen, 1968): KCl-washed ribosomes; monoribosomes derived by incubating purified polyribosomes in the cell-free protein-synthesizing system for 90 min. ('derived monoribosomes'); ribosomal extract as the supernatant extract of ribosomes treated with 0.6M-KCl. To allow layering on sucrose gradients 0.5–0.8 ml. of the ribosomal extract was passed through a 7 cm. × 1 cm. Sephadex G-25 column equilibrated and eluted with 0.1M-KCl–2mm-tris-HCl buffer, pH 7.5, at 22°. All of the red (haemoglobin-containing) eluate was collected (designated 'G-25 extract').

The reaction mixture for the measurement of amino acid incorporation has been described previously (Cohen, 1968), the only difference being that in this work protamine sulphate treatment in the preparation of the enzyme fraction was omitted completely.

Sucrose gradient centrifugation. Samples (1.0–1.5 ml.) of G-25 extract were applied to 19 ml. 5–20% (w/v) sucrose gradients adjusted to 0.1M-KCl–0.5mm-2-mercaptoethanol–2mm-tris-HCl buffer, pH 7.5, at 22°. The gradients were centrifuged for 11–12 hr. at 95000g_{av} in the 3 × 23 ml. swinging bucket rotor of the MSE Superspeed 65 centrifuge. After centrifugation 40 drop fractions (equal to 0.93 ml.) were collected through the bottom of the tubes and assayed for their ability to stimulate KCl-washed ribosomes or derived monoribosomes in the cell-free protein-synthesizing system. It is convenient to mention here that two peaks of activity were observed, designated 'fast fraction' and 'slow fraction' (see Fig. 1).

Concentration of fast and slow fractions. Several samples of ribosomal extract were fractionated into the fast and slow fractions as described above and the most active fractions stored at –15° (in Fig. 1 these would correspond approximately to tubes 11–14 and tubes 18 and 19). The tubes of each were pooled and concentrated by pressure

dialysis against 2×21 of 0.1M-KCl - $0.5\text{mM-2-mercaptoethanol}$ - 0.05M-tris-HCl buffer, pH 7.5, at 20° . Ribosomal extract (12 ml.) yielded 1.3 ml. of fast fraction at a protein concentration of 2 mg./ml. and 0.6 ml. of slow fraction at a concentration of 8 mg./ml.

Analytical centrifugation. The Spinco model E ultracentrifuge was used for examining the homogeneity of preparations and for determining sedimentation coefficients. Where possible schlieren optics were used but when haemoglobin was present u.v. optics had to be used. The runs were carried out at 20° with the $2^\circ 12\text{mm}$. Kel F cell in the AN-D rotor.

Fig. 1. Sucrose gradient centrifugation of G-25 extract. G-25 extract (1 ml.) was centrifuged for 12 hr. and fractionated as described in the Materials and Methods section. A portion (0.08 ml.) of each fraction was added to the cell-free incubation mixture to give a final volume of 0.18 ml. and which contained L - $[^{14}\text{C}]$ valine of specific radioactivity 9.6 c/mole and 0.14 mg. of KCl-washed ribosomes (●) or 0.28 mg. of derived monoribosomes (○). After incubation at 37° for 1 hr. samples (0.1 ml.) were removed for measurement of the amino acid incorporation. The incorporations in controls with the sucrose gradient were 7 and 223 c.p.m. for derived monoribosomes and KCl-washed ribosomes respectively, and these have been subtracted. The highest value in the figure corresponds to a stimulation of incorporation of 0.65 nmole of valine/mg. of KCl-washed ribosomes. Hb, Haemoglobin.

Measurement of ribosomal concentrations. All ribosomal concentrations were estimated by assuming $E_{1\text{cm}}^{1\%}$ 120 at 260 nm.

Measurement of protein concentrations. Protein concentrations were determined by the method of Warburg & Christian (1941).

In the experiments with trypsin and ribonuclease, purified rabbit haemoglobin was added to the fast and slow fractions to give an E_{415} reading of approx. 4. This increased their thermal stability at 37° .

Fig. 2. Effect of trypsin on the slow (a) and fast (b) fractions of ribosomal extract. G-25 extract was fractionated and assayed as described in the legend to Fig. 1. Samples (0.06 ml.) of the fast peak or slow peak tubes along with $0.033\text{ }\mu\text{g.}$ of trypsin (○) in a final volume of 0.07 ml. were incubated at 37° . After the times indicated 0.01 ml. of soya-bean trypsin inhibitor at a concentration of 1 mg./ml. was added and the tubes were chilled. Potassium chloride-washed ribosomes (0.125 mg.), L - $[^{14}\text{C}]$ valine of specific radioactivity 12 c/mole and the other components of the cell-free incubation mixture were added to give a final volume of 0.18 ml., and the tubes reincubated at 37° for 1 hr. Samples (0.1 ml.) were removed and the incorporation was measured. Appropriate controls are indicated in the figure: ●, trypsin omitted; Δ, trypsin and trypsin inhibitor omitted; ---, KCl-washed ribosomes alone present. The highest value in the figure corresponds to an incorporation of 0.88 nmole of valine/mg. of ribosomes.

Table 1. *Effect of fast and slow fractions on protein synthesis by derived monoribosomes and potassium chloride-washed ribosomes*

Samples (0.18 ml.) of the standard amino acid-incorporation mixture were incubated at 37° for 1 hr. In addition, the reaction mixture contained L-[¹⁴C]valine of specific radioactivity 9.6 c/mole. In Expts. 1 and 2 0.28 mg. of derived monoribosomes was added and in Expts. 3 and 4 0.14 mg. of KCl-washed ribosomes. Other additions are indicated in the table. *I*, incorporation; *S*, stimulation expressed as nmole of valine incorporated/mg. of ribosomes.

Expt. no.	...	...	1		2		3		4	
Additions to system			<i>I</i>	<i>S</i>	<i>I</i>	<i>S</i>	<i>I</i>	<i>S</i>	<i>I</i>	<i>S</i>
None			0.032		0.021		0.391		0.208	
Fast fraction (0.02 ml.)			0.102	0.070	0.124	0.103	0.449	0.058	0.328	0.120
Slow fraction (0.06 ml.)			0.241	0.209	0.069	0.048	0.656	0.265	0.352	0.144
Both			0.400	0.368	0.258	0.237	0.831	0.440	0.759	0.551
Fast fraction (0.04 ml.)			0.128	0.096	0.131	0.110	0.562	0.171	0.430	0.222
Slow fraction (0.04 ml.)			0.205	0.173	0.059	0.038	0.555	0.164	0.280	0.072
Both			0.414	0.382	0.329	0.308	0.828	0.437	0.785	0.577
Fast fraction (0.06 ml.)			0.137	0.105	0.174	0.153	0.600	0.209	0.447	0.239
Slow fraction (0.02 ml.)			0.156	0.124	0.047	0.026	0.493	0.102	0.204	-0.004
Both			0.367	0.335	0.363	0.342	0.743	0.352	0.645	0.437

RESULTS

Sucrose-density-gradient centrifugation of Sephadex G-25 extract. Sephadex G-25 extract was sedimented through a sucrose gradient, fractions were collected and assayed for their ability to stimulate amino acid incorporation in cell-free systems with either KCl-washed ribosomes or derived monoribosomes (Fig. 1). The activity was resolved into two fractions designated 'fast' and 'slow' on the basis of their relative sedimentation rates. Apart from those fractions containing haemoglobin, none of the fractions had any significant extinction above background.

To determine whether the fast and slow fractions performed the same role in stimulating protein synthesis, they were assayed together and separately in the cell-free system with either derived monoribosomes or KCl-washed ribosomes (Table 1). Although each fraction alone stimulated incorporation, a marked enhancement was observed when the two fractions were mixed. This enhancement suggests that the fractions perform distinct and complementary functions.

Preincubation of fast and slow fractions with trypsin. Both the slow and fast fractions were preincubated with trypsin and the tryptic activity was stopped by the addition of trypsin inhibitor. The fractions along with controls were then assayed in the cell-free system with potassium chloride-washed ribosomes (Fig. 2). The stimulatory activity of both the fast and slow fractions was destroyed by trypsin. Thus the nature of the activity in each fraction is probably protein or partly protein.

Preincubation of fast and slow fractions with

ribonuclease. The two fractions were incubated with a low activity of ribonuclease and then assayed in the cell-free system with potassium chloride-washed ribosomes (Fig. 3). With both fractions the amino acid incorporation was not significantly decreased by preincubation with ribonuclease. This suggests that RNA is not an active component of either fraction.

Analytical centrifugation. (a) Schlieren optics. The concentrated fast fraction was analysed by ultracentrifugation (Fig. 4). One main component was observed with a sedimentation coefficient of 15.5 s (the average value of two determinations). The sharpness of the peak decreased during the run. This might have been caused by degradation during the run.

(b) U.v. optics. The concentrated slow fraction was shown to contain two main components, one of which had a sedimentation coefficient of 10.5 s. The coefficient of the other component was 4 s, which is the coefficient of haemoglobin (Fig. 5).

DISCUSSION

It is clear that the ribosomal extract described previously (Miller *et al.* 1967; Miller & Schweet, 1968; Bonanou *et al.* 1968; Cohen, 1968) can be fractionated by sucrose gradient centrifugation into a fast-sedimenting and a slow-sedimenting fraction. The treatment of these fractions with ribonuclease or trypsin suggests that the activity is due to protein. This is supported by the absorption spectra, which show little or no nucleic acid to be present (unpublished results).

One way of interpreting the result of analysis of

Fig. 3. Effect of ribonuclease on the slow (a) and fast (b) fractions of ribosomal extract. G-25 was fractionated and assayed as described in the legend to Fig. 1. Samples (0.06 ml.) of the fast peak or slow peak tubes were incubated at 37° with $0.02 \mu\text{g.}$ of ribonuclease in a final volume of 0.08 ml. (○). After the times indicated the tubes were chilled and the components of the cell-free incubation mixture added along with 0.125 mg. of KCl-washed ribosomes and L- ^{14}C valine of specific radioactivity 12 c/mole to give a final volume of 0.18 ml. The tubes were reincubated at 37° for 1 hr. Samples (0.1 ml.) were removed and the incorporation was measured. Appropriate controls are indicated in the figure: ●, ribonuclease omitted; ----, KCl-washed ribosomes alone present. The highest value in the figure corresponds to an incorporation of 0.59 nmole of valine/mg. of ribosomes.

the two fractions in the ultracentrifuge is to assume that the fast fraction consists of one large component of 15.5s, which possibly dissociates into two during centrifugation. This is supported by the fact that the fast fraction can be resolved into two components by starch-gel electrophoresis (unpublished result). Alternatively it might be that the fast fraction consists of two components of different electrophoretic mobilities but similar sedimentation coefficients. Centrifugation showed the slow fraction to contain, as well as haemoglobin, another component of 10.5s. This component was not detectable by starch-gel electrophoresis but was

Fig. 5. Analytical centrifugation of the slow fraction. Concentrated slow fraction was diluted with buffer to 1 mg./ml. and centrifuged at 59780 rev./min. Photographs were taken every 8 min., starting approximately when the top speed was reached. The photographs were scanned in a Joyce-Loebl microdensitometer. The tracings shown show the boundary distributions after 16 and 32 min. Sedimentation is from left to right.

Fig. 4. Analytical centrifugation of the fast fraction. Fast fraction concentrated to 2 mg./ml. was centrifuged at 44770 rev./min. Photographs were taken every 8 min., starting approximately when the top speed was reached: (a) 8, (b) 16 and (c) 32 min. Sedimentation is from right to left.

evident with polyacrylamide (unpublished result). It is unfortunate that at present there is no way of correlating the components observed in these analyses with biological activity so that the possibility exists that none of them has any activity. However, it may be tentatively concluded that the two active components in ribosomal extract are protein in nature and have sedimentation coefficients of 10·5s and 15·5s.

Crude ribosomal extract converts monoribosomes into polyribosomes during protein synthesis *in vitro* (Cohen, 1968). The specific role of each of the two fractions described here has not yet been determined, but since they act synergistically in stimulating cell-free systems containing potassium chloride-washed or derived monoribosomes, it would appear that they have separate functions. Three protein factors have been isolated from *Escherichia coli* ribosomes after treatment with a high concentration

of ammonium chloride (Iwasaki, Sabol, Wahba & Ochoa, 1968). These were shown to stimulate polypeptide chain initiation and it might be supposed that the two fractions described here have similar functions.

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REFERENCES

- Bonanou, S., Cox, R. A., Higginson, B. & Kanagalingam, K. (1968). *Biochem. J.* **110**, 87.
Cohen, B. B. (1968). *Biochem. J.* **110**, 231.
Iwasaki, K., Sabol, S., Wahba, A. J. & Ochoa, S. (1968). *Arch. Biochem. Biophys.* **125**, 542.
Miller, R., Hamada, K., Yang, P. C., Cohen, B. & Schweet, (1967). *Fed. Proc.* **26**, 1125.
Miller, R. L., & Schweet, R. (1968). *Arch. Biochem. Biophys.* **125**, 632.
Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.