Association of Nascent Polypeptide with 30S Ribosomal Subunits

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1. Crude extracts of *Escherichia coli* were used to synthesize nascent peptides under the direction of endogenous mRNA and in the presence of radioactive amino acids. Analysis of such extracts by sucrose-gradient centrifugation in low Mg^{2+} concentration has shown that after 2 min of incubation approximately 14% of the total labelled protein recovered on the gradient, in association with whole ribosomes, sediments with 30S ribosomal subunits; this value rises to approximately 24% after 30 min of incubation. The labelled protein associated with 30S ribosomal subunits is insoluble in hot trichloroacetic acid. 2. Similar results were also obtained in extracts that synthesized polypeptides under the direction of either of the synthetic polyribonucleotides poly(A) or poly(A,G,C,U). In contrast, however, analysis of crude extracts programmed in protein synthesis by poly(U) has indicated that under these conditions 30S ribosomal subunits have no associated polyphenylalanine; similarly there is little associated peptide after programming of extracts by poly(U,C).

Early studies on protein synthesis, programmed by poly(U), in extracts of *Escherichia coli*, showed that those ribosomes actively synthesizing polyphenylalanine readily dissociated into their constituent subunits when exposed to low Mg^{2+} concentration (0.1 mM). Under these conditions all nascent polyphenylalanine remains tightly associated with 50S ribosomal subunits (Gilbert, 1963). In contrast, when endogenous mRNA is the template for protein synthesis most nascent polypeptide remains associated with a class of 70S ribosomes ('active' ribosomes) resistant to dissociation in low Mg^{2+} concentration (Tissières *et al.*, 1960). A small fraction of the nascent protein is localized on both 50S and 30S ribosomal subunits under these conditions, but amounts bound have been difficult to quantitate because of poor resolution of the particles (Schlessinger & Gros, 1963). Attempts to dissociate 'active' ribosomes by extensive dialysis against low Mg^{2+} concentration have also been unsatisfactory since this treatment released many of the nascent polypeptide chains previously bound to ribosomes (Schlessinger & Gros, 1963).

In the present work we have successfully dissociated, in low Mg^{2+} concentration, a large proportion of the 'active' ribosomes present in *E. coli* extracts programmed in protein synthesis by endogenous mRNA. Further, by using isokinetic sucrose

gradients we have obtained a clearer separation of the various ribosome types and have been able to analyse more precisely the distribution of nascent peptides between these particles. We have also examined the distribution where different mRNA species were used to direct protein synthesis. Our results suggest that association of nascent peptides with both ribosomal subunits could well be characteristic of cell-free protein synthesis, in *E. coli* extracts, directed by most mRNA species. Nascent peptides containing a large percentage of phenylalanine seem to associate solely with 50S ribosomal subunits.

Materials and Methods

Materials

Cells. *E. coli* B163 (requiring histidine, leucine and methionine) was grown at 37°C as described by Cannon (1967). The cells were harvested, without cooling, during exponential phase (E_{550} , 0.5–0.6) and were washed in 5 mM-Tris-HCl buffer, pH 7.4, containing 10 mM-magnesium acetate. The cells were stored frozen until use.

Crude extracts. These were prepared from frozen cells as described previously (Cannon, 1968) and were adjusted to contain 8 mg of ribosomes/ml.

Methods

Incorporation of amino acids into nascent protein. The composition of reaction mixtures for the incorporation of amino acids into protein has been

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described previously (Cannon, 1968, 1970). Reaction mixtures contained approx. 4mg of ribosomes/ml. For incorporations directed by endogenous mRNA, mixtures were supplemented with ^{14}C -labelled *Chlorella* protein hydrolysate and incubation was at 30°C for 10min before chilling. For incorporations directed by poly(U), poly(A), poly(U,C) and poly(A,G,U,C), reaction mixtures initially contained no radioactive amino acids. After preincubation for 15min at 30°C to degrade natural mRNA the Mg^{2+} concentration was raised to 17.5mM, mixtures were supplemented with extra ATP and phosphoenolpyruvate (Cannon, 1970) and the required polyribonucleotide was added as mRNA, along with the suitable radioactive amino acid (see the figure legends). Incubations were then continued for 10min at 30°C before chilling.

Preparation of ribosomes. After chilling, reaction mixtures (1ml) were diluted with 9ml of ice-cold 5mM-Tris-HCl buffer, pH7.4, containing 10mM-magnesium acetate and 500mM- NH_4Cl , and ribosomes were sedimented by centrifugation at 165000g for 45min. The pellets were rinsed gently in the same buffer and the liquid was discarded. Ribosomes were resuspended in the same buffer and sedimented again and this procedure was repeated once more. Ribosomes were finally resuspended in 1ml of 5mM-Tris-HCl buffer, pH7.4, containing 0.1mM-magnesium acetate (low Mg^{2+} concentration) and were dialysed against this buffer in the cold for 18h. Ribosome suspensions were then centrifuged (10min at 5000g) to remove aggregates and adjusted to a concentration of approx. 2mg/ml before analysis on sucrose gradients.

Sucrose gradients. Ribosome suspensions (1ml) were applied to 15–30% isokinetic sucrose gradients (Noll, 1969) prepared in 5mM-Tris-HCl buffer, pH7.4, containing 0.1mM-magnesium acetate (volume of gradient, 16ml). Centrifugation was for 16.5h at 22500rev./min at 4°C in the Spinco Model L-2 ultracentrifuge in the SW25-3 rotor. Samples (8 drops) were collected (see Cannon *et al.*, 1963), diluted with water (2.5ml) and the absorbancy of the samples recorded at 260nm. Fractions were then mixed with 50% (w/v) trichloroacetic acid (0.5ml). Bovine serum albumin (2 drops, 5mg/ml) was added as carrier and the tubes kept at 90 – 95°C for 15min. After cooling, precipitates were filtered on to 3cm glass-fibre filters (Whatman) and washed three times with 3ml portions of ice-cold 5% (w/v) trichloroacetic acid. The filters were glued to aluminium planchets and counted at approx. 30% efficiency in a gas-flow counter for at least 1000 counts.

Synthetic polyribonucleotides. Poly(U) was obtained from the Sigma (London) Chemical Company, London S.W.6, U.K. Poly(A), poly(A,G,U,C) and poly(U,C) were kindly provided by Dr. W. Gratzer of the Department of Biophysics, King's College, University of London.

Labelled compounds. ^{14}C -labelled *Chlorella* protein hydrolysate (52mCi/matom of carbon) was obtained from the Radiochemical Centre (Amersham, Bucks., U.K.) as were L-[U- ^{14}C]lysine (270mCi/mmol) and L-[U- ^{14}C]phenylalanine (450mCi/mmol).

Results and Discussion

Ribosomes were prepared from extracts incubated with the ATP-generating system and ^{14}C -labelled amino acids. The extent of incorporation was similar to values reported previously (Cannon, 1967) and under the conditions used, in the absence of a preincubation step, all protein synthesis was directed by the endogenous mRNA of the crude extract. Ribosomes were washed thoroughly in buffer containing 10mM-magnesium acetate and 500mM- NH_4Cl , a critical step which ensures removal of any protein associated nonspecifically with ribosomes. Fig. 1 shows an analysis, on gradients run in low Mg^{2+} concentration, of ribosomes from extracts labelled with radioactive amino acids for different time-intervals. After only 2min incubation (Fig. 1a) approx. 14% of the total ribosome-bound radioactive material is associated with 30S ribosomal subunits. Approximately 80% of the labelled nascent peptide recovered on the gradient is ribosome-bound, and much of this protein sediments with the 'active' 70S fraction. After 10min of incubation (Fig. 1b) more label has been incorporated into protein. Approximately 77% of the recovered counts are ribosome-bound and of these counts 17% are associated with 30S ribosomal subunits. The 'active' 70S fraction is less evident than in Fig. 1(a) and a marked radioactive peak sediments at 50S. After 30min of incubation (Fig. 1c) there has been little additional protein synthesis beyond the 10min incubation period but the 'active' 70S fraction apparently dissociates more easily in low Mg^{2+} concentration, into its constituent subunits. Approximately 73% of the recovered label is ribosome-bound and of the counts bound 24% are associated with 30S ribosomal subunits.

Ribosomes carrying natural nascent peptides have been reported to resist dissociation in low Mg^{2+} concentration (Tissières *et al.*, 1960). Schlessinger & Gros (1963) attempted to overcome this problem by dialysing ribosomes against buffer containing 0.05mM- Mg^{2+} . However, although all 'active' ribosomes appeared to dissociate, approx. 80% of the original ribosome-bound radioactivity was released from the particles. In our experiments (Fig. 1c) we have obtained an effective dissociation of 'active' ribosomes by dialysis against buffer containing 0.1mM- Mg^{2+} . Our calculation that approx. 24% of the total counts bound to ribosomes are associated with 30S ribosomal subunits under the analytical conditions used

Fig. 1. Distribution of nascent peptides between ribosomal subunits; effect of incubation time

Crude extract (1.5 ml) was mixed with $3 \mu\text{Ci}$ of ^{14}C -labelled *Chlorella* protein hydrolysate and the ATP-generating system in a total volume of 3 ml as described in the Materials and Methods section. The mixture was subdivided into $3 \times 1 \text{ ml}$ portions and incubation was at 30°C for 2, 10 and 30 min respectively. Ribosomes, prepared as described in the Materials and Methods section, were analysed on sucrose gradients in low Mg^{2+} concentration. (a) shows 2 min incubation; (b) 10 min incubation and (c) 30 min incubation. $\bullet$, E_{260} ; $\blacksquare$, ^{14}C radioactivity (c.p.m.).

must be nearly representative of the true subunit distribution. It is impossible to estimate such a distribution when many of the nascent peptides are associated with 'active' 70S ribosomes which resist dissociation (Fig. 1a). The rapid appearance and high proportion of labelled peptide associated, in low Mg^{2+} concentration, with ribosomal subunits suggest that this material is nascent protein. The increasing proportion on ribosomal subunits of the total bound counts with increasing incubation times reflects the increased tendency towards dissociation of 'active' 70S ribosomes from which, presumably, the labelled ribosomal subunits derive. The bound counts do not differ significantly if ice-cold 10% (w/v) trichloroacetic acid is substituted for hot trichloroacetic acid in the precipitation and washing procedure. Further, a similar distribution of radioactivity is observed if extracts are labelled with a single amino acid instead of the complete protein hydrolysate.

The association of nascent peptide with 30S ribosomal subunits is of particular interest. The early work of Gilbert (1963) showed that in *E. coli* extracts synthesizing protein as directed by poly(U), polyphenylalanine chains associate solely with 50S ribosomal subunits and we have repeated these experiments (Fig. 2). In low Mg^{2+} concentration ribosomes carrying polyphenylalanine appear as 50S and 30S subunits and polyphenylalanine associates entirely

with the former. Few, if any, 'active' 70S ribosomes appear on the gradient profile.

We have examined protein synthesis in extracts programmed by each of the three synthetic mRNA species poly(U,C), poly(A) and poly(A,G,U,C). Fig. 3(a) shows that for the extract programmed by poly(U,C) nascent protein sediments mainly with the 50S ribosomal subunit and only a small shoulder in the count profile represents nascent peptide on the 30S ribosomal subunit (cf. Figs. 1 and 2). Figs. 3(b) and 3(c) represent gradient analyses of ribosomes from extracts programmed in protein synthesis by poly(A) and poly(A,G,U,C) respectively. In both cases dialysis in low Mg^{2+} concentration has removed a fairly large percentage (approx. 37%) of nascent protein from ribosomes (cf. Figs. 1 and 2). The nascent peptides that remain ribosome-bound are associated with an 'active' 70S region and with both 50S and 30S ribosomal subunits. It can be calculated that approx. 16% of the total ribosome-bound polylysine (Fig. 3b) and approx. 14% of the ribosome-bound nascent peptides produced under the direction of poly(A,G,U,C) (Fig. 3c) associate with 30S ribosomal subunits (cf. Fig. 1). In both these experiments, however, since there are 'active' ribosomes present the proportion of the total label, on 30S ribosomal subunits, may well be higher than the values quoted since dissociation of those ribosomes remaining in the

Fig. 2. Phenylalanine incorporation directed by poly(U)

Crude extract (0.5ml) was incubated at 30°C with the ATP-generating system in a total volume of 1 ml for 15 min as described in the Materials and Methods section. The Mg^{2+} concentration was raised to 17.5 mM and the mixture was supplemented with extra ATP and phosphoenolpyruvate and with 50 μ g of poly(U) and [^{14}C]phenylalanine (1.25 μ Ci). Incubation was continued for 10 min before chilling and dilution as described under Fig. 1. Ribosomes were prepared and analysed as described under Fig. 1. The result shown illustrates the sedimentation profile of the nascent polyphenylalanine formed in the extract. ●, E_{260} ; ■, ^{14}C radioactivity (c.p.m.).

'active' 70S form could well lead to additional 30S subunit labelling.

From our results it appears that peptidyl-tRNA rich in phenylalanine may well have particular affinity for some part of the larger ribosomal subunit whilst having no corresponding affinity for the smaller subunit (Figs. 2 and 3a). It seems possible that association of nascent peptides with 30S ribosomal subunits could be characteristic of protein synthesis directed by most mRNA species.

In our experiments with endogenous mRNA we do not know why we have obtained a more effective dissociation of 'active' ribosomes in low Mg^{2+} concentration relative to earlier work (see Schlessinger & Gros, 1963, for references). Conceivably, our routine washing of ribosomes in 0.5M-NH₄Cl may inadvertently aid the dissociation. However, the experiments of Figs. 3(b) and 3(c) suggest perhaps that ease of dissociation of 'active' ribosomes may depend upon the nature of the associated peptidyl-tRNA.

Some of our results are similar to those of Phillips (1966) who studied protein synthesis on reticulocyte ribosomes. Under conditions where all 80S particles dissociate into subunits, approximately one-half of the labelled nascent peptide originally associated with these particles is released; the remaining label remains bound to both ribosomal subunits. Phillips (1966) claims that the larger nascent polypeptide chains are bound to the larger ribosomal subunit. We have not determined the size of the peptide chains synthesized under the conditions of our experiments. However, the peptide chains associated with both ribosomal subunits are of sufficient size to remain precipitable in hot trichloroacetic acid.

Fig. 3. Amino acid incorporations directed by poly(U,C), poly(A) and poly(A,G,U,C)

A preincubated crude extract (final vol. 3 ml) was prepared as described under Fig. 2; samples (1 ml) were incubated for 10 min at 30°C with (a) 50 μ g of poly(U,C) and [^{14}C]phenylalanine (1.25 μ Ci), (b) 100 μ g of poly(A) and [^{14}C]lysine (0.5 μ Ci), (c) 25 μ g of poly(A,G,U,C) and ^{14}C -labelled *Chlorella* protein hydrolysate (0.5 μ Ci). Ribosomes were processed and analysed as described for Fig. 2. ●, E_{260} ; ■, ^{14}C radioactivity (c.p.m.).

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