Association of Nascent Polypeptide and Transfer Ribonucleic Acid with 30S Ribosomal Subunits

By M. AMIN A. MIRZA,* MICHAEL CANNON and MARGARET L. M. ANDERSON†

*Department of Biochemistry, King's College, University of London,
Strand, London WC2R 2LS, U.K.*

(Received 1 May 1973)

1. Crude extracts of *Escherichia coli* programmed in protein synthesis by endogenous mRNA have incorporated amino acids into protein. Analysis of such extracts by sucrose-gradient centrifugation in low Mg^{2+} concentration has revealed that 30S ribosomal subunits carry associated radioactive material of which a considerable proportion can be removed from ribosomes by treatment of pre-labelled extracts with puromycin. 2. Gradient analyses of incorporations carried out in the additional presence of added ^{32}P -labelled tRNA have indicated that tRNA sediments in the regions of the newly synthesized nascent protein and that both labels are associated with all ribosomal components detected on the gradients under the experimental conditions employed. 3. 30S ribosomal subunits carrying both ^{32}P and ^{14}C labels have been isolated, disrupted with sodium dodecyl sulphate, and analysed by chromatography on Sephadex G-200 columns. Both labels elute closely together and well away from a tRNA marker analysed under identical conditions. 4. It is proposed that 30S ribosomal subunits, isolated from extracts which have synthesized nascent peptides under the direction of endogenous mRNA, carry associated peptidyl-tRNA.

Early work showed that polyphenylalanine chains, synthesized by extracts of *Escherichia coli* programmed by poly(U), are covalently linked to tRNA molecules; these complexes are bound to the 50S subunit of the 70S ribosome (Gilbert, 1963). Similar observations have been made with *E. coli* extracts synthesizing nascent protein as directed by endogenous mRNA, where again peptidyl-tRNA becomes associated with the larger ribosomal subunit (Cannon, 1967). We have demonstrated (Cannon *et al.*, 1973) by careful analysis of *E. coli* extracts programmed in protein synthesis by a variety of mRNA species, that nascent peptides may be found also in association with 30S ribosomal subunits. This confirms earlier suspicions of such an association (Schlessinger & Gros, 1963; Cannon, 1967) and agrees also with work involving the reticulocyte cell-free protein synthesizing system (Phillips, 1966).

In the present work we have shown that many of these nascent peptides, which become associated with 30S ribosomal subunits can react with puromycin under certain conditions. In addition, by analysing incorporation mixtures supplemented with ^{32}P -labelled tRNA we have attempted to find out if these nascent peptides are linked covalently to tRNA and are bound to 30S ribosomal subunits as peptidyl-tRNA.

* Present address: Syntex Institute of Molecular Biology, Syntex Research, 3401, Hillview Avenue, Palo Alto, Calif. 94304, U.S.A.

† Present address: Imperial Cancer Research Fund, Burtonhole Lane, Mill Hill, London N.W.7, U.K.

Materials and Methods

Methods

Conditions for preparation of cells and extracts and for incorporation of amino acids into protein are described by Cannon *et al.* (1973). All incorporations were directed by the endogenous mRNA of the extract.

Treatment of incorporation mixtures with puromycin. After incubating incorporation mixtures for 10 min, puromycin (0.1 mM) was added along with unlabelled amino acids. Incubation was continued for 5 min before chilling. Puromycin dihydrochloride was obtained from the Nutritional Biochemicals Corporation (Cleveland, Ohio, U.S.A.).

Preparation of ^{32}P -labelled tRNA. Whole cells of *E. coli* grown in the presence of [^{32}P]orthophosphate (Cannon, 1967) were extracted with phenol to isolate transfer RNA (tRNA) as described by Cannon & Richards (1967). The product (2 mg) was freed of 5S RNA on a Sephadex G-100 column (210 cm $\times$ 1.8 cm diam.) by elution with 15 mM-sodium citrate–150 mM-NaCl (500 ml), fractions (4 ml) were collected. Samples (20 μ l) of each fraction were assayed for radioactivity to locate the 4S peak. Fractions containing tRNA were pooled and freeze-dried. The specific radioactivity of tRNA was approx. 10^8 c.p.m./mg of tRNA.

Preparation of ribosomal subunits carrying ^{14}C -labelled nascent peptides and ^{32}P -labelled tRNA. Amino acid incorporation mixtures were prepared (Cannon *et al.*, 1973) with the further addition of ^{32}P -labelled tRNA (250 000 c.p.m./ml of incorpora-

tion mixture). Ribosomes were isolated and washed as described in the figure legends, and analysed on sucrose gradients as previously described (Cannon *et al.*, 1973). Radioactivity was determined by precipitation and washing with ice-cold 5% (w/v) trichloroacetic acid. Samples containing both ^{14}C and ^{32}P labels were first assayed to determine total counts. Planchets were then covered with filter-paper discs (Whatman 3MM) and re-counted. The filters absorbed approx. 99% of ^{14}C radioactivity and 17% of ^{32}P radioactivity. Corrections were made to estimate the amounts of each isotope in the samples.

Isolation and characterization of double-labelled material from 30S subunits. Ribosomes carrying ^{14}C -labelled nascent peptides and ^{32}P -labelled tRNA were analysed on sucrose gradients in low Mg^{2+} concentration (0.1 mM-magnesium acetate). Subunit peaks were located by measuring absorbance of samples at 260 nm and fractions corresponding to 30S ribosomal subunits were pooled and freeze-dried. The residue was dissolved in 5 mM-Tris-HCl buffer, pH 7.4 (1 ml), made 2% with respect to sodium dodecyl sulphate and kept at room temperature for 30 min. The sample (from approx. 600 μg of 30S ribosomal subunits) was loaded on a Sephadex G-200 column (30 cm $\times$ 1.5 cm diam.) previously equilibrated with 10 mM-Tris-HCl buffer, pH 7.4, containing 100 mM-NaCl and 0.25% sodium dodecyl sulphate, and eluted with this buffer; fractions (1 ml) were col-

lected. Samples were prepared for assay of radioactivity by precipitation and washing in ice-cold 10% (w/v) trichloroacetic acid.

Materials

^{14}C -labelled *Chlorella* protein hydrolysate (52 mCi/atom of carbon) was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.).

Results and Discussion

Fig. 1 shows an analysis, on gradients run in low Mg^{2+} concentration, of ribosomes from extracts labelled with radioactive amino acids. As expected (Cannon, 1967) the particles are present almost entirely as 50S and 30S subunits and the resolution in this experiment is excellent. Approximately 80% of the labelled nascent protein recovered on the gradient remains associated with ribosomes and a large fraction of this protein sediments with 50S and 30S subunits. Of the total nascent protein bound to ribosomes approx. 12% is associated with the 30S subunit.

Fig. 1(b) shows a gradient analysis of an experiment in which an extract, after synthesizing protein under the direction of endogenous mRNA, was incubated with puromycin. At the time of addition of the puromycin, the reaction mixture was supplemented with unlabelled amino acids as described in the figure

Fig. 1. Amino acid incorporation directed by endogenous mRNA and the effect of puromycin treatment

Crude extract (0.5 ml) was mixed with 1 μCi of ^{14}C -labelled *Chlorella* protein hydrolysate and the ATP-generating system in a total volume of 1 ml as described in the Materials and Methods section. The mixture was incubated for 10 min at 30°C. Ribosomes, prepared as described in the Materials and Methods section were analysed on sucrose gradients in low Mg^{2+} concentration. The result shown in (a) illustrates the sedimentation profile of the nascent protein formed in the extract; (b) shows the effect of puromycin on a prelabelled extract. The extract was incubated for 10 min as described above, puromycin (0.1 mM) was added along with a mixture of 18 common amino acids (500 μg total) and the incubation continued for 5 min before chilling and treatment as for (a) above. ●, E_{260} ; ■, ${}^{14}\text{C}$ radioactivity (c.p.m.).

legend. This 'chase' procedure ensures that any new polypeptides synthesized during incubation with puromycin have negligible specific activity. Puromycin removes approx. 80% of the nascent protein associated with ribosomes at the time of its addition. The remaining 20% is associated entirely with the 50S and 30S subunits. This result is similar to one obtained by Cannon (1967) but has hitherto not been further investigated. Relative to the total radioactive counts associated with ribosomes in the control (Fig. 1a) approx. 7.5% remains associated with 30S ribosomal subunits after puromycin treatment. In addition the specific radioactivity of the 50S and 30S ribosomal subunits is approximately equal after puromycin treatment. The counts remaining on the ribosomes are on a population of particles which dissociate readily into subunits upon exposure to low Mg^{2+} concentration. Thus after puromycin treatment it is not necessary to dialyse ribosome populations to ensure that the bound counts sediment with the subunits. It suffices to suspend the ribosomes in low Mg^{2+} concentration and analyse them on gradients immediately. A further point of interest in Fig. 1 concerns the prominent heavy shoulder on the 50S subunit. We are of the opinion that this represents undissociated 70S ribosomes, presumably 'active'.

If extracts synthesizing polyphenylalanine as directed by poly(U) are incubated with puromycin a different result from the one shown in Fig. 1 is ob-

tained. Puromycin removes approx. 96% of the nascent chains formerly associated with ribosomes, and the 4% that remains sediments almost entirely with the 50S ribosomal subunit in low Mg^{2+} concentration.

We have carried out experiments to determine if the radioactive peptide on 30S ribosomal subunits is linked covalently to tRNA (Gilbert, 1963). Ribosomes, from crude extracts incubated with radioactive amino acids and ^{32}P -labelled tRNA were washed in buffer containing 10mM-magnesium acetate and 500mM- NH_4Cl , and were then analysed on a sucrose gradient as illustrated in Fig. 2. The tRNA sediments in the region of the newly-synthesized nascent protein and is associated with all ribosomal particles, including the 30S ribosomal subunit, as are nascent peptides. Since the two components follow each other so closely, it seems likely that they are linked covalently as peptidyl-tRNA.

In the original incubation for the experiment of Fig. 2 approx. 10% of the input ^{32}P -labelled tRNA becomes bound to those ribosomes not engaged in protein synthesis (Cannon *et al.*, 1963). This binding requires a high Mg^{2+} concentration (0.01M) and 1 mol of tRNA is bound/mol of 70S ribosome in a tight but non-specific association. However, the binding is weak in the presence of K^+ , or NH_4^+ (Cannon, 1967) and washing in NH_4Cl (Fig. 2) decreases the amount of tRNA bound. A second binding reaction involving

Fig. 2. Analysis on sucrose gradients of ribosomes carrying ^{14}C -labelled nascent peptides and ^{32}P -labelled tRNA

Crude extract (1 ml) was mixed with $2\mu Ci$ of ^{14}C -labelled *Chlorella* protein hydrolysate, ^{32}P -labelled tRNA (approx. 500000 c.p.m.) and the ATP-generating system in a total volume of 2 ml as described in the Materials and Methods section. Ribosomes were isolated and washed and then analysed on sucrose gradients as described under Fig. 1. ●, E_{260} ; ■, ^{14}C radioactivity (c.p.m.); ○, ^{32}P radioactivity (c.p.m.).

Fig. 3. Chromatography on Sephadex G-200 of radioactive material isolated from 30S ribosomal subunits

30S ribosomal subunits carrying ^{14}C -labelled nascent peptides and ^{32}P -labelled tRNA were isolated and treated with sodium dodecyl sulphate as described in the Materials and Methods section. A sample (1 ml) was analysed by chromatography on Sephadex G-200 as described in the Materials and Methods section. The elution position of a sample of the ^{32}P -labelled tRNA used in all the experiments is plotted (arbitrary units). The arrows indicate where two protein markers, (1) bovine serum albumin and (2) haemoglobin, elute when chromatographed under the same conditions. ■, ^{14}C radioactivity (c.p.m.); ○, ^{32}P radioactivity (c.p.m.); ----, elution position of marker ^{32}P -labelled tRNA.

tRNA takes place during protein synthesis in crude extracts. The 'active' ribosomes involved in peptide-bond formation (Tissires *et al.*, 1960) carry nascent chains linked covalently at their carboxy-termini to tRNA molecules (Cannon *et al.*, 1963). The two binding reactions can be easily distinguished. When the Mg^{2+} concentration is lowered to 0.1 mM, as in the experiments of Fig. 2 before gradient analysis, all tRNA bound non-specifically dissociates from ribosomes although 'active' ribosomes still carry associated peptidyl-tRNA under these conditions (Cannon *et al.*, 1963). In the present work, however, we have shown that even when dissociation of 'active' ribosomes is induced much tRNA and nascent peptide remains associated with ribosomal subunits. It seems reasonable to conclude therefore that any tRNA remaining in association with ribosomes, treated under our conditions, must be bound as peptidyl-tRNA.

This interpretation is substantiated by our experiments illustrated in Fig. 3. The 30S ribosomal subunits carrying ^{14}C -labelled nascent peptides and ^{32}P -labelled tRNA were broken up with sodium dodecyl sulphate and analysed on Sephadex G-200 (Gilbert, 1963). The pore size of this gel allows tRNA to be retarded although ribosomal RNA

leaves the column with the last of the void volume. Both radioactive labels follow each other very closely on the column and elute in a position well clear of a sample of ^{32}P -labelled tRNA run as a marker under identical experimental conditions. Much of the material elutes between molecular-weight markers of 62500 (bovine serum albumin) and 32000 (haemoglobin) suggesting that the peptide is a heterogeneous mixture of different chain lengths. The Sephadex column provides a test for the association of the ^{32}P and ^{14}C labels, and the results obtained, in conjunction with those of Fig. 2, argue strongly that the two labels isolated from 30S ribosomal subunits represent tRNA and nascent peptide linked covalently as peptidyl-tRNA.

During protein synthesis each incoming amino acyl-tRNA is selected by mRNA and binds to the ribosomal A site, which is a complex one, probably overlapping between both ribosomal subunits. The amino acyl-tRNA then reacts with peptidyl-tRNA bound at the ribosomal P site, which is located mainly, if not entirely, on the larger ribosomal subunit (Cannon, 1967). The resultant peptidyl-tRNA is bound at the A site and must be translocated from this site to the P site to allow binding to the former of the next aminoacyl-tRNA. Therefore at any stage of protein synthesis all ribosome-bound peptidyl-tRNA will be distributed between A and P sites, possibly equally (Cannon, 1968). The ribosomal P site is well characterized for both tRNA and peptidyl-tRNA binding (Cannon *et al.*, 1963; Gilbert, 1963; Cannon, 1967). Little is known, however, about A site binding for peptidyl-tRNA.

Ribosomes carrying polyphenylalanyl-tRNA dissociate easily in low Mg^{2+} concentration (Cannon *et al.*, 1973) and all polyphenylalanyl-tRNA is associated with 50S ribosomal subunits (Gilbert, 1963). It has not been determined whether or not polyphenylalanyl-tRNA binds to a single site on the subunit. Certain types of peptidyl-tRNA, however, can travel with 30S ribosomal subunits upon dissociation of the 70S particle. Thus for Fig. 2 approx. 16% of the total ribosome-bound nascent peptide associates with the smaller subunit. In Fig. 2, however, the distribution of ^{32}P -labelled tRNA is quite different since the comparable value for binding is about 32%. If 1 mol of tRNA is bound/mol of active ribosome and each tRNA carries a protein chain (Gilbert, 1963; Cannon *et al.*, 1963), then 32% represents also the number of protein chains associated with 30S ribosomal subunits relative to 100% chains bound to total ribosomes. The difference in the amounts of ^{14}C radioactivity can be explained in two ways. Peptides on the 30S ribosomal subunit may have shorter chain lengths than those bound elsewhere, an interpretation favoured by Phillips (1966) from work with the reticulocyte cell-free system. Alternatively, the chains could have a wide range of sizes

but may have incorporated little radioactivity during the actual incubation period. This could happen if a given ribosome carried a damaged piece of mRNA and incorporated few amino acids into the protein it was synthesizing. This idea is attractive since our extracts, prepared by alumina grinding and having few if any polyribosomes, are likely to contain ribosomes attached to fragmented pieces of mRNA. The results of Fig. 3 might also support this view, since the elution profile of peptidyl-tRNA on Sephadex G-200 indicates a whole range of sizes for the protein associated with tRNA.

Puromycin strips growing peptide chains from ribosome-bound peptidyl-tRNA associated with the ribosomal P site, and when added to crude extracts actively synthesizing protein (Fig. 1*b*) it released only 80% of the nascent protein, the remainder being associated with 50S and 30S subunits upon analysis of dissociated ribosomes. Since the puromycin reaction is carried out in the presence of excess of unlabelled amino acids the label associated with subunits is not short-chain peptidyl-tRNA synthesized during puromycin treatment, and presumably represents peptidyl-tRNA bound at the ribosomal A site from where it has not moved during incubation with the drug. Before puromycin treatment, ribosomes have approx. 12% of their nascent protein associated with 30S subunits in low Mg^{2+} concentration, although this value is an underestimate because there are undissociated 'active' 70S ribosomes present (Fig. 1*a*). After puromycin treatment the absolute amount of nascent protein associated with 30S ribosomal subunits is approx. 7% relative to that bound to total ribosomes in the control. Thus much of the material that associates potentially with 30S ribosomal subunits in the control can react with puromycin and is presumably biologically active.

Some peptidyl-tRNA may remain blocked in the ribosomal A site when ribosomes reach the end of a fragmented mRNA (see above), as the absence of the next intact mRNA codon would preclude selection of the next amino acyl-tRNA. When such ribosomes dissociate some peptidyl-tRNA could travel with the

smaller subunit. This interpretation seems reasonable as the A site is undoubtedly complex and probably overlaps both ribosomal subunits and it could explain our results (Fig. 1*b*) where peptidyl-tRNA, presumably derived from the A site, distributes itself between both subunits, selection being determined by several factors, including chain length and/or amino acid composition of the peptide. For example, polyphenylalanyl-tRNA never associates with 30S subunits because polyphenylalanine may have strong affinity for the 50S subparticle. In Fig. 2, where approx. 32% of the total ribosome-bound peptidyl-tRNA chains associate in low Mg^{2+} concentration with 30S ribosomal subunits, these may all derive from the ribosomal A site and travel with the 30S sub-particle because of the nature of their peptide. Of the total ribosome-bound peptidyl-tRNA some may be blocked at the A site but most is probably biologically active (see above). This value of 32% is high, however, so we cannot exclude the possibility that some peptidyl-tRNA originally bound at the ribosomal P site could travel with 30S subunits when ribosomes dissociate, selection again being determined by the nature of the peptide itself.

In some of these experiments Mrs. Susan Smith has rendered expert technical assistance. Part of this work was aided by a research grant from the Wellcome Trust awarded to M. A. A. M.

References

- Cannon, M. (1967) *Biochem. J.* **104**, 934-946
- Cannon, M. (1968) *Eur. J. Biochem.* **7**, 137-145
- Cannon, M. & Richards, E. G. (1967) *Biochem. J.* **103**, 23c-25c
- Cannon, M., Krug, R. & Gilbert, W. (1963) *J. Mol. Biol.* **7**, 360-378
- Cannon, M., Mirza, M. A. A. & Anderson, M. L. M. (1973) *Biochem. J.* **136**, 859-863
- Gilbert, W. (1963) *J. Mol. Biol.* **6**, 389-403
- Phillips, G. R. (1966) *J. Mol. Biol.* **15**, 587-599
- Schlessinger, D. & Gros, F. (1963) *J. Mol. Biol.* **7**, 350-359
- Tissières, A., Schlessinger, D. & Gros, F. (1960) *Proc. Nat. Acad. Sci. U.S.A.* **46**, 1450-1463