Differences between Newly Formed and Recycled Free Small Ribosome Subunits in Liver Cytoplasm

By RICHARD H. HINTON and BARBARA M. MULLOCK

Wolfson Bioanalytical Centre, University of Surrey, Guildford GU2 5XH, Surrey, U.K.

(Received 3 December 1975)

Heterogeneity among the free small ribosomal subunits of rat liver was studied. Newly made free small subunits differ from the bulk of the free subunits in the following ways. (1) The newly made free small subunits sediment slightly faster than most free small subunits and form a band at slightly lower densities. (2) Mature free small subunits, prepared under conditions which minimize the amount of loosely bound protein, readily dimerize in solutions of low ionic strength. Little dimerization is found with newly formed subunits. This permits the separation of fractions enriched in newly formed subunits. (3) The fraction enriched in newly formed subunits contains a distinctive additional protein as compared with most of the free small subunits.

The small ribosome subunits found free in rat liver cytoplasm appear to be heterogeneous as judged by sedimentation rate (Mullock *et al.*, 1971; Mullock & Hinton, 1974) and by isopycnic banding density (Henshaw & Loebenstein, 1970; Sugano *et al.*, 1971). When the native small subunits were separated by isopycnic banding, the bulk of the small subunits were separated into two fractions with densities of 1.45 and 1.53 g/ml respectively; newly formed subunits formed a distinct band at a density of 1.49 g/ml. Sugano *et al.* (1971) believe that the band at density 1.45 g/ml may be an artifact owing to the presence of adsorbed protein, but Henshaw and his colleagues have argued that a similar low-density band of free small subunits from Ehrlich ascites cells represents small subunits with specifically bound initiation factors (Henshaw *et al.*, 1973; Hirsch *et al.*, 1973; Ayuso-Parilla *et al.*, 1973*a,b*; Smith & Henshaw, 1975). However, in mouse L cells, Perry & Kelley (1968) found no heterogeneity among mature free small ribosome subunits, although they did demonstrate that newly formed subunits band at a lower density than do mature free small ribosome subunits (1.41 and 1.45 g/ml respectively).

Various explanations have been advanced for the heterogeneity among free small ribosome subunits. The differences in density between mature and newly formed subunits suggest that small ribosome subunits are released into the cytoplasm carrying additional protein. However, the small ribosome subunits in reticulocytes have also been shown to be heterogeneous (Baglioni *et al.*, 1969) and this heterogeneity has been shown to be due to the complexing of the small subunit with initiator tRNA and initiation factors (Baglioni, 1972; Darnbrough *et al.*, 1973). Small ribosome subunits separated after incubation

of polyribosomes in the presence of puromycin and 0.88M-KCl have also been found to differ in properties, depending on whether or not they contain deacylated tRNA (Wettenhall *et al.*, 1973). Free complexes of mRNA and small ribosome subunits have been reported by Zehavi-Willner & Danon (1973). Further confusion may be caused by the presence, free in the cytoplasm, of informosomes containing mRNA (Olsen *et al.*, 1972; Jacobs-Lorena & Baglioni, 1972; Buckingham & Gros, 1975), by the non-specific binding of cytosol protein to ribonucleoprotein particles during isolation (Warner & Pene, 1966) and by possible differences between free and membrane-bound small ribosome subunits (Hulse & Wettstein, 1972).

For liver free small ribosome subunits, one may exclude explanations involving the presence of complexes containing mRNA. Very little poly(A)-containing RNA is found in liver ribonucleoprotein particles sedimenting at about 40S, indicating that free informosomes or complexes between mRNA and small ribosome subunits are not present in a significant concentration (Mullock & Hinton, 1975). Loosely bound proteins may be removed by treatment with iso-osmotic NaCl or KCl (Warner & Pene, 1966; Olsnes, 1971), and explanations based on differences between free and membrane-bound subunits may be tested by examining preparations of small subunits prepared with and without the use of detergents. In the present paper we demonstrate that, if prepared under conditions in which contamination by loosely bound protein is minimized, the majority of rat liver free small ribosome subunits form a single band on CsCl-gradient centrifugation, but that newly formed small subunits form a band at a lower density and appear to possess a distinctive additional protein.

Materials and Methods

[6-¹⁴C]Orotic acid and [³H]orotic acid were obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Native small ribosome subunits were separated from the livers of rats fed *ad libitum* by centrifugation in a B-XIV zonal rotor as described by Mullock & Hinton (1975). The gradient solutions contained 5 mM-triethanolamine/HCl, pH 7.8, and 1 mM-MgCl₂. The starting material was either a 12000 g/10 min supernatant or purified ribonucleoprotein particles prepared by treating such a supernatant with Triton X-100 and then either pelleting through 1 M-sucrose in the presence of 0.15 M-KCl (Mullock & Hinton, 1975) or precipitating the particles with 0.05 M-MgCl₂ by the method of Leitin & Lerman (1969). The region containing the small ribosome subunits was identified from the *E*₂₆₀ trace obtained during displacement of the gradient and was concentrated by filtration through a Diaflo PM30 membrane (Amicon Ltd., High Wycombe, Bucks., U.K.).

Further fractionation of the small subunits

Small ribosome subunits separated as described above were further fractionated by rate sedimentation either in a B-XIV zonal rotor or in a 3 × 23 ml swing-out rotor (MSE Ltd., Crawley, Sussex, U.K.) or by isopycnic banding in CsCl gradients after fixing with formaldehyde (Perry & Kelley, 1968).

Re-fractionation of the small-subunit fraction in the B-XIV zonal rotor was carried out in exactly the same way as the initial separation. Rate sedimentation in the tubes of the swing-out rotor was performed essentially as described by Mullock *et al.* (1971). For this, 16 ml gradients of 0.5–1.0 M-sucrose were prepared by using an MSE small-tube gradient maker. The gradient solutions contained 5 mM-Tris/HCl, pH 7.4, 1 mM-MgCl₂ and, in some cases, 0.15 M-KCl. A portion (0.5 ml) of the concentrated particles was layered on each gradient. Treatment with KCl was carried out by the addition of an appropriate volume of the concentrated solution of the reagent to the particle suspension and incubating for about 5 min at 0°C before layering on to the gradient. Centrifugation was carried out in an MSE Super-Speed 65 ultracentrifuge, which had been modified to permit slow acceleration of the rotor. After centrifugation, the rotor was decelerated without use of the brake. The gradients were fractionated by upward displacement by using an MSE gradient-displacement device. The *E*₂₆₀ was monitored by using a Unicam SP.500 spectrophotometer, and fractions of equal volume (about 0.5 ml) were collected. The fractions were diluted with 1.5 ml of water and counted for radioactivity by using either a Triton X-100-based scintillant, which contained butyl-PBD [5-(4-biphenyl)-2-(4-*t*-butylphenyl)-1-oxa-3,4-diazole] as sole scintillant

(Dobrota & Hinton, 1973) or a scintillation mixture containing the neutral detergent Synperonic NXP (I.C.I. Ltd., Billingham, Cleveland, U.K.), PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] (Wood *et al.*, 1975). Counts were corrected for quenching of ³H radioactivity in macromolecules or small particles by the gradient material (Dobrota & Hinton, 1973).

Isopycnic banding

Immediately after concentration, particles were fixed by the addition of sufficient 28% (v/v) formaldehyde dissolved in 0.02 M-triethanolamine/HCl/0.05 M-KCl/1 mM-MgCl₂ (Perry & Kelley, 1966) to give a final formaldehyde concentration of 6% (v/v). After fixation for 18 h at 0°C, excess of formaldehyde was removed by dialysis for 24 h against 0.01 M-triethanolamine/HCl (pH 7.8)/0.05 M-KCl/1 mM-MgCl₂. Isopycnic density-gradient centrifugation was carried out by layering a 0.5 ml portion of the fixed particles over 2.5 ml of 54% (w/w) CsCl and 2.5 ml of 38% (w/w) CsCl. A linear gradient formed by diffusion during centrifugation. Both CsCl solutions were prepared in 0.01 M-triethanolamine/HCl (pH 7.8)/0.01 M-NaCl/0.001 M-MgCl₂/0.8% Brij 58 (Baltimore & Huang, 1969). Centrifugation was for at least 14 h at 50000 rev./min in the tubes of an MSE 3 × 6.5 ml swing-out rotor. The rotor was slowed without the brake. Fractions of equal volume (about 0.15 ml) were collected and prepared for scintillation counting as described above. Before dilution, the volume of every fifth fraction was measured and a 0.05 ml portion removed for measurement of the refractive index in an Abbé refractometer (Billingham and Stanley Ltd., London N.15, U.K.).

Separation of proteins of subribosomal particles

Ribonucleoprotein particles were precipitated from fractions separated by zonal centrifugation by the addition of MgCl₂ to a final concentration of 8 mM and of ethanol to a final concentration of 40% (v/v). After storage overnight at -20°C, the precipitates were collected by low-speed centrifugation, and redissolved in a mixture containing 0.06 M-Tris/HCl, pH 6.7, 0.1% 2-mercaptoethanol, 1% (w/v) sodium dodecyl sulphate, 10% (v/v) glycerol and about 0.002% Bromophenol Blue. To ensure release of all proteins from the RNA, the solutions were heated for 1 min in a boiling-water bath (Maizel, 1971). The proteins were then analysed by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate-containing gels by using the discontinuous buffer system described by Maizel (1971). Electrophoresis was continued until the Bromophenol Blue reached the base; this meant in practice about 3 h at a current of 2 mA/gel. After electrophoresis the gels were stained overnight with 0.2% Coomassie Brilliant Blue R

(Gurr and Co., High Wycombe, Bucks., U.K.) in 50% (v/v) methanol containing 7% (v/v) acetic acid and then de-stained electrophoretically.

Results

Native small ribosome subunits isolated directly from rat liver post-lysosome supernatant sediment in a broad band; the faster-sedimenting particles contain a slightly lower proportion of rapidly labelled RNA (Fig. 1a; see also Mullock & Hinton, 1975). The addition of 0.15M-KCl to the sample and gradient sharpens the band and removes the shoulder of slowly labelled material (Fig. 1b). An experiment in which a gradient containing 0.15M-KCl was run in parallel with a gradient lacking KCl indicated that treatment with KCl decreases the sedimentation rate of the subunits. If the sedimentation coefficient of the small subunits in the absence of KCl was 45S, then the value in the presence of KCl would be about 40S.

Ribosome subunits separated directly from the post-lysosomal supernatant are contaminated by cytosol proteins. This contamination may be prevented by separating the small subunits from purified

ribonucleoprotein particles prepared either by precipitation with 0.05M-MgCl₂ (Leitin & Lerman, 1969) or by sedimentation through 1M-sucrose containing 0.15M-KCl (Mullock & Hinton, 1975). Re-sedimentation in the absence of KCl of free small subunits separated by zonal centrifugation from either preparation shows there to be considerable dimerization; two peaks are found at about 40S and 57S respectively (Fig. 1c). Both peaks are heterogeneous, rapidly labelled RNA being concentrated on the more rapidly sedimenting side of the peak in each case, but the ¹⁴C/³H ratio is higher in the monomer (40S) peak than in the dimer (57S) peak. If 0.15M-KCl is added to the sample and gradient, the 57S peak is abolished and a pattern very similar to that obtained with native small subunits separated directly from the post-lysosomal supernatant is obtained (Fig. 1d).

The experiments described in the preceding paragraph show that newly made small ribosome subunits differ in their properties from the bulk of the small subunits and that the properties of the latter are influenced by the method of separation. These differences were further explored by measuring the

Fig. 1. Pattern obtained after centrifugation of the small subunit (40S) fraction separated from either (a, b) the 12000 g/10 min supernatant fraction of rat liver by centrifugation in a B-XIV zonal rotor, or (c, d) from rat liver ribonucleoprotein particles precipitated with 0.05M-MgCl₂

The rats were injected with [³H]orotic acid 24h before death and with [¹⁴C]orotic acid 50min before death. The particles were treated as described below and layered over a 0.5–1.0M-sucrose gradient containing 5 mM-Tris/HCl, pH 7.4, and 1 mM-MgCl₂ in the tubes of a 3×23 ml swing-out rotor and centrifuged for 4.5 h at 93 000 g. The gradients in the experiments illustrated in (b) and (d) contained, in addition, 0.15M-KCl. The samples layered on to the gradients were (a) and (c) untreated, or (b) and (d) treated, with KCl to a final concentration of 0.15M. Fractions (0.5 ml) collected from the gradient were counted for radioactivity directly. Hence the radioactivity on the left-hand side of the Figure is due to acid-soluble nucleotides. The movement of particles in the gradients is not directly comparable. —, ¹⁴C; —, ³H.

density of the particles on CsCl gradients. Banding of formaldehyde-fixed particles isolated from whole cytoplasm gives a shoulder at a density of 1.56 g/ml and a broad peak between densities of 1.41 and 1.47 g/ml. Particles containing rapidly labelled RNA are concentrated in the less dense fractions of the main peak (Fig. 2). Small free ribosome subunits separated from preparations of ribonucleoprotein particles made by Mg^{2+} precipitation formed much sharper bands (Fig. 3). Most of the free small subunits banded at a density of 1.55 g/ml, whereas the newly formed subunits banded at a density of 1.52 g/ml. The density of the mature native small ribosome subunits is the same as that found by Hamilton *et al.* (1971) for active small subunits separated from polyribosomes.

The results described in the preceding paragraph suggest that the newly made small ribosome subunits contain more protein than the bulk of the cytoplasmic pool of small subunits, which have been recycled from polyribosomes. The experiment illustrated in Fig. 1(c) showed that, in the absence of extraneous protein and in solutions of low ionic strength, the newly formed subunits dimerize much less readily than do most of the subunits. We therefore decided

Fig. 2. Pattern obtained after isopycnic banding after fixation with 6% (v/v) formaldehyde of particles from the 40S region separated from the 12000 g/10 min supernatant fraction of rats injected with [3H]orotic acid and [^{14}C]orotic acid as in Fig. 1

Centrifugation was for 18 h at 205000 *g* on preformed CsCl gradients in the tubes of an MSE 3 $\times$ 6.5 ml swing-out rotor. Fractions (0.15 ml) were collected, and their density and radioactivity determined as described in the text. ----, ^{14}C ; —, 3H ; — · — · —, density.

Fig. 3. Pattern after isopycnic banding after fixation with 6% (v/v) formaldehyde of particles from the 40S region separated from rat liver ribonucleoprotein particles precipitated with 0.05 M-MgCl₂

The rats were injected 24 h before death with [3H]orotic acid and 50 min before death with [^{14}C]orotic acid. Centrifugation was for 18 h at 205000 *g* on preformed CsCl gradients in the tubes of an MSE 3 $\times$ 6.5 ml swing-out rotor. Fractions (0.15 ml) were collected, and their density and radioactivity determined as described in the text. ----, ^{14}C ; —, 3H ; — · — · —, density (20°C).

to investigate the proteins of the small ribosome subunits separated under these conditions. A zonal B-XIV rotor was used to obtain sufficient material; the separation (Fig. 4) was essentially identical with that obtained with the swing-out rotor illustrated in Fig. 1(c). Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate (Fig. 5) showed that one peak (marked with an arrow on Fig. 5) was very much higher in the non-dimerized subunits than in the dimers. In addition there were small differences apparent in the minor components which are to be found on the left-hand side of Fig. 5. These differences are probably not significant, since proteins which migrate in this position are present in less than one copy per ribosome and do not seem to be genuine ribosomal proteins (King *et al.*, 1971). The suggestion that these proteins are due to contaminants of the small-subunit preparation is strengthened by the peaks being most apparent in fraction (i), in which the total amount of ribosomal subunits, as indicated by the 3H counts in Fig. 4, are very low, and which is the fraction most likely to be contaminated by soluble protein.

The change in the peak marked with an arrow on Fig. 5 is much more significant. The height of the peak in gels (i)–(iii) indicates that it is due to a protein or proteins present in at least one copy per subunit in the non-dimerized subunits, whereas the results in gels (iv)–(vi) show that it is almost absent from the dimerized subunits. The protein could not be detected in small ribosome subunits derived from polyribosomes and is not visible in published electrophoretograms of native small ribosome subunits from

Fig. 4. Preparative subfractionation of particles from the small-subunit (40S) fraction separated from ribonucleoprotein particles prepared by pelleting through 1 M-sucrose in the presence of 0.15M-KCl

Centrifugation was for 4 h at 47000 rev./min in a B-XIV zonal rotor. The shape of the gradient is indicated in the Figure; other conditions were as in the experiment illustrated in Fig. 1(a). Groups of fractions (each 20 ml) were pooled as indicated, and the ribonucleoprotein particles precipitated by 8 mM-MgCl₂ and 40% (v/v) ethanol. ○, ¹⁴C radioactivity; ●, ³H radioactivity; — · — · —, density.

reticulocytes (Martini & Gould, 1971). Comparison with the results of the latter authors would indicate that the mol. wt. of the additional protein found in our experiments was about 24000.

Discussion

Taken together, the results in the present paper indicate that small ribosome subunits that have just entered the cytoplasm differ from free small subunits recycled from polyribosomes in the possession of at least one additional protein. When rats are injected intraperitoneally with labelled orotic acid, labelled 18S RNA is found in free subunits after only 20 min (B. M. Mullock & R. H. Hinton, unpublished work), but even after 45 min labelling, only a small proportion of the newly formed subunits have been incorporated into polyribosomes (Mullock *et al.*, 1971). Similar kinetics of labelling have been found in mouse L cells (Perry & Kelley, 1968). Given that the synthesis of a protein molecule takes several minutes, one may

conclude that 50 min after the injection of a radioactive precursor few of the labelled free small subunits will have been recycled from polyribosomes. On the other hand, 24 h after injection of a labelled precursor, the label will be uniformly spread through all ribonucleoprotein particles. As most free small subunits have been recycled from ribosomes so, 24 h after injection of a radioactive precursor, most of the labelled free small subunits will be those recycled from ribosomes.

In our experiments we found, in agreement with Henshaw & Loebenstein (1970) and Sugano *et al.* (1971), that isolated small subunits containing rapidly labelled RNA banded at lower densities in CsCl gradients than did uniformly labelled subunits. Although McConkey (1974) has argued against there being a strict proportionality between the banding density in CsCl gradients and the proportions of RNA and protein in ribonucleoprotein, we know of no instance where this lack of proportionality has been shown among particles banded on the same gradient.

Fig. 5. Fractionation of proteins of the ribonucleoprotein particles separated in the experiment illustrated in Fig. 4

Electrophoresis was carried out in 10cm-long 13% (w/v) polyacrylamide gels containing 1% sodium dodecyl sulphate. Details of procedures for preparing the gels are given in the text. The gels were stained with Coomassie Brilliant Blue R and destained electrophoretically. After destaining, the gels were scanned with a Vitatron TLD 100 scanner (Fisons Ltd., Loughborough, Leics., U.K.). Migration is from left to right. It should be noted that in gel (v) the scanner has failed to resolve the three components of the first large peak from the left. These could clearly be distinguished in the original. The numbering of the gels refers to the numbering of the regions on Fig. 4. The arrow marks the position of the protein found mainly in the newly synthesized subunits.

The density difference between newly formed and recycled subunits was found whether the subunits were isolated directly from the post-lysosomal fraction or from purified ribonucleoprotein particles. As, in the former case, the membranes were not solubilized by Triton treatment, we conclude that the differences that we observe are not due to differences between free and membrane-bound ribonucleoprotein particles.

When analysed in the presence of 0.15M-KCl, the rapidly labelled subunits were found to sediment more rapidly than the 'uniformly labelled' particles. Rate sedimentation in the absence of KCl gives more confusing results, but with free small subunits separated from purified ribonucleoprotein particles it is clear that there is preferential dimerization of the recycled subunits. The pattern with small subunits isolated directly from the post-lysosomal supernatant is more complicated, but analysis of the specific radioactivity of the 18S RNA (Mullock & Hinton, 1975) after such a separation indicated that the faster-sedimenting subunits contain less of the rapidly labelled RNA. The results may be explained by proposing that there is some dimerization of recycled subunits, but that this is restricted by the attachment of loosely bound proteins under conditions of low ionic strength. Our earlier results did, however, show heterogeneity among small ribosome subunits on re-centrifugation in sucrose gradients, with the more slowly sedimenting fractions being the more rapidly labelled (Mullock *et al.*, 1971; Mullock & Hinton, 1975). It is clear from our present experiments that this was due to partial dimerization of the small subunits. The experiments illustrated in Mullock *et al.* (1971, Fig. 7) therefore illustrate that 20min after injection of labelled orotic acid, radioactivity is restricted to subunits which do not dimerize.

Thus all the results are consistent with the newly formed subunits containing additional proteins which cover the site responsible for dimerization of the small subunits. We would thus argue that, as the newly formed subunits appear from their density to possess additional protein, and as the fraction of purified subunits that is resistant to dimerization is enriched in newly formed subunits, the protein species that is present in much higher concentrations in the non-dimerized subunits than in the dimers is derived from the newly formed subunits.

The present results provide evidence for at least one distinctive protein in the newly formed small ribosomal subunits, but give no direct evidence for its function. In the last few years there have been several reports of heterogeneity among the free small subunits. Henshaw and his colleagues (Henshaw *et al.*, 1973; Hirsch *et al.*, 1973; Ayuso-Parilla *et al.*, 1973a,b; Smith & Henshaw, 1975) presented evidence for a very-low-density ($\rho = 1.40\text{g/ml}$) fraction of free small subunits that is enriched in initiation factors. The

free small subunits were separated after precipitating ribonucleoprotein particles with 0.05 M-MgCl₂, which should minimize contamination by non-specifically bound proteins. In our experiments with liver we could find no evidence for such a light fraction among 40S particles separated from purified ribonucleoprotein particles. The 'smear' of low-density material that we found on banding liver native small subunits separated directly from the post-lysosomal fraction probably corresponds to the peak at $\rho = 1.40$ g/ml found in similar preparations by Henshaw & Loebenstein (1970); in neither case is a significant proportion of the rapidly labelled RNA associated with the low-density material. Hence we would conclude that the extra protein that we find associated with the newly formed subunits in liver is quite different from those that bind to recycled small subunits to give rise to the complex that bands at a density of 1.40 g/ml.

Although most of the proteins of ribosomal subunits are common to ribosomes in all tissues of the body, certain variations have been noted. In particular the amounts of one protein in the small subunit and three in the large subunit have been found to vary between normal and regenerating liver (Schumbuks *et al.*, 1974), and a protein found in the large subunit of kidney ribosomes is not present in the large subunit of liver ribosomes (Kaulenas & Unsworth, 1974). There also seems to be some variation between the proteins of small subunits of free polyribosomes, of membrane-bound polyribosomes and of free small subunits (McConkey & Hauber, 1975). These differences were, however, in the higher-molecular-weight proteins, whereas the additional protein that we find in newly formed small subunits is of relatively low molecular weight. Probably more relevant to the present discussion is the observation that some of the proteins of the nucleolar precursors of ribosome subunits differ from those present in the subunits found in the cytoplasm (Olson *et al.*, 1974; Prestayko *et al.*, 1974). We therefore postulate that the additional protein that we find in the newly formed subunits is a 'remnant' that is removed when the small subunit is first used in protein synthesis. As the newly formed subunits do not dimerize readily, one might speculate that the role of such a protein is to prevent non-specific association with other nucleoprotein particles in the nucleus.

We thank Dr. E. Reid for his interest in this work, and Dr. B. J. Gould and Mrs. K. Cromwell for help in our early attempts at electrophoresis of ribosomal proteins. Mr. B. Shipton advised on the scanning. Financial assistance was provided by the Cancer Research Campaign.

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