Reconstruction of the smaller subparticle of rabbit ribosomes from core-particle and split-protein fractions

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The smaller subparticle of rabbit reticulocyte ribosomes was shown to yield core-particle and split-protein fractions on treatment with 2.5 M-NH₄Cl/61 mM-MgCl₂. The core-particle fraction was inactive in poly(U)-directed polyphenylalanine synthesis, but activity was restored after recombination with the split-protein fraction. Optimum ionic conditions for the reconstruction of active subparticles were found to be 0.75 M-NH₄Cl/19 mM-MgCl₂ at 0°C. Improved extents of reconstruction were obtained when the core-particles were isolated by methods that avoided pelleting. Core-particles isolated from subparticles pretreated with either proteinases or ribonucleases had diminished capacity to become re-activated.

The ability to take bacterial ribosomes apart and to reassemble them has proved invaluable in establishing relationships between the structure and function of individual components (for review see Nomura, 1973; Nomura & Held, 1974). Eukaryotic ribosomes do not respond in the same way as bacterial ribosomes, and the technology for their disassembly and reassembly remains rudimentary. Few of the factors that impede the reassembly of eukaryotic ribosomal subparticles have been identified.

The larger subparticle (L-subparticle) of rabbit ribosomes was shown to yield core-particle and split-protein fractions on treatment with 2.75 M-NH₄Cl/69 mM-MgCl₂ at 0°C (Cox & Greenwell, 1976). The core-particles themselves were inactive in both poly(U)-directed polyphenylalanine synthesis and in the puromycin reaction, but were re-activated after recombination with split-proteins from rabbit (Cox & Greenwell, 1976) or from *Neurospora crassa* (Cox & Greenwell, 1980).

The present paper describes the partial reconstruction of the smaller subparticle (S-subparticle) of rabbit ribosomes from core-particles and split-proteins produced by treatment with 2.5 M-NH₄Cl/61 mM-MgCl₂. The isolated core-particles were not active in poly(U)-directed polyphenylalanine synthesis, but activity was restored by recombination with the split-protein fraction. The extent to which

reconstruction took place depended on both the method used for isolating the core-particles and the medium used for the reconstruction step. The effects of pretreatment of S-subparticles with nucleases and proteinases were also examined. The performance of core-particle and split-protein fractions from pretreated S-subparticles on reconstruction was investigated.

Methods

General methods

U.v. spectrophotometry. Unicam SP.500 and SP.1800 spectrophotometers were used for routine measurements with 1 cm cells (3.0 or 0.5 ml capacity). Concentrations of L-subparticle solutions were measured by using the value $A_{260}^{1\%} = 140$ (Cox & Greenwell, 1976).

Ribosome buffers. The pH of buffer solutions was adjusted to 20°C. 'Storage buffer' for stock solutions of L-subparticles was 0.1 M-NH₄Cl/2 mM-MgCl₂/1 mM-dithiothreitol/15% (v/v) glycerol/20 mM-Tris/HCl, pH 7.6 (Cox *et al.*, 1976). 'Sample buffer' for dilution of L-subparticle and core-particles before assay for polyphenylalanine synthesis was 25 mM-KCl/1 mM-MgCl₂/1 mM-dithiothreitol/0.25 M-sucrose/50 mM-Tris/HCl, pH 7.6. 'Activation buffer' for reconstruction of core-particles (Cox & Greenwell, 1976) was 0.3 M-NH₄Cl/15 mM-MgCl₂/1 mM-dithiothreitol/15% (v/v) glycerol/20 mM-Tris/HCl, pH 7.6. Dialysis buffer for the dialysis of S-subparticles in salt-shock buffer solutions or of reconstructed S-subparticles was 0.3 M-NH₄Cl/7.5 mM-MgCl₂/1 mM-dithiothreitol/

Abbreviations used: S-subparticle, the smaller ribosomal subparticle; S-rRNA, the RNA component of S-subparticles; L-subparticle, the larger ribosomal subparticle; SDS, sodium dodecyl sulphate; RNAase, ribonuclease.

15% (v/v) glycerol/1% aprotinin/20 mM-Tris/HCl, pH 7.6. Salt-shock buffer was 2.5 M-NH₄Cl/63 mM-MgCl₂/1 mM-EDTA/1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6.

Purification of S-subparticles

A BXIV MSE zonal rotor was used for the purification step.

S-subparticles (200 A_{260} units) in storage buffer (20 ml) were loaded on to a 15–45% (w/v) concave sucrose gradient (670 ml) containing 0.3 M-KCl/10 mM-MgCl₂/20 mM-Tris/HCl/1 mM- β -mercaptoethanol, pH 7.6. The sample and overlay displaced 250 ml of cushion. The gradient was centrifuged for 3 h at 45 000 rev./min at 15°C. The first four 40 ml fractions were discarded and 38 fractions (15 ml) were collected. The S-subparticle fraction comprised a single peak. The combined fractions were centrifuged for 17 h at 40 000 rev./min (MSE 8 $\times$ 25 rotor) and the pelleted S-subparticles were resuspended in storage buffer, frozen quickly in a solid-CO₂/ethanol bath and stored at -80°C. Values of $s_{20,w}$ of the RNA moiety of the purified S-subparticles were measured by analytical centrifugation (see below) in 0.025 M-KCl/1 mM-MgCl₂/0.05 M-Tris/HCl, pH 7.6, before and after salt-shock treatment. The sedimentation profile of the RNA moiety was obtained by the addition of SDS (0.5% final concn.) to a sample of the stock solution of S-subparticles. The SDS-treated solution was then diluted with sucrose-free sample buffer to a final concentration of 0.8 A_{260} unit of S-subparticle/ml. A single boundary of $s_{20,w}$ approx. 18S was found for the RNA moiety of non-treated S-subparticles. Salt-shock treatment of S-subparticles led to limited hydrolysis of the RNA moiety, since approx. 70% of S-rRNA sedimented with $s_{20,w}$ of approx. 18S and the rest as approx. 8–12S species.

Isolation of ribosome, polyribosome and enzyme fractions

Preparation of rabbit reticulocyte polyribosomes and supernatant enzyme fractions. These were prepared from phenylhydrazine-treated rabbits as described previously (Cox & Hirst, 1976; Arnstein *et al.*, 1964).

Separation of ribosomal subparticles. The polyribosome preparations were dissociated by incubation at 37°C for 30 min in 0.5 M-KCl/3 mM-MgCl₂/1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6, together with 0.2 mM-GTP and 0.2 mM-puromycin. The subunits were then separated by zonal centrifugation in a sucrose gradient containing 0.3 M-KCl/4 mM-MgCl₂/1 mM-dithiothreitol/1 mM-EDTA/20 mM-Tris/HCl, pH 7.6, as described by Cox & Hirst (1976); the profile of the gradient within the MSE B XIV titanium rotor increased parabolically with radius from 15% to 37% (w/v) sucrose, and

ended with a cushion of 45% (w/v) sucrose. The pooled fractions of subparticles were pelleted by centrifuging at 105 000 g_{av} for 17 h at 5°C (MSE 8 $\times$ 25 ml rotor), resuspended in storage buffer at 250 A_{260} units/ml, rapidly frozen in a solid-CO₂/ethanol bath and stored at -80°C.

Poly(U)-directed polyphenylalanine synthesis

The incorporation of [¹⁴C]phenylalanine (sp. radioactivity 50 mCi/mmol) into acid-insoluble material by L-subparticles in the presence of S-subparticles, supernatant factors etc. at 37°C was measured as described by Cox & Hirst (1976). Samples were generally 50 μ l of a 2.0 A_{260} units/ml solution (i.e. 10 μ g of L-subparticles) in an assay mixture of 0.5 ml. The assay blank, the incorporated radioactivity equivalent to zero synthesis, was determined by omitting the energy source (pyruvate kinase, phosphoenolpyruvate, ATP and GTP) from a complete control assay mixture. Control L-subparticles incorporated 18–54 phenylalanine residues/ribosome during the assay.

Separation of core-particles and split-protein fractions by pelleting

S-subparticles at 250 A_{260} units/ml in storage buffer were mixed at 0°C with salts solution such that the final composition was 2.5 M-NH₄Cl/61 mM-MgCl₂/0.75 mM-EDTA/1 mM-dithiothreitol/3% (w/v) glycerol/20 mM-Tris/HCl, pH 7.6. After 15 min at 0°C, the reaction mixture was centrifuged for 70 min at approx. 0°C and 40 000 rev./min in the MSE 8 $\times$ 10 ml rotor (2 ml portions in 10 ml polycarbonate tubes, g_{max} = 145 000). The supernatant containing the split-proteins was pipetted off, and the pellets were homogenized in a volume of storage buffer equal to the volume of salt-shock mixture, to yield a suspension of core-particles.

Separation of core-particles on short-column preparative sucrose gradients

It was considered desirable to prepare soluble monomeric core-particles for use in reconstruction experiments. This was achieved by ultracentrifugation of the salt-shock mixture through a short-column sucrose gradient, which effected the separation in about the same time as pelleting and allowed recovery of the separated fractions at concentrations suitable for use in reconstruction experiments. The sucrose gradients were prepared in polyallomer tubes for the Beckman SW50.1 6 $\times$ 5 ml swing-out rotor by layering of the following pre-cooled solutions. First a cushion of 1.0 ml of 60% (w/v) sucrose in reconstruction buffer was pipetted in, followed by 0.3 ml of each of 45, 35, 25 and 20% (w/v) sucrose. This last layer was made up in reconstruction buffer when the sample (0.75 ml) contained control S-subparticles, but in salt-shock buffer (2.75 M-NH₄Cl

etc.) when the sample was salt-shocked S-subparticles. The later precaution was necessary to avoid partial reconstruction of the S-subparticle by dilution of the salt-shock mixture (50 A_{260} units/ml) at the top of the gradient. Centrifugation was at 50000 rev./min (120000 $g_{av.}$) and 5°C for 2 h in a Beckman L2-65B ultracentrifuge. This was just sufficient to separate the split proteins from the compact band of core-particles, but not enough to take the latter to the top of the step concave gradient, where zone destabilization was found to occur with subsequent loss of particles into the sucrose cushion. The gradients were pumped out of the tubes (with a Pharmacia P3 peristaltic pump) through an LKB Uvicord II instrument to record percentage transmission at 254 nm, and 150 μ l fractions were collected manually.

Analytical ultracentrifugation

An MSE Centriscan instrument was used for measurements of $s_{20,w}$. The concentration of rRNA or L-subparticles was 25 mg/litre and the sedimenting boundary was followed by means of u.v. optics.

Estimation of protein by precipitation as Coomassie Blue complex with trichloroacetic acid

A simple procedure was developed to measure the concentration of ribosomal proteins dissolved in concentrated solutions of NH_4Cl or urea. The sample (0–10 μ g of protein) was diluted to 200 μ l in a 1.5 ml polypropylene micro-centrifuge tube, and was then mixed with 500 μ l of a saturated solution of Coomassie Blue R250 in 15% (w/v) trichloroacetic acid (this reagent was made by stirring 50 mg of the dyestuff in 100 ml of the acid solution and filtering out the undissolved solid). The mixture was kept at room temperature for 15 min or more, then centrifuged in a micro-centrifuge (Jobling 320) at approx. 10000 g for 3 min. The supernatant was removed, the pellet surface and tube were rinsed with 3 $\times$ 1.0 ml of 15% (w/v) trichloroacetic acid, and the pellet was dissolved in 1.0 ml of a solution containing 1% SDS and 1% $NaHCO_3$ in ethanol/water (1:1, v/v). The A_{570} was then measured (generally between 0.05 and 1.20) to estimate the protein content by comparison with standard samples containing 0–10 μ g of cytochrome *c*. The standards were adjusted to approximately the same concentration of NH_4Cl or urea as the sample solutions.

Analysis of ribosomal proteins by two-dimensional polyacrylamide-gel electrophoresis

Ribosomal proteins were recovered from subparticles and split-protein fractions by acetic acid extraction and trichloroacetic acid precipitation respectively, as described by Cox & Greenwell (1976). The samples, dissolved in 8 M-urea/10 mM-

2-mercaptoethanol, were then analysed in the gel system used by Cox & Greenwell (1976), in which the first dimension was a 4% (w/v) polyacrylamide tube gel, pH 5.5 (Knopf *et al.*, 1975), and the second dimension was an 18% (w/v) polyacrylamide slab, pH 4.5 (Kaltschmidt & Wittmann, 1970). The slabs were stained overnight in 500 ml of 0.0125% Coomassie Blue R250 in methanol/acetic acid/water (81:15:104, by vol.), and excess stain was allowed to diffuse into 500 ml of the same solvent.

Polyacrylamide-gel electrophoresis of ribosomal proteins in the presence of SDS

Ribosomal proteins were separated according to molecular size in the discontinuous-buffer system of Laemmli (1970). The separating gel contained 12.5% (w/v) acrylamide and 0.1% SDS in a vertical slab 10 cm high. The stacking gel (1 cm high) contained 5% (w/v) acrylamide. Ribosomal proteins isolated as precipitates or urea solutions (see above) were dissolved or diluted 5-fold in the sample buffer, which consisted of 0.001% (w/v) Bromophenol Blue/2.0% (w/v) SDS/10.0% (v/v) glycerol/0.1 M-dithiothreitol/80 mM-Tris/HCl, pH 6.8. The sample mixtures (50–100 μ l) were heated at 100°C for 2 min, cooled, and then electrophoresed into the gel at 50 V for the first 1 h, and at 100 V until the tracking dye reached the bottom (approx. 3.5 h). The electrode buffer was 0.1% (w/v) SDS/192 mM-glycine/25 mM-Tris, pH 8.3. Each gel slab was stained overnight in 250 ml of methanol/acetic acid/water (81:15:104, by vol.) with 0.0125% (w/v) Coomassie Blue R250, and destained by diffusion in 250 ml of the same solvent. The apparent molecular weights of the ribosomal proteins were estimated by comparison of their mobilities with those of a mixture of standard marker proteins. The calibration curve obtained by plotting the logarithms of the molecular weights of the standards against their migration distances was drawn as a shallow sigmoid curve, not a straight line (cf. Neville, 1971).

Materials

Materials were as described by Cox & Greenwell (1976). Molecular-weight marker proteins were from Sigma Chemical Co., Poole, Dorset, U.K. Trypsin (type XI), soya-bean trypsin inhibitor and RNAase I were all obtained from Sigma.

Results

Effect of salt-shock treatment on the activity of S-subparticles in polyphenylalanine synthesis

The first experiments were designed to find the conditions needed to produce core-particles that were inactive but which were re-activated after

recombination with split-proteins. The ionic conditions explored were similar to those used in the partial reconstruction of L-subparticles (Cox & Greenwell, 1976, 1980). The experiments were based on the notion that the native rRNA conformation is essential for ribosome function and that it is maintained over a wide range of NH_4Cl (or KCl) concentrations provided that the molar ratio $\text{Mg}^{2+}/\text{NH}_4^+$ (or K^+) is maintained at (or greater than) 1/40 (Cox & Hirst, 1976). At sufficiently high concentrations of NH_4Cl , proteins are removed from ribosomal subparticles.

Earlier work (Cox & Hirst, 1976) revealed that the activity of rabbit S-subparticles in poly(U)-directed polyphenylalanine synthesis is diminished after treatment at 0°C with 2–3 M- NH_4Cl /50–75 mM- MgCl_2 . After salt-shock for 2 h at 0°C the mixtures were diluted 15-fold into sample buffer and assayed for the ability to synthesize polyphenylalanine in the standard cell-free system. This procedure detected only those changes in S-subparticles that were not reversed during dilution or subsequent storage at 0°C or during the assay at 37°C . It was found that 50% of activity was retained after treatment with 3 M- NH_4Cl /75 mM- MgCl_2 (Cox & Hirst, 1976).

The results presented in Fig. 1 show the activity of core-particles isolated by differential centrifuging after 15 min at 0°C from salt-shock mixtures covering the range 0.1–3 M- NH_4Cl /2.5–75 mM- MgCl_2 . The capacity of core-particles to function in polyphenylalanine synthesis diminished after salt-shock treatment covering the range 1.8–2.5 M- NH_4Cl /61 mM- MgCl_2 . Samples of the salt-shock solutions were also dialysed against dialysis buffer to test the capacity of core-particles and split-proteins to recombine. S-subparticles active in polyphenylalanine synthesis were readily obtained from salt-shock solutions either by dialysis (Fig. 1) or simply by diluting out the excess salt as described previously (Cox & Hirst, 1976).

Comparison of the effects of salt-shock on S- and L-subparticles

S-subparticles were found to be slightly more sensitive to salt-shock than L-subparticles. S-subparticle cores were produced over the range 1.75 M- NH_4Cl /45 mM- MgCl_2 to 2.5 M- NH_4Cl /57 mM- MgCl_2 (Fig. 1), whereas L-subparticle cores were produced over the range 2 M- NH_4Cl /50 mM- MgCl_2 to 2.5 M- NH_4Cl /63 mM- MgCl_2 (Cox & Greenwell, 1976). The proportion of proteins removed by treatment with 2.5 M- NH_4Cl /63 mM- MgCl_2 was 30% from S-subparticles, compared with 17% from L-subparticles. However, S-subparticles were more readily reconstructed by dialysis (Fig. 1) or by dilution of salt-shock mixtures than were L-subparticles (Cox & Greenwell, 1976).

Fig. 1. Activity in polyphenylalanine synthesis of high-salt core-particles and of reaction mixtures of salt-shocked S-subparticles

The results of at least eight independent experiments are summarized. S-subparticles (20 A_{260} units/ml) were treated for 15 min at 0°C with 0.1–3 M- NH_4Cl /2–75 mM- MgCl_2 /15% (v/v) glycerol/1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6. The molar ratio $\text{Mg}^{2+}/\text{NH}_4^+$ was kept at 1:40. Samples were then centrifuged for 70 min at 40000 rev./min (MSE 10×10 titanium rotor) to separate core-particles from split-proteins. The pellets of core-particles were resuspended in sample buffer, and diluted with sample buffer (final concentration 8 A_{260} units/ml), kept at 37°C for 15 min and assayed in the standard cell-free system. Another sample of the salt-shock mixture was dialysed at 4°C overnight against dialysis buffer, diluted to 2 A_{260} units/ml with sample buffer, heated to 37°C for 15 min and assayed in the standard cell-free system. The control samples incorporated 3 nCi of phenylalanine/assay, equivalent to the polymerization of 18 residues of phenylalanine/ribosome. ○, Core-particles; ●, dialysed salt-shock mixtures. Where appropriate, the range of values obtained in independent experiments is shown by a bar.

Properties of protein-deficient S-subparticle cores

Treatment with 2.5 M- NH_4Cl /61 mM- MgCl_2 for 15 min at 0°C led to the production of core-particles of $s_{20,w}$ approx. 36S, with approx. 70% of the normal complement of proteins and which generally sedimented as a single peak on sucrose gradients. The two-dimensional-electrophoretic protein patterns of core-particle proteins and of split-proteins are compared in Fig. 2. The molecular weights of proteins found mainly in either one or other of the two fractions are given in Table 1. A substantial number of proteins were found in both core-particle and split-proteins (see Fig. 2).

Enumeration of core-particle and split-proteins

The two-dimensional method of Knopf *et al.* (1975) used in the present work is based on the

Fig. 2. Two-dimensional electrophoresis of proteins derived from core-particle and split-protein fractions of rabbit *S*-subparticles

Samples of *S*-subparticle ($20 A_{260}$ units/ml) were suspended in salt-shock buffer for 15 min at 0°C . Core-particle and split-protein fractions were separated by differential centrifuging (see the Methods section). Proteins were isolated from the core-particle and split-protein fractions, and analysed by two-dimensional electrophoresis as described in the Methods section. Non-treated control samples of *S*-subparticle incorporated 3.9 nCi of phenylalanine/assay (each ribosome polymerized 23 phenylalanine residues on average). The core-particle fraction had 12% of the activity of the control. (a) Core-particle proteins; (b) split-proteins.

Table 1. Distribution of proteins between core-particle and split-protein fractions

Proteins are enumerated according to the standard nomenclature of McConkey *et al.* (1979). The assignments were made on the basis of the work of Madjar & Traut (1980). The gel system used (Knopf *et al.*, 1975) does not separate proteins S7 and S7a, or S14 and S17, or S16 and S18. The more acidic proteins Sa, Sb, S12 and S21 (Madjar & Traut, 1980) were found in the core-particle fraction and were absent from the split-protein fraction. Proteins of approx. the same molecular weight (see Madjar & Traut, 1980) were found to be specific to the appropriate fraction (core-particle proteins or split-proteins) by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). Proteins of mol.wt. approx. 11 500 (S30), 15 500 (S26), 23 000 (S7 or S7a) or 27 000 (S8) were specific to the split-protein fraction. Proteins of mol.wt. <10 000 (S28), 31 000 (S3), 37 000 (Sb) and 41 000 (Sa) were specific to core-particle proteins.

Proteins specific to core-particles	S3, S5, S10, S13, S15, S16–S18*, S19, S20, S28, Sa, Sb, S12, S21
Proteins specific to split-proteins	S8, S7 or S7a, S26, S27, S30
Proteins prominent in both fractions	S2, S3a, S4, S6, S9, S11, S14–S17, S15a, S23, S24, S25

* This protein was found mainly but not exclusively in the core-particle protein fraction.

Kaltschmidt & Wittmann (1970) technique; the protein spots may be enumerated according to the commonly agreed nomenclature for eukaryotic ribosomal proteins (McConkey *et al.*, 1979) on the basis of the work of Madjar & Traut (1980). The distribution of proteins between the core-particle and split-protein fractions is given in Table 1. Five proteins were found specifically in the split-protein fraction, 13 proteins were found specifically in the core-particle fraction, including the acidic proteins

Sa, Sb, S12 and S21, and 11 proteins were found in both fractions in roughly equal abundance. Two proteins, S27a and S29, were not detected in either fraction.

The results (not shown) of one-dimensional SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) support the data of Table 1. Proteins with molecular weights of the acidic proteins Sa and Sb and of proteins S3 and S28 were found in the core-particle protein fraction and not in the split-protein fraction.

Proteins corresponding in mass to S8, S26 and S30 were in the split-protein fraction and not in proteins of the core-particle, and the band corresponding to protein S7 or S7a was much more pronounced.

Reconstruction of S-subparticles isolated from isolated core-particles and split-proteins

The demonstration that fully active S-subparticles could be recovered by dialysis after treatment with 3 M-NH₄Cl/75 mM-MgCl₂ at 0°C establishes that the core-particle and split-protein fractions can readily interact under favourable conditions. It was inferred that conditions suitable for reconstruction were attained transiently during dialysis (or during dilution) and need not be the same as those used in the reconstruction of *Escherichia coli* S-subparticles (cf. Nomura, 1973).

At first core-particles were separated from split-proteins by differential centrifuging and the conditions used in the reconstruction step were similar to those found suitable for the partial reconstruction of rabbit L-subparticles (Cox & Greenwell, 1976). Limited success was achieved in this way (see Table 2). Two modifications were then introduced. First, core-particles were separated from split-proteins by means of a short-column sucrose gradient and the appropriate fractions were combined to provide the core-particle and split-protein pools. This procedure was introduced to avoid pelleting the core-particles. Secondly, a range of different NH₄Cl concentrations (0.31 M-NH₄Cl/7.5–25 mM-MgCl₂) was utilized in the reconstruction step. Optimum results were obtained when the final concentration conditions were 0.75 M-NH₄Cl/19 mM-MgCl₂ (see Fig. 3), on mixing one equivalent of split-proteins with one equivalent of core-

particles at 0°C for 15 min. Higher proportions of split-proteins diminished activity to some extent. Additional steps such as dialysis against dialysis buffer either with or without subsequent heating at 37°C led to no more than a marginal improvement. Heating the reconstruction mixture (0.75 M-NH₄Cl/19 mM-MgCl₂) at 37°C often led to a loss of activity of up to 40% on heating for 20 min.

Although substantial reconstruction was generally observed, the extent was variable (between 50 and 100% of control). The highest extents of reconstruction were obtained when S-subparticles were first purified on a second preparative gradient before partial disassembly and reconstruction. A possible explanation is that the variation arises from contamination of S-subparticles with hydrolytic enzymes. Such enzymes could be inactive in 2.5 M-NH₄Cl, since the split-protein fraction can be stored at 4°C for at least 48 h, but may be active during the reconstruction step. As a precaution, the trypsin inhibitor aprotinin was present during the preparation and isolation of core-particles and also throughout the reconstruction procedure. There was no strong indication that this procedure was effective. However, it appears from the experiments described below that proteinase or nuclease action could interfere with reconstruction.

Effects of trypsin on S-subparticle activity

The effect of pretreatment of S-subparticles with trypsin on activity in poly(U)-directed polyphenylalanine synthesis is summarized in Fig. 4. Treatment of S-subparticles with 60 ng of trypsin/A₂₆₀ unit diminished activity by 50%. The proportion of S-subparticles reconstructed on dilution after salt-shock treatment was also decreased (Fig. 4). It

Table 2. *Reconstruction of S-subparticles from pelleted core-particles and split-proteins*

S-subparticles (20 A₂₆₀ units/ml) were kept at 0°C in salt-shock buffer. After 15 min a sample was removed and dialysed at 4°C overnight against activation buffer (cf. Cox & Greenwell, 1976), then heated at 37°C for 1 h. A sample was diluted to 2 A₂₆₀ units/ml for assay in the standard cell-free system. The remainder of the salt-shock solution was centrifuged for 70 min at 40 000 rev./min (MSE 10 × 10 titanium rotor) to separate core-particle and split-protein fractions. The supernatant comprised the split-protein fraction, and the pellet of core-particles was resuspended in storage buffer. The core-particle was mixed with sufficient split-protein fraction to compensate for the proteins that had been removed, then dialysed as described above and a sample was assayed in the standard cell-free system. The non-treated control incorporated 6.1 nCi of [¹⁴C]phenylalanine/assay, equivalent to the polymerization of 36 phenylalanine residues/ribosome.

S-subparticle or derivatives	Split-proteins added	Dialysed and heated	[¹⁴ C]Polyphenylalanine synthesis (% of non-treated control)
Control S-subparticles	None	Yes	100
None	None	Yes	5
Salt-shock cores	None	No	15.4
Salt-shock cores	None	Yes	12.3
Salt-shock cores	1 equivalent	Yes	22.7
Salt-shock solution	None*	Yes	91.4

* Both core-particles and split-proteins were present in this case, since the salt-shock solution was simply dialysed to remove the excess salt, then heated.

Fig. 3. Effect of NH_4Cl concentration on the formation of active S-subparticles from core-particle and split-protein fractions

Washed S-subparticles ($20 A_{260}$ units/ml) were kept at 0°C for 15 min in salt-shock buffer. Core-particles were then separated from split-proteins by means of a short column sucrose gradient (see the Methods section). The fractions containing core-particles were pooled and the salts adjusted so that on addition of the split-protein fraction the final composition was $0.5\text{--}1.0\text{ M-NH}_4\text{Cl}/12.5\text{--}25\text{ mM-MgCl}_2/15\%$ (v/v) glycerol/ 0.1% aprotinin/ $20\text{ mM-Tris}/\text{HCl}$, pH 7.6. The molar ratio of $\text{Mg}^{2+}:\text{NH}_4^+$ was kept at 1:40. The samples were kept at 4°C overnight, diluted to $5 A_{260}$ units/ml with storage buffer and then assayed in the standard cell-free system. The control samples incorporated 4.9 nCi/assay , i.e. each ribosome polymerized 28 phenylalanine residues on average. In the absence of split-proteins the activity of core-particles was 15% of that of the control sample.

Fig. 4. Effect of trypsin on the activity of S-subparticles in polyphenylalanine synthesis

S-subparticles in storage buffer were treated with trypsin at 30°C for 15 min. An excess of trypsin inhibitor was then added and the incubation was continued for a further 15 min. The solution was diluted with sample buffer ($2 A_{260}$ units/ml) and S-subparticles were then assayed in the standard cell-free system both before (O) and after (●) treatment with salt-shock buffer at 0°C for 15 min. Core-particle and split-protein fraction components of salt-shock mixtures recombine spontaneously on dilution into sample buffer.

appears that the salt-shock treatment reveals damage by trypsin that otherwise is hidden. Pretreatment of S-subparticles with 100 ng of trypsin/ A_{260} unit was sufficient to abolish the capacity of approx. 80% of the S-subparticle sample to bind to L-subparticles (Fig. 5). The decreased activity of S-subparticles in polyphenylalanine synthesis as a result of pretreatment with trypsin correlates with an impaired capacity to form 80S couples. L-subparticles pretreated with trypsin had a lower capacity to bind S-subparticles (Cox & Kotecha, 1980). Arpin *et al.* (1975) found that rat liver S-subparticles treated with approx. 80 ng of trypsin/ A_{260} unit had diminished in activity by approx. 50% in poly(U)-directed polyphenylalanine synthesis, whereas the poly(U)-dependent binding of $[^{14}\text{C}]$ phenylalanyl-tRNA was increased. On the basis of the assumption that rat and rabbit S-subparticles are similar in structure, it is inferred that the L-subparticle-binding site(s) is more susceptible than the mRNA-

tRNA-complex-binding site pretreatment with trypsin.

Effect of RNAase on S-subparticle activity

Pretreatment of S-subparticles with up to 10 ng of RNAase/ A_{260} unit for 15 min at 20°C diminished activity in polyphenylalanine synthesis to 50% of the control value (Fig. 6). The RNA moiety was cleaved to 5–11 S fragments (solvent for $s_{20,w}$ measurements was $0.1\text{ M-sodium phosphate}$, pH 7.0), but the sedimentation rate of the S-subparticle was not altered (cf. Huvos *et al.*, 1970). Pretreatment of S-subparticles with RNAase also adversely affected the reconstruction of S-subparticles from core-particle and split-protein fraction on dilution of salt-shock

Fig. 5. Interaction of control and trypsin-treated S-subparticles with L-subparticles

S-subparticles in storage buffer were treated with trypsin (100 ng of trypsin/ A_{260} unit) at 30°C for 15 min and then with trypsin inhibitor for a further 15 min. Control S-subparticles were treated in the same way, except that trypsin was omitted. After trypsin treatment the activity of the S-subparticle sample in polyphenylalanine was 15% of the control. S- and L-subparticles either separately or mixed together were then kept at 30°C for 15 min (the solvent was storage buffer made 5 mM in $MgCl_2$; see Falvey & Staehelin, 1970) and then layered at 0°C on a 20–40% sucrose gradient. The gradient was prepared by layering 0.8 ml of 20% (w/v) sucrose and 1 ml each of 25, 30, 35 and 40% (w/v) sucrose, all containing storage buffer made 5 mM in $MgCl_2$. Polyallomer centrifuge tubes were used: the rotor was a Beckman SW50.1 used in a Beckman L5-50 ultracentrifuge. The gradients were spun at 48000 rev./min for 3 h at 10°C and 0.20–0.25 ml fractions were collected after the gradients were pumped out of tubes (with a Pharmacia P3 peristaltic pump) through an LKB Uvicord II instrument to record percentage transmission at 254 nm. (a) Control S-subparticles (0.4 A_{260} unit) alone; (b)

mixtures (Fig. 6). However, pretreatment with RNAase did not affect the capacity of S-subparticles to bind to L-subparticles. In experiments of the type summarized in Fig. 5, the interaction of control and RNAase-treated S-subparticles with L-subparticles were scarcely distinguishable (results not shown). It appears that the integrity of the RNA

Fig. 6. Effect of pretreatment of S-subparticles with RNAase on activity in polyphenylalanine synthesis

S-subparticles in storage buffer were treated with pancreatic RNAase at 20°C for 1 h. The S-subparticles were washed with 0.5 M- NH_4Cl /13 mM- $MgCl_2$, recovered by differential centrifuging and resuspended in storage buffer (250 A_{260} units/ml). Samples were diluted to 8 A_{260} units/ml with sample buffer either without further treatment (O) or after exposure to salt-shock buffer for 15 min at 0°C (●). Samples were then assayed in the standard cell-free system. Control samples which were kept at 0°C in sample buffer for the duration of the experiment incorporated 3 nCi of [^{14}C]phenylalanine/assay (on average each ribosome polymerized 16 phenylalanine residues).

L-subparticles (0.6 A_{260} unit) alone; (c) control S-subparticles (0.4 A_{260} unit) and L-subparticles (1.2 A_{260} units); (d) trypsin-treated S-subparticles (0.4 A_{260} unit) alone; (e) trypsin-treated S-subparticles (0.4 A_{260} unit) and L-subparticles (1.2 A_{260} units).

moiety is important to the partial reconstruction process, but it is less important to function once the S-subparticle is formed. Some hydrolysis of the rRNA moiety was found to take place during the salt-shock treatment of S-subparticles (see 'Purification of S-subparticles' in the Methods section). Thus nuclease action could contribute to the variation in the activity of reconstructed S-subparticles that is observed.

Discussion

The technique used to prepare core-particles was based on the concept that S-rRNA needs a minimum molar ratio of Mg^{2+} to K^+ or NH_4^+ (1:40) to maintain the 'native' conformation (Cox & Hirst, 1976). In solutions of up to 1.8M- NH_4Cl , S-subparticles may be protected against disassembly by raising the Mg^{2+} concentration to the appropriate value. In solutions more concentrated than 1.8M- NH_4Cl , e.g. 2.5M- NH_4Cl , proteins were lost even at concentrations of Mg^{2+} higher than $1Mg^{2+}:40NH_4^+$. It is inferred that the critical factor is the conformation of S-rRNA, which is mediated through the relative concentration of Mg^{2+} ions. The methods previously used to split proteins from S-subparticles cover a wide range of conditions (for review see Bielka & Stahl, 1978), but they have in common the feature of Mg^{2+} deficiency, i.e. had the concentration of Mg^{2+} ion been raised it is unlikely that proteins would have been lost at that particular concentration of electrolyte.

The partial reconstruction of rat liver S-subparticles was reported previously and in both cases proteins were removed from Mg^{2+} -deficient subparticles. The ionic conditions were 0.4M-HCl/5mM- $MgCl_2$ (Terao & Ogata, 1971) and 1.6M-KCl/3mM- $MgCl_2$ (Reboud *et al.*, 1972). Both NH_4Cl and KCl have similar effects on the rRNA conformation in the presence of Mg^{2+} (Cox & Hirst, 1976), and the production of core-particles by treatment with 1.6M-KCl/3mM- $MgCl_2$ ($1Mg^{2+}:533K^+$) is in contrast with finding that S-subparticles recovered from 1.6M- NH_4Cl /40mM- $MgCl_2$ ($1Mg^{2+}:40NH_4^+$) retained 85% of the activity of control ribosomes (see Fig. 1).

As far as they can be compared, it appears that the split-protein fraction obtained by treating S-subparticles with 2.5M- NH_4Cl /61mM- $MgCl_2$ differs from that produced by treatment with 1.6M-KCl/3mM- $MgCl_2$ in that the proteins of highest molecular weight were absent from the former (see Table 1), but were present in the latter (Reboud *et al.*, 1972).

The principal conclusion is that the partial reconstruction of S-subparticles from core-particle and split-protein fractions takes place readily, especially when handling is minimal. Reconstruction is hindered by technical difficulties that include the adventitious effects of nucleases and proteinases, the handling of core-particles and the identification of the optimum ionic conditions for the attachment of split-proteins.

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