Resistance of the peptidyltransferase centre of rabbit ribosomes to attack by nucleases and proteinases

Robert A. COX and Surendra KOTTECHA

National Institute for Medical Research, Mill Hill, London NW7, 1AA, U.K.

(Received 21 December 1979)

Larger ribosomal subparticles (L-subparticles) of rabbit ribosomes were treated with either ribonucleases (I or T_1) or proteinases (trypsin or chymotrypsin), and their capacity to function in poly(U)-directed polyphenylalanine synthesis and in the puromycin reaction was investigated. The effects of pretreatment of L-subparticles on the reconstruction of active subparticles from core-particles derived by treatment with 2.75 M- NH_4Cl /69 mM- MgCl_2 and split-protein fractions were also examined. The protein moiety of proteinase-treated L-subparticles was analysed by one-dimensional sodium dodecyl sulphate/polyacrylamide- and two-dimensional polyacrylamide-gel electrophoresis. The introduction of 16–100 scissions in the RNA moiety had no effect on the activity of the L-subparticles in polyphenylalanine synthesis, and there was no effect on the stability of L-subparticles to high-salt shock treatment and a marginal effect on the reconstruction of L-subparticles from high-salt-shock core-particles and split-protein fractions. In contrast, L-subparticles treated with low amounts of trypsin (0.56 ng of trypsin/ μg of L-subparticle) were inactive in polyphenylalanine synthesis, and their capacity to function in partial-reconstruction experiments was diminished. Activity in the puromycin reaction was increased by 70% as a result of trypsin treatment (280 ng of trypsin/ μg of L-subparticle). At least two of the acidic proteins implicated in the translocation function were not affected by trypsin treatment. Trypsin-treated L-subparticles had lost their capacity to bind the smaller ribosomal subparticle (S-subparticle). The protein(s) needed for S-subparticle binding were shown to be present in high-salt-shock cores. At least six proteins associated with the core-particles were attacked during trypsin treatment of L-subparticles. An examination of L-subparticles isolated from trypsin-treated polyribosomes showed that the amount of trypsin necessary to decrease the activity of the subparticle by 50% was about twice that needed in the treatment of L-subparticles alone. The largest protein of rabbit L-subparticles (approx. 51 000 daltons) was cleaved in a stepwise fashion by trypsin to fragments of approx. 40 000 daltons. This protein was also cleaved by chymotrypsin.

In order to function in protein synthesis, the L-subparticle of the ribosome must be capable both of binding aminoacyl-tRNA and peptidyl-tRNA and of forming a peptide bond (peptidyltransferase function). Those regions of the L-subparticle that are required for these functions may be regarded as the peptidyltransferase centre, since all three activities are needed to make a peptide bond. The L-sub-

particle must also be capable of transferring the newly formed peptidyl-tRNA back to its original (P-) site (translocation) and of binding to the S-subparticle in order to perform in mRNA-directed protein synthesis. Identification of the ribosomal proteins involved in these individual steps has been the aim of many investigations using specific antibodies, antibiotics and the controlled assembly and disassembly of ribosomes (for review see Brimacombe *et al.*, 1976; Cox, 1977; Bielka & Stahl, 1978).

The value of disassembly and re-assembly of ribosomes as a means of probing structure-function relationships has been amply demonstrated in

Abbreviations used: S-subparticle, the smaller ribosomal subparticle; S-rRNA, the RNA component of S-subparticles; L-subparticle, the larger ribosomal subparticle; L-rRNA, the major (28S) component of L-subparticles; SDS, sodium dodecyl sulphate; RNAase, ribonuclease.

prokaryotic systems (for review see Nomura, 1973). Eukaryotic ribosomes have proved more difficult to re-assemble. Previously we have described the partial reconstruction of rabbit L-subparticles (Cox & Greenwell, 1976). Treatment of L-subparticles with 2.75 M-NH₄Cl/69 mM-MgCl₂ at 0°C yields a core-particle deficient in about eight proteins and a split-protein fraction. The core-particle is inactive both in poly(U)-directed polyphenylalanine synthesis and in the puromycin reaction. Activity is recovered when the core-particle and split-protein fractions are recombined. Split-protein fractions from *Neurospora crassa* and from *Xenopus laevis* L-subparticles were shown to be interchangeable with rabbit split-proteins, since they could re-activate rabbit core-particles (Cox & Greenwell, 1980).

The extent of re-activation of core-particles was found to be variable, e.g. from 10 to 70% of control activity was regained when the split-protein fraction was restored. The activity of L-subparticles was also variable, ranging from approx. 18 to approx. 54 residues of phenylalanine incorporated per ribosome. In an attempt to identify the causes of these variations, the effects of RNAases and of proteinases (trypsin and chymotrypsin) on the functions of the rabbit L-subparticle were examined.

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 at 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-subparticles and core-particles before assay for polyphenylalanine synthesis was 25 mM-KCl/5 mM-MgCl₂/11 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.

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/3 mM-MgCl₂/1 mM-dithiothreitol/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 × 25 ml rotor), resuspended in a storage buffer or Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] storage buffer (Hepes replaced Tris in the usual formulation) 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-subparticle in the presence of S-subparticle, supernatant factors etc. at 37°C was measured as described by Cox & Hirst (1976). Samples were generally 50 μ l of 2.0 A_{260} units/ml (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.

Assay of peptidyltransferase by the puromycin reaction

The transfer of acetyl[³H]leucyl groups from acetyl[³H]leucyl-tRNA to puromycin, catalysed by L-subparticles at 20°C in 30% (v/v) methanol, was carried out and measured in a small scintillation vial as described by Cox *et al.* (1976). All assays were run in duplicate and the results averaged.

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

The production of core-particles and split-proteins from rabbit L-subparticles by salt-shock treatment was described in detail previously (Cox & Greenwell, 1976). Effective conditions for producing extensively inactivated core-particles with good potential for reconstruction were taken from the earlier work.

Thus, L-subparticles at 250 A_{260} units/ml in storage buffer were mixed at 0°C with 4 vol. of salts solution such that the final composition was 2.75 M-

$\text{NH}_4\text{Cl}/69\text{ mM}-\text{MgCl}_2/3.5\text{ mM}-\text{EDTA}/1\text{ mM}-\text{dithiothreitol}/3\%$ (w/v) glycerol/ $20\text{ mM}-\text{Tris}/\text{HCl}$, pH 7.6. After 15 min at 0°C , the reaction mixture was centrifuged for 30 min at approx. 0°C and 40000 rev./min in the MSE 8 $\times$ 10 ml rotor (1.25 ml portions in 10 ml polycarbonate tubes, $g_{\text{max.}} = 145\,000$). 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 cloudy suspension of core-particles.

Separation of subparticles on short-column preparative sucrose gradients

Polyribosomes (5 mg/ml in storage buffer) were dissociated into subparticles by puromycin treatment (see above), and layered (0.35 ml) on a 20–40% sucrose gradient containing 0.3 M-KCl/3 mM-MgCl₂/0.1 mM-dithiothreitol/20 mM-Tris/HCl, pH 7.6. The gradient was prepared by layering 0.8 ml portions of 25, 30, 35 and 40% (w/v) sucrose and 1 ml of 20% (w/v) sucrose solution. The polyribosome fraction was layered on top of the gradient at 0°C . The gradient was centrifuged at 48000 rev./min at 20°C for 3 h (Beckman 50.1 rotor; Beckman L5.50 ultracentrifuge). The gradient was unloaded by displacement with 50% (w/v) sucrose (Pharmacia P3 peristaltic pump), passed through an LKB Uvicord recorder II to register percentage transmission, and fractions (250 μl) were collected manually.

Isolation of split-protein fraction from L-subparticles treated with ethanol (cf. Reyes et al., 1977)

L-subparticles (7.5 mg/ml) were resuspended in storage buffer and the composition was adjusted to 0.4 M- $\text{NH}_4\text{Cl}/20\text{ mM}-\text{MgCl}_2$. Ethanol (0.5 vol.) was added at 0°C and after 10 min more ethanol (a further 0.5 vol.) was then added. After 5 min the core-particles were separated by centrifuging for 10 min at 18000 rev./min. The pellet was again treated with ethanol [to give final concns. 0.2 M- $\text{NH}_4\text{Cl}/10\text{ mM}-\text{MgCl}_2/50\%$ (v/v) ethanol]. The supernatants were recombined, dialysed against 1 M-acetic acid and then freeze-dried.

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 μg of protein) was diluted to 200 μl in

a 1.5 ml polypropylene micro-centrifuge tube, and was then mixed with 500 μl 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 ml $\times$ 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 μg 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 μl) 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, chymotrypsin, phenylmethanesulphonyl fluoride, RNAase I and RNAase T, were all obtained from Sigma.

Results

Effect of pretreatment with RNAases on L-subparticle function

The integrity of L-rRNA is not required for the formation of the L-subparticle in protein synthesis (Delihias, 1970; Cahn *et al.*, 1970; Huvos *et al.*,

1970). However, the effect of damage to L-rRNA on either the stability of the L-subparticle to high-salt treatment or on the capacity of core-particles to recombine with split-protein fractions has not been reported. The effect of RNAase T₁ at 0.1–10 units (as defined by Sigma) of enzyme/*A*₂₆₀ unit of L-subparticles was examined. After treatment with RNAase, L-subparticles were exposed to salt-shock with increasing concentrations of salt up to 2.75 M-NH₄Cl/69 mM-MgCl₂. The activity of RNAase-treated L-subparticles in poly(U)-directed poly-phenylalanine synthesis followed the pattern described for non-treated controls (see Cox & Greenwell, 1976), i.e. activity was scarcely diminished after pretreatment with 2 M-NH₄Cl/50 mM-MgCl₂ (see Table 1). The inactive core-particles produced by treatment with 2.75 M-NH₄Cl/69 mM-MgCl₂ were re-activated by recombination with split-proteins (see Table 1). Treatment with pancreatic RNAase had a marginal effect on both the stability of L-subparticles to high-salt treatment and on the capacity of the cores to recombine with split-proteins to form functional L-subparticles (see Table 1).

Treatment of L-subparticles with low amounts of RNAase led to RNA fragments sedimenting in the range 3–7S, compared with 28S for the control. Increasing the amount of RNAase did not appreci-

Table 1. *Activity in poly(U)-directed polyphenylalanine synthesis of L-subparticles previously treated with RNAases*
L-subparticles in storage buffer were kept at 20°C for 1 h in the presence of RNAase. Samples were then assayed in the standard cell-free system. Other samples were first kept at 0°C for 15 min in either 2M-NH₄Cl/50mM-MgCl₂ or 2.75 M-NH₄Cl/69 mM-MgCl₂, diluted into assay buffer and then added to the cell-free system. The amount of RNAase introduced into the cell-free system by this procedure was shown to have no influence in the amount of polyphenylalanine synthesized. The control sample was treated as described above, except that RNAase was omitted. The radioactivity of polyphenylalanine synthesized by control L-subparticles was 3000 c.p.m./assay (or 9.3 nCi/assay), corresponding to the polymerization of 54 phenylalanine residues/ribosome. Results are expressed as a percentage of this control. Exposure to 2M-NH₄Cl/50mM-MgCl₂ provides a measure of the stability of L-subparticles to high-salt concentrations (Cox & Greenwell, 1976). In 2.75 M-NH₄Cl/69 mM-MgCl₂ approximately eight proteins are removed from the L-subparticle. The core-particles have 10 ± 5% of the control activity, but can be re-activated by recombination with split-protein fraction. Some reconstruction takes place simply when the high salt is diluted out.

		Activity in poly(U)-directed polyphenylalanine synthesis after pretreatment with RNAase (%)					
Previous high-salt treatment	Enzyme added (units/ A_{260} unit of L-subparticle) ...	RNAase T ₁					RNAase I (0.5 ng of RNAase/ A_{260} unit of L-subparticle)
		0	0.1	0.3	3	10	
None		100	97	101	97	96	106
2 M-NH ₄ Cl/50 mm-MgCl ₂		68	88	70	60	73	47
2.75 M-NH ₄ Cl/69 mm-MgCl ₂		2	66	47	45*	31	35†

* Activity of core-particles was 15% of control, and activity increased to 24% of control on the addition of the split-protein fraction.

† Activity of core-particles was 12.5% of control, and activity increased to 25% on the addition of the split-protein fraction.

ably alter the sedimentation profile. Since $s_{20,w}$ is proportional to the square root of the molecular weight (for review see Cox, 1968), then 16–100 scissions had been introduced into L-rRNA without significantly affecting the functioning of the L-subparticle. Throughout this investigation the ratio $Mg^{2+}:NH_4^+$ was kept at approx 1:40, conditions favouring the preservation of rRNA tertiary structure (Cox & Hirst, 1976).

Effect of trypsin treatment on the activity of L-subparticles

Whereas RNAase treatment did not affect the activity of L-subparticles (see Table 1), mild trypsin treatment led to inactivation (see Fig. 1). The effects of pre-treatment with trypsin (0–40 ng of trypsin/ A_{260} unit of L-subparticle, i.e. 0–0.56 ng of trypsin/ μg of L-subparticle) was even more apparent after L-subparticles were treated with 2.75 M- NH_4Cl /69 mM- $MgCl_2$ (see Fig. 1), suggesting that hidden damage was exposed by the disassembly process. Evidently proteinase action is far more likely than RNAase action to give rise to variations in the activity of L-subparticles. Although trypsin treatment has been shown to diminish the activity of L-subparticles in poly(U)-directed polyphenylalanine synthesis, the lesion(s) have not been identified (Arpin *et al.*, 1975). For this reason the effects of trypsin was further explored.

Although trypsin-treated L-subparticles failed to function in polyphenylalanine synthesis, their capacity to function in the puromycin reaction was enhanced. Thus raising the concentration of trypsin more than 10-fold, to 4 ng/ μg of L-subparticle, led to an increase in activity from 100 (non-treated ribosomes) to 170% after trypsin treatment (see Fig. 2).

Treatment of L-subparticle with high salt, i.e. 2.75 M- NH_4Cl /69 mM- $MgCl_2$, yields a core-particle and a split-protein fraction (Cox & Greenwell, 1976). The core-particles alone are inactive in both the puromycin reaction and polyphenylalanine synthesis. Active subparticles can be reconstructed by recombining the core-particle and split-protein fractions (Cox & Greenwell, 1976). The core-particles from trypsin-treated L-subparticles were shown to be inactive in the puromycin reaction (Fig. 2). This shows that high-salt treatment splits off protein(s) important to the peptidyltransferase centre, but that these proteins are protected by their environment from attack by trypsin.

Trypsin-treatment (40 ng/ A_{260} unit) of L-subparticles did not alter the stability of the peptidyltransferase centre to high salt. When control L-subparticles were exposed to increasing concentrations of salt by the addition of increasing proportions of 4 M- NH_4Cl /0.1 M- $MgCl_2$ at 0°C, the

Fig. 1. *Effect of trypsin treatment on the activity of L-subparticles in polyphenylalanine synthesis and on the response to high-salt shock*

L-subparticles in storage buffer were treated with trypsin at 30°C for 15 min. An excess of trypsin inhibitor was added and the incubation was continued for another 15 min. The L-subparticles were then assayed in the standard cell-free system. The activity/assay of the control corresponds to the incorporation of approx. 8 nCi/assay, i.e. each ribosome polymerized 47 phenylalanine residues on average. ○, L-subparticles after trypsin treatment (curve I); ●, L-subparticles treated first with trypsin and then with 2.75 M- NH_4Cl /69 mM- $MgCl_2$ (curve II). The treatment with high salt removes approximately eight proteins and the activity in polyphenylalanine synthesis depends on the reconstruction of L-subparticles when the salt is diluted out (cf. Cox & Greenwell, 1976).

Fig. 2. Effect of trypsin treatment on the activity of L-subparticles in the puromycin reaction

L-subparticles in storage buffer were treated with trypsin at 30°C for 15 min and then with trypsin inhibitor for a further 15 min and were then assayed in both the puromycin reaction (see the Methods section) and in polyphenylalanine synthesis (cf. Fig. 1). After trypsin treatment the L-subparticles were treated with 2.75 M-NH₄Cl/69 mM-MgCl₂ and protein-deficient core-particles were obtained by centrifugation (Cox & Greenwell, 1976). □, Activity of L-subparticles in polyphenylalanine synthesis; O, activity of L-subparticles in the puromycin reaction; ●, activity of high-salt core particles in the puromycin reaction.

capacity to function in the puromycin reaction was unimpaired after treatment with 1.5 M-NH₄Cl/37.5 mM-MgCl₂. However activity of the L-subparticles in the puromycin assay diminished to 10% of the control over the range 1.5 M-NH₄Cl/35 mM-MgCl₂ to 2.25 M-NH₄Cl/56 mM-MgCl₂ (cf. Cox & Greenwell, 1976). Essentially the same profile was observed when trypsin-treated L-subparticles were treated in the same way. Thus trypsin treatment of L-subparticles had little effect on the stability of the peptidyltransferase centre to high-salt treatment.

Interaction of trypsin-treated L-subparticles with S-subparticles

The observation that trypsin pre-treatment of L-subparticles enhanced peptidyltransferase function but diminished the capacity to function in polyphenylalanine synthesis reveals that trypsin treatment affects proteins implicated in either translocation or binding S-subparticles, or both. The ability of L-subparticles to bind S-subparticles was tested. A mixture of S- and L-subparticles in storage buffer made 5 mM in MgCl₂ (cf. Falvey & Staehelin, 1970) was kept at 30°C for 15 min and the products were analysed by sedimentation through sucrose gradients. Non-treated control L-subparticles were

Fig. 3. Effect of trypsin treatment on the interaction of L-subparticles with S-subparticles

L-subparticles in storage buffer were treated with trypsin (50 ng of trypsin/ A_{260} unit) at 30°C for 15 min and then with trypsin inhibitor for a further 15 min. Control L-subparticles were treated in the same way, except that trypsin was omitted. 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 MgCl₂) and then layered at 0°C on a 20–40% sucrose gradient. The gradient was prepared by layering 1 ml each of 25, 30, 35 and 40% (w/v) sucrose and 0.8 ml of 20% (w/v) sucrose, all containing storage buffer made 5 mM in MgCl₂. Polyallomer centrifuge tubes were used; the rotor was a Beckman SW50.1 in Beckman L5-50 ultracentrifuge. The gradients were spun at 38000 rev./min for 3 h at 10°C and 0.2–0.25 ml fractions were collected. (a) S-Subparticles (0.4 A_{260} unit) alone; (b) control L-subparticles (0.6 A_{260} unit) alone; (c) S-subparticles (0.4 A_{260} unit) and control L-subparticles (1.2 A_{260} units); (d) S-subparticles (0.4 A_{260} unit) and trypsin-treated L-subparticles (1.2 A_{260} units).

found to have a high capacity for forming ribosomes (S- and L-subparticle complex). In contrast, trypsin-treated L-subparticles (0.7 ng of trypsin/ μ g of

L-subparticle) scarcely interacted with S-subparticles (see Fig. 3). The inability of trypsin-treated L-subparticles to bind to S-subparticles is sufficient to account for their failure to function in polyphenylalanine synthesis. This observation also demonstrates that the integrity of a particular protein (or proteins) is essential for the interaction between subparticles. Rabbit L-subparticles can also form active ribosomes by binding S-subparticles from moulds and amphibia (Cox & Greenwell, 1980; cf. Martin *et al.*, 1970; Cammarano *et al.*, 1972). Thus the protein(s) implicated in S-subparticle binding has structural features that are highly conserved.

Interaction of high-salt core-particles with S-subparticles

Treatment of L-subparticles with 2.75 M-NH₄Cl/69 mM-MgCl₂ yields core-particles that are inactive in both polyphenylalanine synthesis and the puromycin reaction. These core-particles ($s_{20,w}$ approx. 60S) form dimers and higher aggregates, but they are capable of binding S-subparticles (see Fig. 4). We infer that the protein(s) implicated in S-subparticle binding are present in high-salt core-particles.

Effect of trypsin treatment on polyribosomes

The L-subparticle protein(s) involved in the interaction with S-subparticles are susceptible to trypsin treatment when L-subparticles alone are exposed to enzyme. The sensitivity of these proteins to trypsin could be altered once the S-subparticle becomes bound. This possibility was investigated by examining the effects of trypsin treatment on the polyribosome fraction. It was found (see Table 2) that, although the rate of attack by trypsin was decreased by approx. 50%, the protein(s) involved in S-subparticle binding were attacked.

The ribosome fraction of rabbit reticulocytes was separated into ribosomes and polyribosomes by zonal centrifuging. The polyribosome fraction was isolated, and its capacity to function in protein biosynthesis was diminished by trypsin treatment (see Fig. 5a). An initial fast inactivation was followed by a slower reaction rate. The globin synthesis that is measured in the cell-free system arises both from the completion of globin chains and from the re-initiation of globin synthesis when ribosomes are released on the completion of globin chains and are recycled. We show below that the initiation of globin synthesis is more sensitive to trypsin treatment than is the completion of globin chains.

The contribution of the process of completing globin chains to globin synthesis was estimated as approx. 75% by measuring the incorporation of

Fig. 4. Interaction of S-subparticles with L-subparticle high-salt-shock core-particles

L-subparticles were treated with 2.75 M-NH₄Cl/69 mM-MgCl₂ at 0°C for 15 min. The core-particle fraction was separated by centrifugation (see the Methods section) and the pellet was resuspended in storage buffer. In the puromycin reaction the core-particle fraction had 4% of the activity of control L-subparticles. S-subparticles, L-subparticles, core-particles and appropriate mixtures were kept at 30°C for 15 min in storage buffer made 5 mM-MgCl₂ and then layered at 0°C on a 20–40% sucrose gradient, and centrifuged for 3 h at 48 000 rev./min as described in the legend to Fig. 3, and 0.2–0.25 ml fractions were collected. (a) S-subparticles alone (0.4 A_{260} unit); (b) core particles (0.6 A_{260} unit); (c) S-subparticles (0.4 A_{260} unit) and core-particles (0.6 A_{260} unit); (d) S-subparticles (0.2 A_{260} unit) and L-subparticles (0.5 A_{260} unit).

¹⁴C-labelled leucine when poly(U) was present to compete with globin mRNA for run-off ribosomes (see Fig. 5b). The capacity of the polyribosome fraction to complete globin chains was halved after treatment with approx. 100 ng of trypsin/ A_{260} unit of polyribosomes.

Table 2. Comparison of the effects of trypsin on the activity of polyribosomes, ribosomes, S-subparticles and L-subparticles in poly(U)-directed polyphenylalanine synthesis

The Table summarizes the data given in Figs. 1 and 5.

Species	Concn. of trypsin (ng/ A_{260} unit of nucleoprotein) during pre-treatment at 30°C for 15 min that decreases activity in protein biosynthesis to 50%
Polyribosomes (completion of globin chains)	100 ± 20
Ribosomes (40S–60S couples*; and run-off ribosomes)	60 ± 10
S-subparticles†	55 ± 10
L-subparticles	25 ± 5

* Results (not shown) were similar in form to curve II, Fig. 5.

† Results (not shown) were similar in form to curve I, Fig. 1.

Fig. 5. Effect of pretreatment with trypsin on the activity of the polyribosome fraction in cell-free protein biosynthesis. The ribosome fraction was isolated from rabbit reticulocytes by precipitation at pH 5.7–6.0 (Arnstein *et al.*, 1964). The precipitate was collected by centrifugation and suspended in storage buffer made 5 mM in $MgCl_2$. A 15–45% sucrose gradient containing the same buffer was prepared in a BXIV MSE zonal rotor, and the sample (18 ml; 11 mg of ribosomes/ml) was introduced. Sample and overlay displaced 250 ml of gradient. The sample was centrifuged for 1 h at 43 000 rev./min at 15°C (MSE Superspeed 65 centrifuge). The first 160 ml of the gradient was discarded and then 38 fractions (each 15 ml) were collected. Fractions containing polyribosomes were pooled and centrifuged at 40 000 rev./min at 4°C (MSE 8 × 25 rotor). The pellets were resuspended in storage buffer. Samples were treated with trypsin at 30°C for 15 min. An excess of trypsin inhibitor was added and the solution was kept at 30°C for a further 15 min. The polyribosome fraction was assayed in the standard cell-free system in the presence and absence of poly(U) by using either [^{14}C]phenylalanine or [^{14}C]leucine as the radiotracer. For reference, L-subparticles were also treated with trypsin and assayed for their capacity to synthesize polyphenylalanine. The activity of the non-treated polyribosome fraction corresponds to an average incorporation of 16 phenylalanine residues/ribosome, compared with the L-subparticle fraction, which gave an average incorporation of 27 phenylalanine residues/ribosome. (a) ○, Activity of polyribosome fraction in globin synthesis measured by incorporation of [^{14}C]phenylalanine (curve I); ●, activity of polyribosome fraction in the presence of poly(U), i.e. in globin and polyphenylalanine synthesis (curve II); ■, activity of L-subparticles in polyphenylalanine synthesis (curve III); (b) ○, activity of polyribosome fraction in the completion of globin chains measured by the incorporation of [^{14}C]leucine in the presence of poly(U) (curve IV); ●, activity of run-off ribosomes (curve V) measured by the difference curve II—curve I (Fig. 5a); □, activity of polyribosome fraction in globin synthesis ([^{14}C]leucine incorporation).

The activity of run-off ribosomes was followed by measuring the incorporation of ^{14}C -labelled phenylalanine into protein when poly(U) was present. The capacity to synthesize polyphenylalanine measures the capacity of run-off ribosomes to become programmed with poly(U). The radioactivity of polyphenylalanine is measured to a first approximation by the difference in radioactivity that is incorporated into acid-insoluble protein in the presence and absence of poly(U), because completed globin chains have 5% of the specific radioactivity of polyphenylalanine. The activity of run-off ribosomes in protein synthesis was halved after treatment with approx. 50 ng of trypsin/ A_{260} unit of polyribosomes.

The observation that trypsin treatment adversely affects the ability of run-off ribosomes to recycle more rapidly than it diminishes the capacity of polyribosomes to complete globin chains is sufficient to account for the observed dependence of the activity of the polyribosome fraction in globin synthesis (Fig. 5). The initial more rapid effects correspond to the inactivation of run-off ribosomes.

The response of the polyribosome fraction to trypsin treatment accords with the view that trypsin treatment diminishes the capacity of L-subparticles to bind S-subparticles. We infer that run-off ribosomes have diminished activity because the

capacity of L-subparticles to combine is diminished by trypsin treatment of polyribosomes. The relative resistance of polyribosomes to trypsin treatment that is shown by their capacity to complete globin chains can be explained if additional factors such as the presence of the nascent polypeptide chain stabilize the interaction between subparticles. This would allow the subparticles to remain in contact for the completion of chains even though the subparticles themselves may no longer interact. In other words, the interaction between subparticles that is essential for the initiation of protein synthesis may be less important for the propagation of the polypeptide chains once a programmed ribosome has been formed. In support of this view, it is known that the removal of the nascent polypeptide chain by puromycin treatment renders polyribosomes more sensitive to dissociation into subparticles (Blobel & Sabatini, 1971).

Subparticles were isolated from trypsin-treated polyribosomes, and their properties are summarized in Table 3. The conclusion is that the L-subparticle protein(s) involved in binding S-subparticles are protected to some extent once ribosomes are formed. Treatment of L-subparticles with 25 ng of trypsin/ A_{260} unit diminished activity in polyphenylalanine synthesis by 50%, whereas polyribosomes require

Table 3. *Activity in protein synthesis of subparticles isolated from trypsin-treated polyribosomes*

Polyribosomes in storage buffer were treated at 30°C for 15 min with trypsin (either with 40 ng/ A_{260} unit as in Expt. 1 or 100 ng/ A_{260} unit as in Expt. 2), then for 15 min with trypsin inhibitor. At the same time, control polyribosomes were incubated first without trypsin and then with trypsin inhibitor. Both trypsin-treated and control ribosomes were dissociated into subparticles by puromycin treatment (see the Methods section). S- and L-subparticles were then isolated by means of sucrose gradients (see the Methods section). The incorporation of phenylalanine into acid-insoluble protein (either haemoglobin or polyphenylalanine) by polyribosomes and the derived subparticles was measured by utilizing the standard cell-free system (see the Methods section), —, Results not available; however, there is negligible incorporation by subparticles of phenylalanine into acid-insoluble protein in the absence of poly(U).

	Trypsin treatment (ng/ A_{260} unit)	Derived subparticles	Radioactivity of haemoglobin		Radioactivity of polyphenylalanine	
			(c.p.m./assay)	(% of control)	(c.p.m./assay)	(% of control)
Polyribosome (Expt. 1)	None	—	475	100	—	—
		S-subparticle	—	—	3050	100
		L-subparticle	—	—	1580	100
Polyribosome (Expt. 1)	40	—	290	61	—	—
		S-subparticle	—	—	437	14
		L-subparticle	—	—	735*	45
Polyribosome (Expt. 2)	None	—	175	100	—	—
		S-subparticle	—	—	1300	100
		L-subparticle	—	—	1505	100
Polyribosome (Expt. 2)	100	—	91	51	—	—
		S-subparticle	—	—	58	5
		L-subparticle	—	—	498†	33

* Activity of L-subparticle in the puromycin reaction was 120% of the non-treated control.

† Activity of L-subparticle in the puromycin reaction was 150% of the non-treated control.

Table 4. *Activity of L-subparticle and ethanol core-particles in the puromycin reaction and in polyphenylalanine synthesis*
The activity of L-subparticles and of cores produced by either ethanol treatment [0.2M-NH₄Cl/10mM-MgCl₂/50% (v/v) ethanol] at 0°C or high-salt treatment (2.75M-NH₄Cl/69mM-MgCl₂) at 0°C was measured in both the puromycin reaction and in poly(U)-directed polyphenylalanine synthesis as described in the Methods section.

Species	Trypsin treatment (ng/A ₂₆₀ unit)	Activity in the puromycin reaction		Activity in polyphenylalanine synthesis	
		Radioactivity (c.p.m./assay)	(% of control)	Radioactivity (c.p.m./assay)	(% of control)
L-subparticle	None	5990	100	1649*	100
Ethanol cores	None	2695	45	184	11
High-salt core	None	300	5	83	5
L-subparticle	50	6039	110	900	55
Ethanol cores	40	2784	46	140	8.5

* 5nCi of phenylalanine incorporated, i.e. 30 phenylalanine residues incorporated/ribosome.

Table 5. *Summary of properties of split-protein fractions examined*

The molecular-weight values that are given were obtained by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) and are the average of five determinations. The protein bands examined could contain more than one protein.

Conditions for preparation of split-protein fraction	10 ⁻³ × Mol.wt. of split-protein components											
	38.6	35.1	33.0	31.1	28.4	25.8	23.0†	20.1	17.5	15.0*	13.0*	9
2.75mM-NH ₄ Cl/69mM-MgCl ₂	38.6	35.1	33.0	31.1	28.4	25.8	23.0†	20.1	17.5	15.0*	13.0*	9
0.5M-NH ₄ Cl/10mM-MgCl ₂ /50% (v/v) ethanol	—	—	—	31.1	28.4	25.8	—	20.1	17.5	15.0*	13.0*	9
0.25M-NH ₄ Cl/10mM-MgCl ₂ /50% (v/v) ethanol	—	—	—	31.1§	—	—	—	20.1‡	17.5	15.0*	13.0*	9

* Putative acidic proteins.

† This protein is slowly eluted during high-salt shock of L-subparticles and the intensity of the band increases with the length of the high-salt shock treatment.

‡ This band could comprise two proteins, one of which is affected by trypsin.

§ This protein was attacked by 120ng of trypsin/A₂₆₀ unit of L-subparticles, but not by 40ng of trypsin/A₂₆₀ unit of L-subparticles.

Fig. 6. *Effect of trypsin treatment of L-subparticles on the acidic proteins*

L-subparticles were kept in storage buffer for 15 min at 30°C. One sample was treated with trypsin (120ng/A₂₆₀ unit of L-subparticles for 15 min at 30°C. Trypsin inhibitor was then added and the incubation continued for a further 15 min. The trypsin-treated and control samples were treated with ethanol; the final composition of the solution was 50% (v/v) ethanol/0.2M-NH₄Cl/10mM-MgCl₂/10mM-Tris/HCl, pH 7.6. The proteins were analysed by two-dimensional electrophoresis as described in the Methods section. (a) Split-protein fraction from control L-subparticles; (b) split-protein fraction from trypsin-treated L-subparticles. †, putative acidic proteins.

treatment with 50 ng of trypsin/ A_{260} unit in order to decrease the activity of run-off ribosomes by 50% (Table 2).

Effect of trypsin treatment of L-subparticles on the acidic proteins implicated in translocation

Translocation of peptidyl-tRNA from the amino-acyl-tRNA site to the peptidyl-tRNA site is one step in the elongation of the nascent polypeptide chain. Acidic proteins analogous to proteins L7 and L12 of *Escherichia coli* are implicated in this process (for review see Bielka & Stahl, 1978). These acidic proteins are dissociated from the L-subparticle by the method of Reyes *et al.* (1977) (see also Leader & Coia, 1978). The core-particles produced have diminished activity in poly(U)-directed polyphenylalanine synthesis, but still function in the puromycin reaction (see Table 4). The split-protein fraction derived by this treatment is comparatively simple in composition (see Table 5) and contains the analogues of proteins L7 and L12. The molecular mass of these analogues lies in the range 13 500–14 500 daltons, and they are phosphorylated (Leader & Coia, 1978). Proteins of this size were also found in the standard high-salt-shock split-protein fraction (see Table 5). The two-dimensional gel system used by Leader & Coia (1978) allows all the proteins (whether acidic or basic) to migrate in the same direction in the gel used for the first dimension. Our two-dimensional system also has this property. The two-dimensional patterns of the split-protein fraction obtained by ethanol treatment were examined, and the patterns of proteins obtained from control and trypsin-treated L-subparticles are given in Fig. 6. No differences were detected when low amounts (40 ng of trypsin/ A_{260} unit) of trypsin were used. Two of the spots corresponding to acidic proteins were still present after L-subparticles had been treated with 120 ng of trypsin/ A_{260} unit.

Analysis of the protein moiety of L-subparticles after trypsin treatment

The effects of trypsin on the protein moiety of L-subparticles were investigated by polyacrylamide-gel electrophoresis by using both SDS/one-dimensional and two-dimensional systems. To simplify the protein mixtures, protein-deficient core-particles were prepared from trypsin-treated L-subparticles by treatment with either high salt (2.75 M- NH_4Cl /69 mM- MgCl_2) or ethanol [0.25 M- NH_4Cl /50 (v/v) ethanol]. Proteins were then isolated from core-particles. Both core-particle and split-protein fractions were analysed.

The principal results are summarized in Figs. 7 and 8 and in Tables 5–7. The largest protein (approx. 51 000 daltons) was found to be progressively hydrolysed first to 45 000, then to 43 000, 42 000 and finally 40 000 daltons (see Fig. 7). This

Fig. 7. Progressive hydrolysis of the largest protein of L-subparticles by trypsin treatment as shown by SDS/polyacrylamide-gel electrophoresis

After treatment with trypsin, L-subparticles were subjected to high-salt shock and proteins were isolated from the core-particle and split-protein fractions. The proteins were then analysed by the discontinuous buffer system of Laemmli (1970) by using a 5% (w/v) acrylamide stacking gel and a 12.5% (w/v) separating gel. The proteins were stained with Coomassie Brilliant Blue R250. Gels: (a) core-particle proteins from control L-subparticles [the largest protein (shown by the arrow) is already partly degraded]; (b) core-particle proteins from L-subparticles treated with 33 ng of trypsin/ A_{260} unit; (c) molecular-weight marker proteins, respectively phosphorylase (90 000), transferrin (78 000), bovine serum albumin (67 000), ovalbumin (45 000), carboxypeptidase (34 300), lactoglobulin (18 400), myoglobin (17 000) and cytochrome c (12 500); (d) core-particle proteins isolated from L-subparticles treated with 83 ng of trypsin/ A_{260} unit; (e) core-particle proteins isolated from L-subparticles treated with 200 ng of trypsin/ A_{260} unit; (f) core-particle proteins isolated from L-subparticles treated with 500 ng of trypsin/ A_{260} unit. Trypsin treatment was for 15 min at 30°C. Activity of the L-subparticles in the puromycin reaction is given in Fig. 2.

Table 6. *Molecular weights of proteins of high-salt shock core-particles attacked by trypsin*

After treatment of L-subparticles with low concentrations of trypsin (30–50ng of trypsin/ A_{260} unit) high-salt core-particles were prepared, and the proteins were isolated and analysed by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970). The molecular weights (an average of at least five determinations) were obtained of those protein bands that were clearly and reproducibly affected by the trypsin treatment. The standard deviations are given. Because the split-proteins do not seem sensitive to the trypsin treatment, the sensitive core-particle proteins represent all of the L-subparticle proteins of molecular weight greater than 18000 that are sensitive to trypsin. For comparison, the proteins of rat liver L-subparticles judged by Henkel *et al.* (1974) to be very sensitive to trypsin treatment are also included. The molecular weights of the rat liver proteins were taken from Bielka & Stahl (1978).

$10^{-3} \times \text{Mol. wt of trypsin-sensitive proteins}$		
Rabbit high-salt core-particles	Rat liver L-subparticles	
$52 \pm 2^*$	L2	55.5
	L4	45.5†
35.5 ± 1	L5	35.5
33.5 ± 1	L6	35.5
32.5 ± 0.5	L29	31.5
29.5 ± 1	L20	30.5
24.0 ± 1	L27	23.5
21.0 ± 1	L35a	22.0
18.5 ± 0.5	L24	18.5
	L35b	14.5

* This protein was found to be partly degraded in many preparations of L-subparticles that had not been deliberately exposed to trypsin. In these cases a new band of 45000 daltons was observed. This protein was then degraded in a stepwise fashion to 40000 daltons as the dosage of trypsin was increased (see Fig. 9).

† In rabbit L-subparticles a protein of this size, sensitive to trypsin, corresponds to a cleavage product of the largest protein analogous to protein L2 of rat liver.

stepwise hydrolysis suggests that approx. 110 amino acid residues at the end(s) of the protein are accessible to trypsin. One site, approx. 60 amino acids from one end, seems especially vulnerable, because in several preparations of L-subparticles this protein was already degraded to a 45000-dalton fragment in the non-trypsin-treated controls. These L-subparticles were still active in polyphenylalanine synthesis. Inspection of one-dimensional SDS/polyacrylamide gels (see Fig. 7 and Table 6) reveals that six other protein bands ranging in mass from 18000 to 3500 daltons were also affected by trypsin

treatment (40ng of trypsin/ A_{260} unit of L-subparticle). Several proteins were found to be missing from two-dimensional gels (Fig. 8). The larger proteins of the split-protein fractions were not affected by trypsin. The results observed for the split-proteins are summarized in Table 5.

Proteins of rabbit reticulocyte L-subparticles were enumerated by Martini & Gould (1975), who used SDS in the second dimension, and by Howard *et al.* (1975), who used the method of Kaltschmidt & Wittmann (1970). We have used a modification of the Kaltschmidt & Wittmann (1970) method that gives patterns close to those obtained by the original method (Knopf *et al.*, 1975; Kenny *et al.*, 1975). Some correlations can be made between these studies based on the molecular weights of the proteins and their positions in two-dimensional gels. Some of the properties of the six largest proteins of the L-subparticle established in the present study are summarized in Table 7. These six proteins correspond (within experimental error) in mass to the six largest proteins (proteins L1, L2, L3a, L3b, L4 and L5) described by Martini & Gould (1975). The first cleavage product of the largest protein appears to correspond both in mass and in its position in two-dimensional gels to protein L17 of Howard *et al.* (1975). Protein L5 of Howard *et al.* (1975) corresponds in mass to a protein that was isolated from trypsin-treated but not from control L-subparticles, and protein L29 is tentatively identified as a protein that is attacked by trypsin. Further correlations are hampered by the difficulties of distinguishing unambiguously between proteins of similar mass and basicity.

Two-dimensional patterns of ribosomal proteins isolated from different mammalian species closely resemble one another (for review see Bielka & Stahl, 1978). The close correlation in the molecular weights of proteins of rabbit reticulocytes and rat liver sensitive to trypsin (Table 6) is to be expected if the two sets of proteins are closely related.

Chymotrypsin treatment of L-subparticles led to results similar to those found for trypsin treatment. Activity in polyphenylalanine synthesis was diminished, activity in the puromycin reaction was enhanced, and the proteins that were seen to be cleaved by trypsin were also cleaved by chymotrypsin. The largest protein was shown to be sensitive to low amounts of chymotrypsin (results not shown).

Analysis of the protein moiety of L-subparticles isolated from trypsin-treated polyribosomes

Polyribosomes were treated with trypsin and L-subparticles were then isolated (see above). The protein moiety of L-subparticles was then analysed by SDS/polyacrylamide-gel electrophoresis. No evidence for the protection of particular

Fig. 8. Analysis of the protein moiety of trypsin-treated L-subparticles by two-dimensional gel electrophoresis. Core-particles were prepared from L-subparticles by treatment with 0.25 M-NH₄Cl/50% (v/v) ethanol as described in the Methods section. Proteins were then isolated and resolved by two-dimensional gel electrophoresis as described by Cox & Greenwell (1976). First dimension, migration (left to right) in 4% (w/v) acrylamide at pH 5.5; second dimension, migration from top to bottom in 18% (w/v) acrylamide at pH 4.5. (a) Proteins from control core-particles; (b) proteins from core-particles derived from L-subparticles treated with 50 ng of trypsin/A₂₆₀ unit. †, Protein spots that are absent after trypsin treatment. The white lines in the photograph arise from hairline cracks in the gel.

Table 7. Summary of the properties of several of the larger proteins of rabbit L-subparticles. Molecular weight (average of five determinations) were measured by SDS/polyacrylamide-gel electrophoresis (see Tables 5 and 6). Rabbit reticulocyte ribosomal proteins were enumerated by Martini & Gould (1975), who used SDS in the second dimension, and by Howard *et al.* (1975), who followed the method of Kaltschmidt & Wittman (1970). Tentative correlations between the two sets of data were presented by Martini & Gould (1975). Abbreviation: n.d., no effect of trypsin treatment (approx. 40 ng of trypsin/A₂₆₀ unit) was detected.

10 ⁻³ × Mol.wt.	Location after high-salt shock (2.75 M-NH ₄ Cl/69 mM-MgCl ₂ , 15 min at 0°C)	Sensitivity to trypsin (approx. 40 ng/A ₂₆₀ unit)	Correlation with proteins enumerated by Martini & Gould (1975)	
			Protein	10 ⁻³ × Mol.wt.)
52 ± 2*	Core	Yes	L1	53.3
47.8 ± 1	Core	n.d.	L2	48.8
38.0 ± 0.8	Split	n.d.	L3a	40.5
36.8 ± 0.8	Core	n.d.	L3b	39.9
35.5 ± 0.8	Split	n.d.	L4	36.0
35.0 ± 0.8	Core	Yes	L5	35.6

* This protein appears to yield a hydrolysis product of 442 000 ± 800 daltons that corresponds to protein L17 enumerated by Howard *et al.* (1975) that on further digestion by trypsin leads to a product similar in molecular weight to protein L5 of Howard *et al.* (1975).

proteins was obtained (see Fig. 9). Although the data of Table 2 indicate that their location in poly-ribosomes tends to shield L-subparticles from attack by trypsin, this is not reflected in the electrophoretograms.

Discussion

The results (Table 1) show that although the rRNA moiety of ribosomal subparticles may be fragmented by RNAase treatment (i.e. RNAase I

Fig. 9. Analysis of the protein moiety of L-subparticles isolated from trypsin-treated polyribosomes by SDS/polyacrylamide-gel electrophoresis

Polyribosomes were treated with trypsin (40 or 120 ng of trypsin/ A_{260} unit) and subparticles were prepared and isolated by means of sucrose gradients. The isolated L-subparticles were subjected to high-salt shock with 2.75 M- NH_4Cl /69 mM- MgCl_2 , core-particles were isolated and proteins were prepared by the standard method and then analysed by SDS/polyacrylamide-gel electrophoresis. (a) Gels 1 and 6, molecular-weight marker proteins (see legend to Fig. 7); gel 2, proteins from high-salt cores of trypsin-treated L-subparticles (40 ng of trypsin/ A_{260} unit); gel 3, proteins from high-salt cores derived from L-subparticles isolated from trypsin-treated polyribosomes (40 ng of trypsin/ A_{260} unit) [the derived L-subparticles had diminished activity in poly(U)-directed polyphenylalanine synthesis (45% of control, see Table 4) and enhanced activity in the puromycin reaction (120% of control)]; gel 4, core-particle proteins from L-subparticles isolated from control polyribosomes; gel 5, core-particle proteins from a standard large-scale preparation of L-subparticles; (b) gel 1, core-particle proteins of L-subparticles isolated from trypsin-treated polyribosomes (100 ng of trypsin/ A_{260} unit); the derived L-subparticles had 150% of control activity in the puromycin reaction and 33% of control activity in poly(U)-directed polyphenylalanine synthesis (see Table 3); gel 2, core-particle proteins from L-subparticles isolated from control polyribosomes; gel 3, core-particle proteins from a standard large-scale preparation of L-subparticles; gel 4, molecular-weight marker proteins (see legend to Fig. 7).

and RNAase T_1), the subparticles remain functional in poly(U)-directed polyphenylalanine synthesis. The ability of core-particles derived from L-subparticles by high-salt treatment (Cox & Greenwell, 1976) to recombine with split-proteins was scarcely altered by previous RNAase treatment. The introduction of up to 16–100 scissions in the RNA moiety of L-subparticles could not account for the variation in the activity of L-subparticles in reconstruction experiments.

However, trypsin treatment had a marked effect on both the capacity of L-subparticles to function in poly(U)-directed polyphenylalanine synthesis and on the reconstruction of L-subparticles from high-salt cores and split-protein fractions (Fig. 1). It appears as though hidden damage to proteins is revealed by

high-salt-shock treatment of trypsin-treated L-subparticles. Chymotrypsin also diminished the activity of L-subparticles, and so it is likely that adventitious proteinase action could account for the variations in the activity of L-subparticles that we have observed.

The peptidyltransferase centre of L-subparticles is protected from the effects of both RNAase and trypsin. Indeed, trypsin treatment enhances the activity of L-subparticles in the puromycin reaction, possibly by making the tRNA-binding sites more accessible to isoleucyl-tRNA and puromycin. The sites themselves are not attacked by hydrolytic enzymes, either because the sensitive bonds are protected by their secondary and/or tertiary structure, or because the sites themselves are not accessible to enzymes, or because the active centre is

devoid of sensitive sites. Further work is needed to distinguish between these possibilities.

The marked effect of trypsin treatment of L-subparticles on the capacity to function in poly(U)-directed polyphenylalanine synthesis is attributed to an impairment of the binding site(s) for S-subparticles. the peptidyltransferase activity is not diminished and the acidic proteins required for translocation are also unaffected. Although it appears that the L-subparticle protein(s) involved in binding S-subparticles remain with core-particles produced by high-salt-shock treatment, we have not further identified the protein(s) involved. However, they are likely to be one or more of the core-particle proteins that are known to be susceptible to trypsin (see Table 6). Some limited protection is afforded to the S-subparticle-binding protein(s) on the formation of polyribosomes as shown above (see Tables 2 and 3 and Fig. 5). The ability of trypsin-treated polyribosomes to complete globin chains, even though the constituent L-subparticles have diminished capacity to bind to S-subparticles, suggests that the nascent polypeptide chain (and/or mRNA) plays a role in the stabilization of the interaction between L- and S-subparticles, as was discussed above. If the nascent polypeptide chain does play a role in subparticle interaction, then it appears that it is less susceptible to trypsin treatment than the L-subparticle protein(s) implicated in S-subparticle binding.

Earlier work has shown (Morgan *et al.*, 1963; Kaji *et al.*, 1966; Nair *et al.*, 1966; Delihis, 1970) that proteinase treatment of ribosomes gives rise to subparticles. The techniques were not then available to investigate the damage incurred by the component subparticles, and the effects of trypsin were attributed to hydrolysis of the nascent polypeptide chain, among other factors. In contrast, we present direct evidence that protein(s) implicated in the binding of S-subparticles are attacked.

The observation that RNAase treatment of L-subparticles had no effect on their activity in protein synthesis *in vitro* shows that this treatment did not affect the S-subparticle-binding sites. Hence, if RNA sequences are important to S-subparticle binding, they are not attacked by RNAases under the conditions of our experiments. The requirement for Mg^{2+} ions for subparticle interaction may arise because a minimum concentration of this ion is needed to maintain the tertiary structure of the rRNA moiety (Cox & Hirst, 1976), which in turn may influence the configuration of particular proteins. Whereas the roles of Mg^{2+} and RNA in the interaction between subparticles are not clear, it is evident that the integrity of a particular L-subparticle protein(s) is essential.

The stepwise hydrolysis by trypsin of the largest protein of rabbit L-subparticles implies that a stretch

of approx. 100 amino acid residues close to one end of the molecule is particularly sensitive to hydrolysis, possibly because this part of the protein is exposed. There is no conflict between the results of this study and earlier work (for review see Bielka & Stahl, 1978), but neither are there many points where direct comparison is possible. The novel features of the present study concern the effects of proteinase treatment on particular functions of L-subparticles.

We thank Betty Higginson for skilled technical assistance.

References

- Arnstein, H. R. V., Cox, R. A. & Hunt, J. A. (1964) *Biochem. J.* **92**, 648–661
- Arpin, M., Reboud, J. P. & Reboud, A. M. (1975) *Biochimie* **57**, 1177–1184
- Bielka, H. & Stahl, J. (1978) *Biochem. Ser. One* **18**, 111–168
- Blobel, G. & Sabatini, D. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 390–394
- Brimacombe, R., Nierhaus, K. H., Garrett, R. A. & Wittmann, H. G. (1976) *Prog. Nucleic Acid. Res.* **18**, 1–36
- Cahn, F., Schachter, E. M. & Rich, A. (1970) *Biochim. Biophys. Acta* **209**, 512–520
- Cammarano, P., Felsani, A., Gentile, M. & Guelerzi, G. (1972) *Biochim. Biophys. Acta* **281**, 625–642
- Cox, R. A. (1968) *Q. Rev. Chem. Soc.* **22**, 499–526
- Cox, R. A. (1977) *Prog. Biophys. Mol. Biol.* **32**, 193–231
- Cox, R. A. & Greenwell, P. (1976) *Biochem. J.* **160**, 533–546
- Cox, R. A. & Greenwell, P. (1980) *Biochem. J.* **186**, 861–872
- Cox, R. A. & Hirst, W. (1976) *Biochem. J.* **160**, 505–519
- Cox, R. A., Greenwell, P. & Hirst, W. (1976) *Biochem. J.* **160**, 521–531
- Delihis, N. (1970) *Biochem. Biophys. Res. Commun.* **39**, 905–910
- Falvey, A. K. & Staehelin, T. (1970) *J. Mol. Biol.* **53**, 1–19
- Henkel, B., Welfe, H. & Bielka, H. (1974) *Acta Biol. Med. Ger.* **33**, 691–698
- Howard, G. A., Traugh, J. A., Croser, E. A. & Traut, R. R. (1975) *J. Mol. Biol.* **93**, 391–404
- Huvos, P., Vereczkey, L. & Gaal, O. (1970) *Biochem. Biophys. Res. Commun.* **41**, 1020–1026
- Kaji, H., Suzuka, I. & Kaji, A. (1966) *J. Mol. Biol.* **18**, 219–234
- Kaltschmidt, E. & Wittmann, H. G. (1970) *Anal. Biochem.* **36**, 401–412
- Kenny, J. W., Sommer, A. & Traut, R. R. (1975) *J. Biol. Chem.* **250**, 9434–9436
- Knopf, U. C., Sommer, A. & Traut, R. R. (1975) *Mol. Biol. Rep.* **2**, 35–40
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Leader, D. P. & Coia, A. A. (1978) *Biochem. J.* **176**, 569–572

- Martin, T. E., Bicknell, J. N. & Kumar, A. (1970) *Biochem. Genet.* **4**, 603–615
- Martini, O. H. W. & Gould, H. J. (1975) *Mol. Gen. Genet.* **142**, 299–316
- Morgan, R. S., Greenspan, C. & Cunningham, B. (1963) *Biochim. Biophys. Acta* **68**, 642–644
- Nair, R., Zak, R. & Rabinowitz, M. (1966) *Biochemistry* **5**, 2674–2680
- Neville, D. M. (1971) *J. Biol. Chem.* **246**, 6328–6334
- Nomura, M. (1973) *Science* **179**, 864–873
- Reyes, R., Vazquez, D. & Ballista, J. P. G. (1977) *Eur. J. Biochem.* **73**, 25–31