A RIBONUCLEOPROTEIN CORE IN THE 50 S RIBOSOMAL SUBUNIT OF
ESCHERICHIA COLI

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1. Introduction

Many methods have been used for investigating the structural organisation of the ribosome. Although steady progress has been made in characterising both the RNA and protein regions that are accessible on the surface of the ribosome and the approximate locations of the RNA binding sites of the proteins, little is known about the internal organisation of proteins and RNA in the ribosomal subunits (reviewed in [1]).

Recently, evidence has accumulated for the presence of a large RNA core in the 5'-one third of 23 S RNA; when protein L24 is bound, based on:

(i) Limited ribonuclease digestion [2].
(ii) Electron microscopy [3].
(iii) Physical chemical studies [4].
(iv) Identification of RNA–RNA interactions between widely separated sequence regions [5] in a complex of protein L24 and 23 S RNA.

There are also indications that a stable protein–RNA core may exist in 50 S subunits which includes this RNA region and a small group of proteins. The evidence derives from three main sources:

(i) In vitro assembly of 50 S subunit proteins to large fragments of 23 S RNA [6].
(ii) Intermediate particles formed during in vitro assembly of 50 S subunits [7].
(iii) Studies on protein-depleted ribosomes prepared in high salt [8–10].

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In the present work we provide additional, and more direct, evidence for such a protein–RNA core using the approach of controlled trypsin and ribonuclease digestion of 50 S subunits. A resistant protein–RNA fragment complex containing proteins L3, L13, L17, L21, L22, and L24 was reproducibly formed.

2. Materials and methods

50 S subunits were isolated from 70 S ribosomes of *Escherichia coli*, strain A19, by sucrose gradient zonal centrifugation [11]. They were concentrated either by high speed centrifugation or by precipitation with 10% polyethylene glycol 6000 in the presence of 10 mM $MgCl_2$. They were dissolved at 2.3 mg/ml in TMA buffer (10 mM Tris–HCl, pH 7.8, 10 mM $MgCl_2$, 30 mM NH_4Cl and 6 mM 2-mercaptoethanol) and stored at −80°C. Total 50 S subunit proteins were isolated by a standard procedure [12].

Aliquots of 50 S subunits were degraded with a series of trypsin concentrations ranging from 0.03–22 enzyme units/mg total 50 S subunit protein for 20 h at 37°C. The highly purified Worthington sterile trypsin (245 enzyme units/mg) was used. It was the only commercially available trypsin that was essentially ribonuclease free [13]. Trypsin-treated 50 S subunits were fractionated by gel filtration on an agarose A 0.5 (Biorad) column (1 × 40 cm). From experience with 16 S RNA fragments it was inferred that only fragments containing ⩽ 300 nucleotides were included in the agarose. Excluded peak fractions were pooled, precipitated with 5% trichloroacetic acid [14], electrophoresed on dodecylsulphate–poly-

acrylamide slab gels [15] and stained for protein with Coomassie brilliant blue.

The state of the RNA was examined by dissolving the precipitate in 1% dodecylsulphate and 8 M urea and electrophoresing in 3–15% gradient polyacrylamide slab gels containing 20 mM Tris–HCl, pH 7.4 and 7 M urea at about 4 V/cm, overnight, with circulating buffer. 16 S RNA, 5 S RNA and tRNA were run as markers.

The proteins in the trypsin-digested product were identified by degrading 50 S subunits at the highest trypsin concentration (22 enzyme units/mg total protein) on a larger scale. The product was isolated by centrifuging for 7 h at 38 000 rev./min. Pellets were dissolved in TMK reconstitution buffer (30 mM Tris–HCl, pH 7.4, 20 mM $MgCl_2$, 350 mM KCl, 6 mM 2-mercaptoethanol) at 11.5 mg RNA/ml.

Part of the degradation product was analysed directly by 2-D gel electrophoresis [16] and stained for protein with Coomassie brilliant blue. The protein samples were prepared by treatment of the ribonucleoprotein with ribonuclease A and T_1 in the presence of sarkosyl and EDTA as in [17]. Aliquots of the remainder of the trypsin-treated product were further digested with either ribonuclease A from Worthington (70 enzyme units/mg RNA) or T_1 from Sankyo, Japan (3200 units/mg RNA), or a mixture of both at the same concentrations. This product was fractionated rapidly on an agarose A 0.5 column (1 X 40 cm). Excluded peak fractions were pooled, precipitated with ethanol and analysed for protein content on 2-D gels.

3. Results

Polyacrylamide gel analyses of the protein contents of the trypsin-treated 50 S subunits, that were excluded by agarose gel filtration, are illustrated in fig.1. Multi-banded patterns were observed at each trypsin concentration tested. Even though the trypsin concentration was varied over almost two orders of magnitude there was little qualitative difference in the protein compositions of the nucleoprotein products (fig.1B–F). At the highest trypsin concentration shown (fig.1F) the strongest bands were observed in the gel region where proteins L9, L11, L17, L18, L19, L22, L23, L24 (fig.1G) and L27 are known to migrate
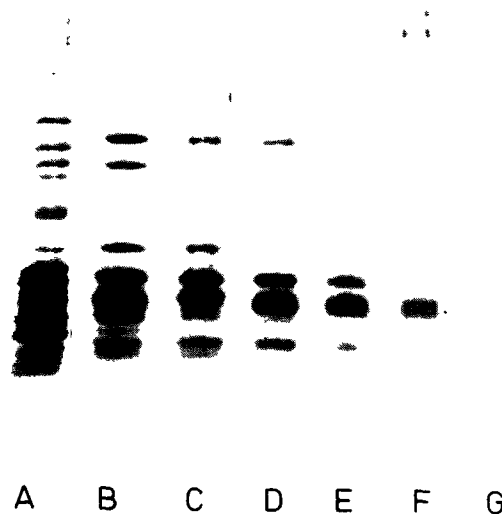

A   B   C   D   E   F   G

Fig.1. Effect of trypsin on the 50 S subunits. Proteins were resolved in a dodecylsulphate–polyacrylamide slab gel. (A) Control total 50 S subunit proteins. (B–F). Increasing trypsin concentrations in enzyme units/mg total 50 S subunit protein were used equivalent to B-0.35, C-0.7, D-2.2, E-4.5, F-22 units, G-L24 protein marker.

[18]; protein aggregates were also observed at the high trypsin concentrations (fig.1F).

The amount of RNA degradation that occurred during the long incubation at 37°C was monitored, qualitatively, by estimating the amount of A_{260} absorbing material that was excluded from the agarose columns. Over the trypsin range tested 65–80% of the A_{260} absorbing material eluted in the excluded volume. In a control sample, where no trypsin was added during the incubation, 90% of the absorbing material was excluded. When the RNA in the excluded peaks was examined by gradient gel electrophoresis, products in the ∼50–500 nucleotides range were found in all trypsin-treated samples. This limited degradation was attributed primarily to the RNA becoming increasingly accessible to trace amounts of endogenous ribonuclease during proteolysis of the proteins.

A 2-D gel of the subunits treated at the highest trypsin concentration is shown in fig.2. The resistant proteins were identified by comparison with a gel of the total 50 S subunit proteins as L3, L13, L17, L21,

Fig.2. 2-D gel electrophoresis of trypsin-digested 50 S subunits. 50 S subunits, 2.3 mg/ml, were digested for 20 h at 37°C with trypsin at 22 enzyme units/mg protein.

L22, L24 and L30. One protein remained at the origin of the second dimension which may correspond to L4. Three well-defined protein fragments were also present. The one at the same level as L3 probably derives from L2; it has been isolated directly from 50 S subunits [18]. The others, adjacent to L13 and slightly above L23, are of unknown origin. A number of less distinct fragments occurred in the region between the proteins L22, L24 and L30.

After trypsin digestion the product was further treated with ribonuclease(s) and fractionated. The %RNA loss was monitored by ΔA_{260} with the following results: ribonuclease A treatment removed 20% and ribonuclease T_1, or both nucleases, displaced about 30% of the RNA. Identical protein contents were found after the different ribonuclease treatments. The results are illustrated in fig.3 and show that proteins L3, L13, L17, L21, L22, L24 and the protein

fragment from L2 all appear as relatively strong spots. L30 was not detected and the fragment adjacent to L13 was absent. Although some staining occurred in the neighbourhood of L23 it was very diffuse and also migrated too slowly for this protein; it probably derived from the unidentified fragment in this position in fig.2. The relative intensities of the different spots in fig.3 suggest the presence of a more homogenous ribonucleoprotein than was observed prior to the nuclease treatment in fig.2.

Kinetic studies on the trypsin and the ribonuclease digestion products, described above, revealed that their protein contents, as shown in fig.2 and 3, respectively, did not change at longer digestion times than those used above; the only detectable change was that the yield of the ribonucleoprotein slowly decreased with time. It was concluded, therefore, that a highly resistant 'end-product' had been formed.
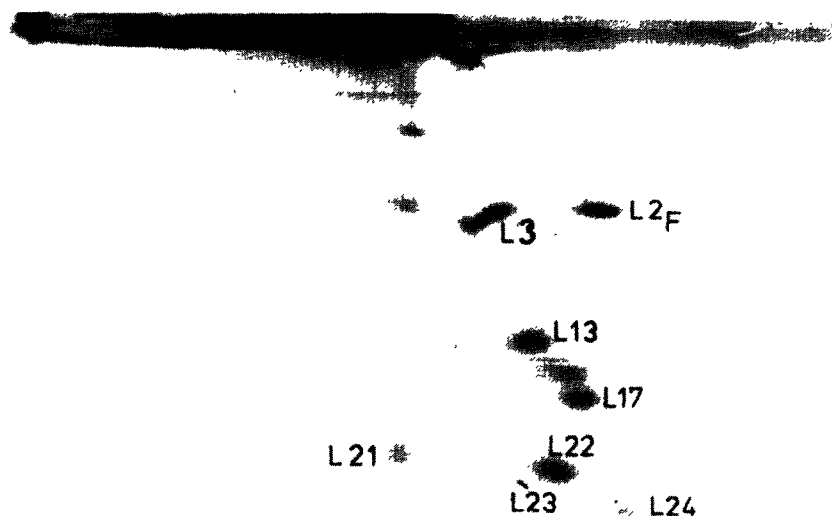
Fig.3. 2-D gel electrophoresis of 50 S subunits after treatment with trypsin (see fig.2) and then T_1 ribonuclease. The trypsin-digested product, at $\sim 35\,A_{260}$ units/ml, was incubated with T_1 ribonuclease for 30 min at 0°C at a ratio of 3200 enzyme units/mg RNA.

Table 1
A summary of data compatible with a protein–RNA core in the 50 S subunit

	L3	L4	L13	L17	L20	L21	L22	L24
Assemble 5'-end 23 S RNA [6,19]	?	+	+		+	+	+	+
Reconstitution intermediate in vitro [7]	+	+	+		+		+	+
Core from 5.5 M LiCl-treated subunits [10]	+	+	+	+			+	
Trypsin/nuclease -treated subunits	+	?	+	+		+	+	+

The data in the first three rows are taken from the literature cited and those of the last row are from the present experiments. The absence of protein L20 in the high salt and enzyme-treated subunits (rows 3 and 4) may reflect that this highly basic protein is very difficult to displace from the 23 S RNA. Also, protein L4, as mentioned in the text, may escape detection if it does not run in the second dimension of the gel electrophoresis

4. Discussion

The results from the present experiments strongly support the presence of a core, consisting of a group of about 6 proteins and an unidentified part of the 23 S RNA, which is strongly resistant to both trypsin and ribonuclease digestion. No smaller, less complex, degradation product was formed, even after much longer digestion times.

Earlier evidence for such a core structure in the 50 S subunit was mainly circumstantial and is summarised below.

(i) Assembly of proteins to large 23 S RNA fragments indicated that a similar group of proteins (see table 1) associates primarily with the 5'-one third of the 23 S RNA [6,19].

(ii) These proteins are amongst the main constituents of ribosomal reconstitution intermediates formed with 23 S RNA both in vivo [9,20] and in vitro [7].

(iii) The proteins are resistant to high-salt dissociation [8,10].

The data are summarised in table 1. The extent of correlation between the results of the different methods is very good and contrasts with the extreme difficulty of correlating the data that were obtained from similar investigations on the 30 S subunit (e.g., [1]).

Although the nucleotide sequence of the RNA moiety was not determined, it almost certainly contains more than the 5'-one third of the 23 S RNA. The evidence is as follows:

(1) Only ~50% RNA was removed from the 50 S subunits.

(2) The RNA 'binding sites' of proteins L2 and L3 occur in the 3'-two thirds of the 23 S RNA [6,19,21].

(3) Branlant et al. [10] recently deproteinised 50 S subunits partially in 5.5 M LiCl, 10 mM MgCl$_2$ (see table 1) and digested the products with ribonuclease. Preliminary sequence analyses indicated that the resultant ribonucleoprotein contained sections of the 3'-two thirds of 23 S RNA in addition to the 5'-region.

Earlier studies on the enzymic digestion of ribosomal protein—RNA complexes have shown that the basis of the resistance depends on two factors:

(1) The inherent resistance of certain protein and RNA structures.

(2) The mutual protection of interacting protein and RNA structures (e.g., [2,22—24]).

Therefore, we cannot infer anything directly, from the present experiments, about the location of the protein—RNA core in the 50 S subunit. However, the observation that none of the 6 'core' proteins were modified in the 50 S subunit by lactoperoxidase, whereas 10 other proteins were [25,26], and that for 3 of them, namely L13, L21 and L24, little or no immunochemical evidence could be found for their accessibility on the 50 S subunit [27], suggests that the protein—RNA core is relatively inaccessible in the 50 S subunit.

In conclusion, the experiments demonstrate that under the appropriate conditions a relatively homogeneous protein—RNA complex can be isolated directly from the 50 S subunit. During its preparation it is important to avoid the ribonuclease contamination that is normally present in the commercial trypsin, otherwise one prepares the protein—RNA product, reported by others [4,28,29], that contains predominantly, but by no means exclusively, protein L24.

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