Fragment of protein L18 from the *Escherichia coli* ribosome that contains the 5S RNA binding site

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ABSTRACT

A fragment of ribosomal protein L18 was prepared by limited trypsin digestion of a specific complex of L18 and 5S RNA. It was characterised for sequence and the very basic N-terminal region of the protein was found to be absent. No smaller resistant fragments were produced. 5S RNA binding experiments indicated that the basic N-terminal region, from amino acid residues 1 to 17, was not important for the L18-5S RNA association.

Under milder trypsin digestion conditions three resistant fragments were produced from the free protein. The largest corresponded to that isolated from the complex. The smaller ones were trimmed slightly further at both N- and C-terminal ends. These smaller fragments did not reassociate with 5S RNA.

It was concluded on the basis of the trypsin protection observations and the 5S RNA binding results that the region extending from residues 18 to 117 approximates to the minimum amount of protein required for a specific and stable protein-RNA interaction.

The accessibility of the very basic N-terminal region of L18, in the L18-5S RNA complex, suggests that it may be involved, in some way, in the interaction of 5S RNA with 23S RNA.

INTRODUCTION

Protein L18 is one of the more interesting proteins of the *Escherichia coli* ribosome. It binds directly to 5S RNA (1) with a binding constant of about 10^7 (2,3). It is elongated at least in its isolated state with an axial ratio of about 6:1 (4) and it exhibits a limited amount of tertiary structure (5). It has an exceptionally basic N-terminal region (5,6).

There are two other proteins, L5 and L25, which bind directly and less strongly to 5S RNA (1-3,7-11). In the presence of these proteins 5S RNA associates stoichiometrically with 23S RNA (12, 13). The mechanism of this association is not yet understood although the presence of the proteins, and especially L18, is

required. Low angle X-ray scattering studies have demonstrated that a significant increase in the radius of gyration of the 5S RNA occurs when L18 is complexed and analysis of the scattering curves indicated that the data were compatible with a model in which a substantial part of the L18 projects out from the 5S RNA, and it would be accessible for interacting with other ribosomal components. However, since at least two of the proteins, namely L18 and L25, may induce conformational changes in the 5S RNA (9, 14), it cannot necessarily be inferred that the proteins directly link the RNA's. Indeed, it has been demonstrated by Noller and co-workers (15) that a conserved sequence region in 5S RNA is complementary to a 12 nucleotide sequence in 23S RNA although, at present, there is no experimental evidence to suggest that these regions interact.

This protein-RNA system containing L5, L18 and L25 and 5S and 23S RNA's may be considered as a microcosm of the ribosome and, because of its relatively simple structure, it may be possible to elucidate its mechanism of assembly by known biochemical methods. In the present study, as a first step, we have set the limited objective of defining the region of protein L18 that binds to 5S RNA and in particular the role of the basic N-terminal region of the protein. The method invoked was that of limited proteolytic digestion of the L18-5S RNA complex using a procedure that was first employed to demonstrate that the 16S RNA binding region of protein S4 was confined to the C-terminal three-quarters of the protein (16,17).

MATERIALS AND METHODS

Preparation of 5S RNA, protein L18, and L18-5S RNA complexes

5S RNA was prepared as described by Monier and Feunteun (18). The predominant presence of the native conformation (greater than 85%) was controlled electrophoretically (18). Protein L18 was fractionated by CM-cellulose and Sephadex gel filtration (19). The procedure was modified slightly to improve the yield of L18 (20). Protein L18 and fragment concentrations were determined by direct amino acid analysis in a Durrum amino acid analyser with 5 to 10 μ g protein. L18 concentrations were also determined with the Folin reagent (21). For L18 the amino acid analysis value

was approximately 75% of that obtained with the Folin reagent such that a correction factor of 0.75 was used instead of the earlier factor of 0.84 (22) that was based on a nitrogen determination of total ribosomal protein.

5S RNA-protein complexes were prepared by incubating 5S RNA with a 3-fold molar excess of protein L18 in a ribosome reconstitution buffer (30 mM Tris-HCl, pH 7.4, 20 mM MgCl₂ and 0.35 M KCl). In a typical experiment 0.5 mg 5S RNA was incubated with 0.45 mg L18 in 1 ml reconstitution buffer for 45 min at 33°C. The sample was cooled slowly and applied to a Sephadex G-100 column (1x150 cm). The main peak, containing the L18-5S RNA complex, was precipitated with 1.7 volumes ethanol for 16 hrs. It was dissolved in about 0.2 ml reconstitution buffer, frozen in liquid N₂ and stored at -80°C.

Trypsin digestion of the L18-5S RNA complex and protein L18

0.3 mg L18-5S RNA complex was dissolved in reconstitution buffer at a protein concentration of 0.2 mg/ml. It was digested with trypsin (Worthington TRL-50S sterile) at a 1:50 enzyme:protein ratio (w/w) and was incubated on ice for 1 hr. Trypsin inhibitor (Boehringer, Germany) was added at a 2:1 inhibitor:trypsin ratio (w/w) and the solution was dialysed against NH₄/Mg buffer (15 mM NH₄ bicarbonate, 10 mM Mg acetate, pH 7.9) overnight to remove salt and small peptides. After lyophilising, the protein fragment was extracted from 5S RNA in 67% acetic acid and separated from any undigested protein on a superfine Sephadex G-50 column (1x80 cm) in 15% acetic acid.

In control experiments 5S RNA was incubated with trypsin under the same conditions as the complex and electrophoresed in 10% polyacrylamide gels containing 40 mM Tris-HCl, pH 7.4 and 8 M urea. No breakdown products were observed on staining with pyronin G (22).

Trypsin-resistant fragments were prepared from the free protein. L18 was dissolved in the NH₄/Mg buffer at a concentration of 0.2 mg/ml. Trypsin was added at a 1:500 ratio (w/w) and the mixture was incubated on ice for 1 hr. The reaction was stopped by adding acetic acid to a final concentration of 10%. After lyophilising, the protein digest was dissolved in 0.2 ml 15%

acetic acid and applied to a superfine Sephadex G-50 column (1x80 cm).

All protein digestion products were characterised on dodecylsulphate-containing polyacrylamide slab gels (23).

Protein-chemical methods

0.15 mg protein L18 or L18 fragments (from the L18-5S RNA complex or the free protein) were dissolved in 0.2 ml of 0.2 M N-morpholine buffer, pH 8. They were digested with 10 µg trypsin for 4 hrs at 37°C, lyophilised, and dissolved in 10 µl electrophoresis buffer (pyridine, acetic acid, acetone and water 40:80:300:1580). Each sample was electrophoresed in the first dimension on microcrystalline cellulose plates (Polygram cel 300) for 1 3/4 hr at 480V in electrophoresis buffer. After drying, the peptides were chromatographed in the second dimension for 6 hrs in pyridine, acetic acid, butan-1-ol, water (50:15:75:60). The peptides were stained with either ninhydrin or fluoescamine (24), extracted in 5.7 M HCl containing 0.02% 2-mercaptoethanol and hydrolysed in sealed tubes under nitrogen for 20 hrs at 110°C. The sample was evaporated to dryness and the amino acid composition determined in a Durrum D-500 analyser.

The N-terminal amino acid of the fragment prepared from the L18-5S RNA complex and those of fragments F1, F2 and F3 from the free protein were determined using dansyl chloride (25).

Binding of the L18 fragment to 5S RNA

The protein fragment F1 was prepared from the L18-5S RNA complex as described. The mixture of the protein fragments was prepared from the free protein, also as described, except that the reaction was stopped by adding trypsin inhibitor at an inhibitor:trypsin weight ratio of 4:1 and the digestion products were not fractionated by gel filtration.

The protein fragment(s) and 5S RNA were incubated at a 3:1 molar protein excess for 40 min at 33°C in reconstitution buffer. The complexes were fractionated by electrophoresis (26) in 5% polyacrylamide slab gels in high salt buffer (30 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 0.25 M KCl) for 12 hrs at 3 V/cm. Gels were stained for RNA with 0.1% toluidine blue in 7.5% acetic

acid, or for protein with Coomassie brilliant blue (22). Control samples in which L18 and S4 were each incubated with 5S RNA at a 3:1 molar excess were co-electrophoresed. After staining, the 5S RNA-protein bands were excised and soaked in 30 mM Tris-HCl, pH 7.4, 20 mM MgCl₂, 0.1 M LiCl for 1 hr to reduce the salt concentration and inserted into the pocket of a 15% polyacrylamide slab gel containing dodecylsulphate (23) and covered in 10% dodecylsulphate. After standing for 12 hrs at room temperature the gel was electrophoresed as described (23) and stained for protein with Coomassie brilliant blue.

The saturation binding characteristics of the L18 protein and fragment to 5S RNA were compared. Samples of the protein or fragment were incubated with 16 µg 5S RNA at increasing molar amounts of protein in the range 0.6:1 to 8:1. The amount of binding was determined by the method described above. A calibration curve was prepared by running increasing amounts of L18 and fragment in the protein gel. The intensities of the stained bands were determined with a Joyce-Loebl micro-densitometer.

RESULTS

Trypsin digestion experiments

The effect of increasing amounts of trypsin on the digestion of the L18-5S RNA complex is shown in Fig. 1A. A single resistant protein fragment was produced in high yield (greater than 90%). Even at much higher trypsin levels (trypsin:L18, 0.2:1) a high yield (>80%) of L18 fragment was obtained. Since no other products were detected it was assumed that the excised region was degraded into small peptides that moved with the front and were not stained. In control experiments the 5S RNA was incubated alone with trypsin under the same conditions as the L18-5S RNA complex. No 5S RNA degradation products were detected after electrophoresing and staining for RNA.

We examined the free protein in order to establish whether it exhibited the same resistance to trypsin. The results illustrated in Fig. 1B demonstrate that the free protein is much less resistant to trypsin. Three main fragment bands F1, F2 and F3 were produced together with some smaller digestion products. F1 migrated with the same R_f value as the fragment prepared from

Fig. 1: The effect of increasing amounts of trypsin on (A) L18-5S RNA complex and (B) free L18 protein. Each sample in (A) contained 19 μ g complex and in (B) 5 μ g protein. Samples were prepared and electrophoresed as described in methods. F1, F2 and F3 denote the resistant protein fragments. R_f values of protein L18 and F1 were 0.68 and 0.76 respectively.

the L18-5S RNA complex (Fig. 1A).

Protein-chemical analyses

Fragments were isolated with a view to determining their sequences. For the L18-5S RNA complex the fragment was separated from the undigested protein. It was then digested with trypsin and subjected to peptide fingerprint analysis. Each peptide was analysed for amino acid composition. By comparison with the re-

sults from the whole L18 protein it was possible to establish that the seven N-terminal peptides T1 to T7 were completely absent from the fragment whereas the remainder, including the C-terminal peptide T22, were present. Three independent experiments were made with complete analyses and the results were identical, except that in one experiment only partial digestion was observed at the arginine residues between T10 and T11 (Arg-30) and at the lysine residues between peptides T14 and T15 (Lys-68) and peptides T18 and T19 (Lys-88) such that three additional weak spots were observed. The peptides absent from the fragment are shaded in Fig. 2B.

The excised sequence region is therefore the N-terminal 16 - 17 amino acid residues. There was some ambiguity in the assignment of the N-terminal amino acid of the protein fragment because both peptide T8a, which resulted from a partial cut at Arg-16 and peptide T8 (Lys-17) were very weak. Therefore, we determined the N-terminal amino acid using the dansyl chloride reagent. Weak lysine and strong leucine spots were detected that correspond to residues 16 and 17 in the sequence, respectively, indicating some heterogeneity in the fragment population. The sequence of the excised part of the protein, the order of the excised peptides, and the N-termini of the L18 fragment are all indicated in Fig. 3. This is the very basic part of the protein in which 9 of the 17 residues are arginine or lysine and only one residue is acidic.

The fragments produced from the free L18 protein could only be partially fractionated on a G-50 Sephadex column. Peptide maps were prepared from each of the partially purified fragments and each peptide was subjected to amino acid analysis. The seven peptides T1 to T7 were completely absent from all three fragments. The peptide map of F1 was indistinguishable from that of the fragment prepared from the L18-5S RNA complex. The peptide map of F2 was similar to that of F1 except that the yields of T8a and T9 were very low, and the latter were assumed to result from cross-contamination with F1. For F3 peptides T8a and T9 were not detected and the yield of the C-terminal peptide T22 was low. It was concluded that the latter was due to cross-contamination such that in F3 the C-terminal peptide of L18 had also been excised.

In order to check these conclusions for the N-termini of

Fig. 2A. Peptide map of the protein L18 in which the tryptic peptides are labelled. B. A summary of the results of the peptide fingerprints obtained with fragment F1 from the 5S RNA-L18 complex and fragments F1, F2 and F3 from the free protein. The dotted peptides were completely absent from F1. The striped peptides T8a and T9 were additionally absent or very weak for fragments F2 and F3. Peptide T22 was weak for fragment F3 (see text for details).

the fragments the N-terminal amino acids was determined for each fragment. Analysis of F1 produced leucine with a trace of lysine (as was found for the fragment prepared from the complex). F2 and F3 both gave leucine corresponding to Leu-26. Therefore, the

Fig. 3: The sequence of the N-terminal region of protein L18. The tryptic peptides, prefixed by T, are numbered as in Fig. 2. A denotes the N-termini of fragment F1 and B denotes the N-terminal cut of fragments F2 and F3. Basic amino acids are striped and acidic ones are dotted.

results support the inferences from the peptide map analyses (Fig. 3).

5S RNA binding studies

The results from the previous experiment suggest that the basic N-terminal region of protein L18 is not important for the interaction with 5S RNA. In order to demonstrate further that the main binding region of the protein lies in its C-terminal part, the protein fragment was isolated from the complex and its binding characteristics to 5S RNA were compared with those of the whole L18 protein. The fragment and L18 protein were incubated with 5S RNA at increasing protein:RNA molar ratios. The complex was separated from unbound protein electrophoretically (as in Fig. 4A), and the amount of protein present in the complex was quantitated electrophoretically (as in Fig. 4B). The saturation binding curves are shown in Fig. 5. The curves are very similar and form plateaux at approximately the same protein-RNA molar ratio. This indicates that both fragment F1 and L18 bind specifically to the 5S RNA and have similar binding properties. The observation that L18 and F1 bind at slightly less than one mole of protein per mole of 5S RNA can be attributable to errors in concentration determination, but it may also be due to a

Fig. 4: A-C. Electrophoresis of 5S RNA and complexes of 5S RNA with L18 and fragment F1 in high salt 8% polyacrylamide gels. A. 5S RNA stained with toluidine blue. B. L18-5S RNA complex stained for protein and C. F1-5S RNA complex stained for protein with Coomassie brilliant blue. D-G. Dissociation and electrophoresis in dodecylsulphate gels of complexes excised from gels B and C. D. L18-5S RNA complex, E. L18 control, F. F1-5S RNA complex and G. F1 control.

small amount of conformational heterogeneity in the RNA or some slight dissociation of the complex. The results reinforce the view that L18 binds to 5S RNA as a monomer (3,27) and not as a dimer (28).

The requirement for a significant molar excess of protein to produce saturation of binding (Fig. 5) is compatible with earlier protein-ribosomal RNA binding studies (e.g. 9,22). It may reflect, partly, the presence of some denatured protein molecules in the protein preparation as was recently inferred for protein S4 (17).

The suitability of the electrophoretic assay for establishing specific protein-RNA interaction was tested earlier (26) when it was demonstrated that no binding of several ribosomal proteins, including L18, occurred to tRNA when incubated under the above conditions. Nor did proteins other than L18, L25 or L5 bind to 5S RNA. In the above experiments a sample of protein S4 was incubated with 5S RNA and invariably showed no binding.

Fig. 5: Binding saturation curves for L18-5S RNA (●—●) and fragment F1-5S RNA (○—○). The binding assay was performed as illustrated in Fig. 4 and described in Methods. Error limits for binding were estimated at about $\pm 20\%$.

The mixture of fragments F1, F2 and F3, from the free protein, were also tested for their capacity to bind to 5S RNA using the electrophoretic procedure. The mixture of fragments (Fig. 1B) was incubated with 5S RNA in the presence of trypsin inhibitor. Only fragment F1 bound to the RNA (data not shown). It was concluded that the presence of the sequence region from residues 18 to 25 (see Fig. 3) was essential for the RNA interaction, although we could not ascertain whether it was directly involved in the interaction and/or essential for stabilising the tertiary structure of the protein fragment.

DISCUSSION

The results which are schematically summarised in Fig. 6 show that the basic N-terminal 16/17 amino acid residues are excised from the L18-5S RNA complex on limited trypsin digestion and that the remainder of the protein whilst exhibiting some intrinsic resistance to trypsin when uncomplexed is strongly protected when bound to 5S RNA. The binding studies indicate that the fragment has a high affinity for 5S RNA, such that its saturation binding characteristics are indistinguishable from those of the whole protein. These results strongly suggest that the primary binding region resides in the C-terminal part of the protein between residues 18 and 117. The incapacity of the smal-

Fig. 6: Summary of the trypsin digestion results on L18-5S RNA complexes and protein L18.

ler F2 fragment (residues 26-117), prepared from the free protein, to bind to 5S RNA suggests that residues 18-25 are important either directly for the protein-RNA interaction or for the tertiary structure of the protein, or both.

Such limited proteolysis studies have been performed on other ribosomal protein-RNA complexes. Protein S4-16S RNA was the first complex to be studied and it was found that the N-terminal 46 amino acids could be removed from both the complex and the free protein without detectably weakening the RNA binding properties of the protein (16,17,29). In addition, evidence was provided from reconstitution experiments that this N-terminal part of the protein was involved in protein-protein interactions (29). It was speculated, on the basis of this and other preliminary evidence (16), that there might be a small group of such proteins that are essential for ribosomal assembly, whose function is to link RNA to proteins (30).

In the free L18 protein, the N-terminal region is unlikely to be structured owing to its high density of positive charges and the presence of a helix-destabilising serine residue. It may therefore constitute a rather flexible part of the protein. The remainder of the protein is relatively trypsin resistant and probably more structured. NMR studies have provided evidence for tertiary structure interactions in L18 involving aromatic residues (5) and they almost certainly occur exclusively within the protein fragment. The recognition of 5S RNA by this part of the protein and its subsequent high resistance to trypsin digestion suggests that the tertiary structure of the protein fragment is

important for the RNA recognition. It is likely, also, that attempts to crystallise the protein-5S RNA complex, with a view to examining the geometry of the protein-RNA interaction by X-ray diffraction, may be more successful for the F1-5S RNA complex than for the more flexible L18-5S RNA complex.

The ready accessibility of the N-terminal region of L18 to trypsin, in the complex, suggests that it may be available for interacting with the other 5S RNA-binding proteins L5 and L25, possibly producing the observed cooperative protein binding effects, or it could be involved in the attachment of the 5S RNA-protein complex to the 23S RNA. These possibilities are currently being investigated.

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