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The characterisation of a fragment of ribosomal protein S4 that is protected against trypsin digestion by 16S ribosomal RNA of Escherichia coli

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ABSTRACT

After mild trypsin treatment of a complex of ribosomal protein S4 and 16S RNA of Escherichia coli, a large homogeneous fragment of the S4 protein was protected against digestion by its RNA binding site. This fragment was isolated and characterised for molecular weight. It was able to rebind specifically to 16S RNA. Preliminary results indicate that protected protein fragments can also be obtained from other proteins that complex specifically with 23S and 5S RNA.

INTRODUCTION

Protein S4 is an important structural protein in the 30S subunit, that binds directly to 16S RNA[1,2,3]. In the free state it has a very elongated structure[4] and recent electron microscopic evidence suggests that it is also elongated in the 30S subunit[5]. It has a large, structurally complex, and compactly organised RNA binding site[6-12].

Although a number of preliminary studies have been reported on the region(s) of protein S4 that interact with 16S RNA, the results only extend to a small number of amino acids that can, or cannot, be modified in the protein-RNA complex[13,14,15]. In order to obtain more precise information about the binding regions of the protein, we investigated whether, by mild trypsin digestion of the S4-16S RNA complex, protected protein fragments could be produced that would constitute RNA binding regions of the protein. A large homogeneous fragment of S4 was reproducibly obtained that rebound specifically with 16S RNA.

MATERIALS AND METHODS

Preparation of RNA, proteins and protein-RNA complexes

16S RNA and 23S RNA were extracted from 30S and 50S subunits, respectively, of Escherichia coli by three extractions with phenol-dodecylsulphate[3]. The RNA was checked for the absence of protein by electrophoresing 50 ug RNA in a 3% polyacrylamide gel, in 40 mM Tris-HCl, pH 7.4, and showing that the RNA band was not stained by Coomassie brilliant blue[3].

Protein S4 was prepared as described earlier[16]. No contaminating proteins were detected electrophoretically[17]. A molecular weight value of 22,550 was taken[18].

In several experiments S4 protein was radioactively labelled by incorporating a low level of [^{14}C]-methyl groups into the lysine residues by the standard method of Rice and Means[19], using [^{14}C]-formaldehyde (NEN, Frankfurt) and Na borohydride. This method results in a random modification of the lysine residues of S4[13]. The modified protein can assemble specifically with 16S RNA[20] and can be reconstituted to form biologically active 30S subunits[20]. Approximately 5 [^{14}C]-methyl groups were incorporated per protein molecule. For each batch of S4 protein used, both unlabelled and the [^{14}C]-methylated protein, saturation curves were prepared by the standard method[3,11] in order to check that the proteins were binding specifically; saturation of binding at a protein:RNA molar ratio of approximately 1:1 was always observed.

Complexes of protein S4 and 16S RNA were formed in TMK reconstitution buffer (30 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 0.35 M KCl and 6 mM 2-mercaptoethanol) at an RNA concentration of 4-5 mg/ml. Protein was added at a 3-5:1 molar excess. The solution was incubated at 40° for 1 hr. Non-bound protein was separated by A 0.5 agarose gel filtration (Biorad, California) on a 2 x 60 cm column. The protein-RNA complex, eluted in the excluded volume, was precipitated with 1.5 vol. ethanol for 36 hrs.

Preparation of the protein fragment

20 mg S4-16S RNA complex was dissolved in 8 ml 15 mM NH_4

bicarbonate and 20 mM Mg acetate, pH 7.9. It was digested with trypsin (TPCK-treated, Merck, Darmstadt) at an enzyme:protein S4 weight ratio of 1:150, at 0°, for 20 min. It was divided into two parts and passed over A 0.5 agarose columns (2 x 60 cm) that had been equilibrated with the above buffer. The RNA peaks were pooled and precipitated with 1.5 vol. ethanol for 36 hrs.

The precipitate was dissolved in 3 ml distilled water and adjusted to 80 mM Mg acetate. The protein fragment was extracted with 67 % acetic acid[21] for 2 hr on ice. Glass tubes were rinsed with 1 ml of a 0.1 % solution bovine serum albumin to reduce adsorption of the protein fragment on glass. After extracting the protein, the supernatant was diluted with 2 volumes distilled water and dialysed overnight against 2 % acetic acid. It was lyophilised, dissolved in 0.2 ml distilled water, and stored at -80°. The final yield of the fragment was approximately 15 % of the theoretical yield.

Each of the commercial trypsin batches tested contained low levels of ribonuclease that caused significant RNA degradation at 37°. Control experiments were performed, therefore, in which 16S RNA was treated with trypsin under the conditions used for digesting the S4-16S RNA complex. The extent of degradation was determined electrophoretically in polyacrylamide gels containing 8 M urea[3]. RNA bands were stained with pyronin G (Merck, Darmstadt) and densitometered[3]. Less than 5 % of the 16S RNA molecules were degraded.

Molecular weight determination

The electrophoretic method of Weber and Osborn[22] that employs polyacrylamide gels containing dodecylsulphate was used in modified form. 15 % polyacrylamide gels were prepared containing 100 mM Na phosphate buffer, pH 6.8, and 0.1 % Na dodecylsulphate, in glass tubes (0.6 cm internal diameter and 7 cm long). Unlabelled and [^{14}C]-methylated S4 fragment was electrophoresed for 9 hr at 7 mA per tube using bromophenol blue as a marker. A series of ribosomal proteins were used for calibration, namely S4 (22,550)[18]; L5 (19,300), L29 (10,100)[23]; S12 (16,500) and S15 (12,300)[24]. Gels were stained with 0.1 % Coomassie brilliant blue in 7.5 % acetic acid overnight. The gels were then

sectioned with a Joyce-Loebl slicer before being dried, burnt in a Packard Sample Oxidiser and counted with Instagel (Packard).

Binding methods

S4 fragment and 16S RNA, or 23S RNA (both 0.86 mg) were incubated together at a protein:RNA molar ratio of about 1:1 in 1 ml TMK reconstitution buffer, under standard conditions.[3] The solution was cooled and passed over an A 0.5 agarose column (1 x 30 cm). The eluate was monitored with an LKB-Uvicord (Sweden). 1 ml fractions were collected and the protein radioactivity was estimated by liquid scintillation using Instagel.

RESULTS

The kinetics of trypsin digestion of protein S4 complexed with 16S RNA was investigated. The S4-16S RNA complex, that had been separated from unbound protein, was digested with trypsin, at increasing times. The digested complex was then separated rapidly by gel filtration from the trypsin and peptides. A typical elution profile showing this separation is given in Figure 1.

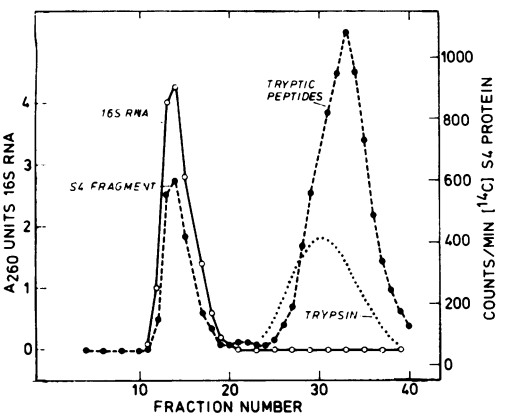

Figure 1. An elution profile, from an agarose column, showing the separation of the [^{14}C]-methylated S4 fragment - 16S RNA complex from tryptic peptides and trypsin. The separation was on an A 0.5 agarose column (1 x 30 cm) in 20 mM Mg^{++}. 1 ml fractions were collected. The RNA peak (o ——o) was measured by ultraviolet absorbance at 260 nm. The protein radioactivity in the fractions (● --- ●) was counted with Instagel. The position of the trypsin peak (·····) was determined by running 5 mg trypsin over the column and measuring the ultraviolet absorbance at 280 nm (absolute absorbance measurements are not given).

The amount of protein that remained attached to the RNA decreased rapidly during the first 20 min of trypsin treatment. Thereafter, a plateau region was reached at which about 30 % of the protein remained bound to the 16S RNA (Figure 2).

In a control experiment designed to show that the resistance to trypsin digestion, exhibited in Figure 2, was not a property of the S4 protein structure, itself, the protein was mixed with 23S RNA and incubated with trypsin for 10 min and 20 min under the conditions described in the legend to Figure 2. Aliquots of the two samples were then electrophoresed at 4°, in 15 % polyacrylamide disc gels containing 6 M urea[16]. All of the [^{14}C]-methylated peptides migrated at the solvent front indicating that no regio of the S4 protein was significantly resistant to trypsin digestion

The most probable interpretation of the formation of the

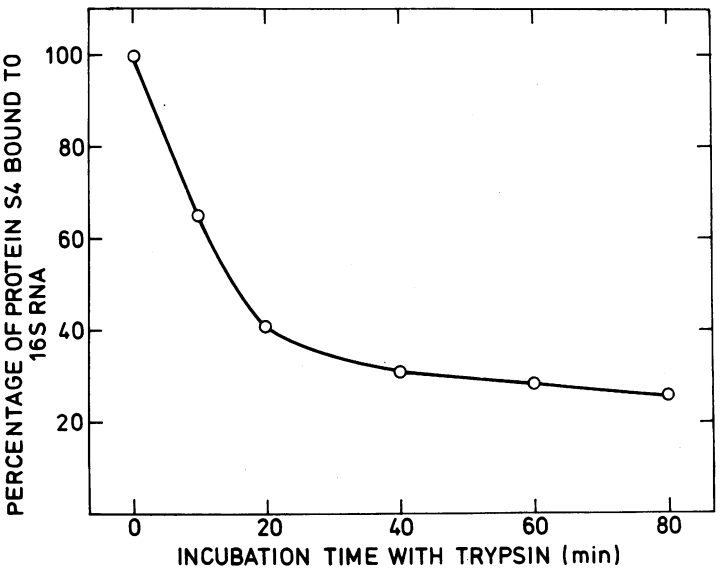

Figure 2. Kinetics of the digestion of a [^{14}C]-methylated S4-16S RNA complex (0.22 mg in 1 ml) by trypsin. Trypsin was added at an enzyme:protein weight ratio of 1:90. The solutions were maintained at 0° for different times and rapidly separated on an A 0.5 agarose column (1 x 30 cm) at 2°. The times given each include 10 min as the estimated separation time of the trypsin. The sample at 0 min (100 % bound S4) is a control complex with no trypsin incubation.

plateau in the kinetics curve in Figure 2 was, therefore, that a certain region of protein S4, that was inaccessible to trypsin molecules, remained bound to the 16S RNA. In order to check this interpretation, and to characterise the protected protein product, the procedure was scaled up for preparing larger quantities of both unlabelled and [^{14}C]-methylated material. The procedure was essentially that used in Figure 2 (at the plateau region), except that the enzyme:protein weight ratio could be reduced to 1:150 because of the higher S4-16S RNA complex concentration (see Materials and Methods).

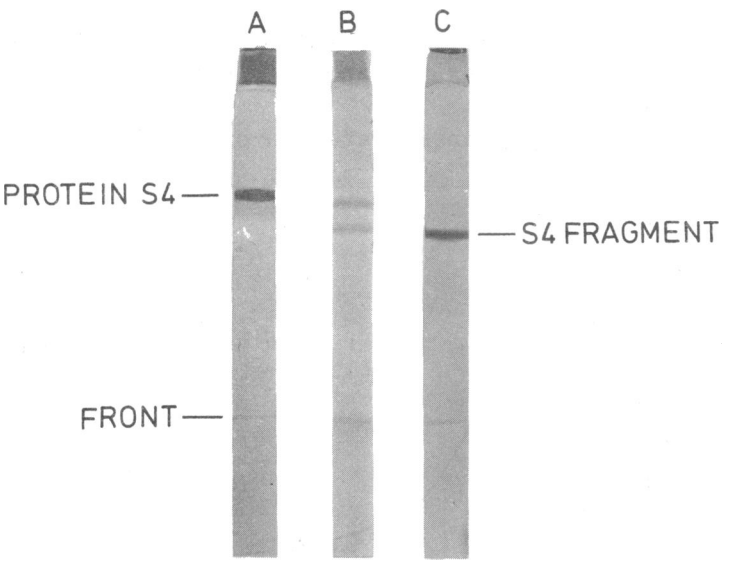

Figure 3. Electrophoresis of (A) S4 protein (B) a mixture of S4 protein and fragment and (C) protein fragment in a polyacrylamide gel (15 % acrylamide, 6 M urea, pH 4.5). The protein bands were stained overnight with 0.1 % Coomassie brilliant blue in 7.5 % acetic acid. The position of the rosaniline hydrochloride marker is given. The R_F value of the fragment was 0.45 and that of the S4 protein was 0.33.

The protected protein product was prepared from 20 mg S4-16S RNA complex. In the early experiments the dialysis step was omitted after the acetic acid extraction, in order to prevent the loss of

any small protein fragments. The extracted material was electrophoresed alone and mixed with whole S4 protein. The result shown in Figure 3 shows that the trypsin digestion product was a large and homogeneous protein fragment that migrated below the S4 protein. In three experiments (out of five) this was the only band observed. In the others, one weak band was detected moving with an R_F value of 0.75. No small protein fragments were detected at the solvent front.

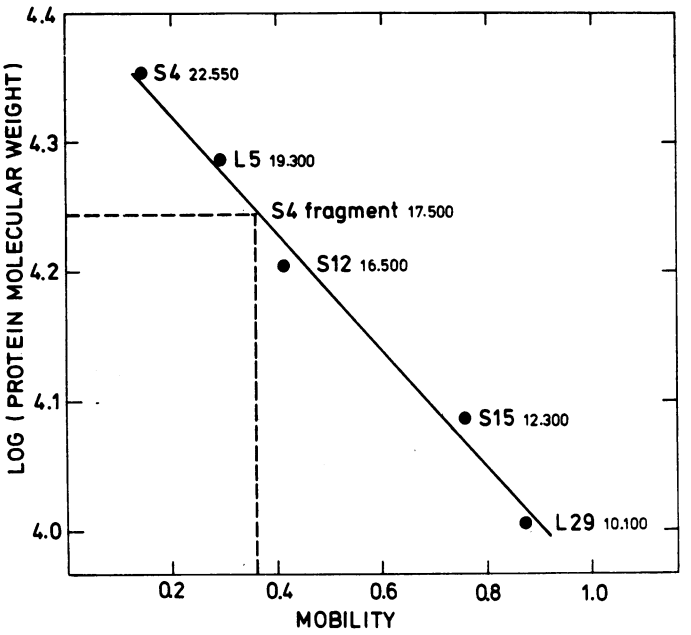

Figure 4. The molecular weight determination of the protein S4 fragment by the dodecylsulphate-polyacrylamide gel method using the ribosomal proteins S4, S12, S15, L5 and L29 for calibration.

The molecular weight of the protein fragment was determined electrophoretically in a polyacrylamide gel containing dodecylsulphate. Ribosomal proteins, of accurately known molecular weight, were selected for calibration. The results, shown in Figure 4, gave a molecular weight of 17,500 (± 1000) for the fragment. Identical values were obtained for both unlabelled and [^{14}C]-methylat-

ed S4 fragments. This result indicates that the trypsin treatment has shortened the protein by about 45 amino acids. The molecular weight of the weak, faster moving band, was estimated at approximately 12,000, about half the length of protein S4. No protein fragment was detected at the solvent front.

As a criterion for its specificity the capacity of the S4

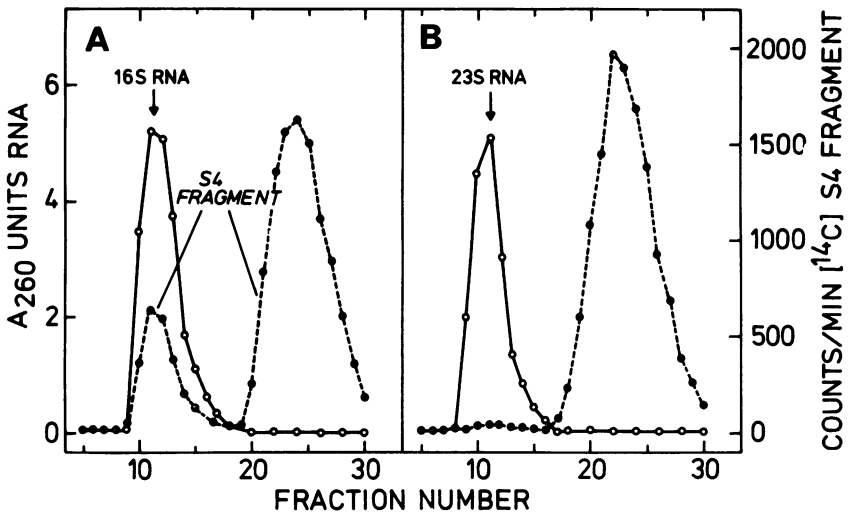

Figure 5. Rebinding of the S4 fragment to 16S and 23S RNA's in TMK reconstitution buffer. Elution profiles from A 0.5 agarose columns (1 x 30 cm) are shown. In (A) the fragment was incubated with an equimolar amount of 16S RNA; the overlap of the S4 fragment peak (● --- ●) with that of the 16S RNA (o —— o) indicated that complex formation had occurred at a molar protein:RNA ratio of 0.2:1. In (B), a control experiment, the S4 fragment (● --- ●) was incubated with 23S RNA (o —— o) and no complex formation was detected.

fragment to rebind preferentially to 16S RNA was investigated. The fragment was incubated,separately, with 16S and 23S RNA under the standard reconstitution conditions. The elution profiles from the agarose column, that are illustrated in Figure 5, indicate that the S4 fragment recognised, exclusively, a binding site on the 16S RNA.

DISCUSSION

This study has demonstrated that it is possible, under carefully controlled conditions, to prepare a region of a ribosomal RNA binding protein that is protected by its RNA binding site against trypsin digestion. The fact that this fragment remains on the RNA, after agarose gel filtration and ethanol precipitation, suggests that it is very strongly bound to the RNA, and, therefore, that it contains the primary binding site(s) of the protein.

Protein S4 is a very elongated protein (very approximately 20 x 20 x 130 Å)[4] that has recently been sequenced.[18] The RNA binding site of protein S4 is structurally very complex[6,7,9] and it has been carefully characterised for nucleotide sequence and secondary structure[10,25,26,27]. Recent work suggests that it, also could have an elongated structure, and that the RNA and protein may lie together with their long axes parallel[26]. This could explain the extensive protection of the RNA by protein S4 and, conversely, it could explain the protection against trypsin digestion of a large part of the protein, by the 16S RNA.

We have recently surveyed the sensitivity to trypsin digestion of most of the possible single protein-ribosomal RNA complexes, using the method described here. The proteins fall into three distinct classes, namely those that were completely resistant; those that produced a plateau as was shown in Fig.2 for S4, and those that were rapidly and completely digested. The proteins that behaved similarly to S4 were L1, L3 and L23 complexed with 23S RNA, and L18 and L25 complexed with 5S RNA. A description of these protected products will be the subject of a subsequent publication.

There is circumstantial evidence to suggest that mainly the N-terminal region of the protein has been excised. The evidence is as follows. (1) The C-terminus of protein S4 is very resistant to attack by carboxypeptidase A and B when complexed with 16S RNA[14]. (2) S4 proteins that were isolated from mutants that were revertants from streptomycin dependence to independence, and were altered at, or near, their C-termini[28] bound very weakly to 16S RNA[29].

We are currently engaged in determining the amino acid sequence of the fragment, although the very low yields that are

obtainable render protein-chemical work difficult.

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