Structural domains of ribosomal protein S8 and their relationship to ribosomal RNA binding

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Escherichia coli ribosomal protein S8 has been subjected to mild proteolytic digestion in order to search for structural domains within the protein [1]. A characteristic fragment produced in high yield after chymotrypsin treatment has been located with the protein sequence. Circular dichroism has shown this domain to be rich in α helix. However, the fragment loses its ability to bind to 16 S rRNA as does a similar fragment produced by trypsin cleavage. The intact protein is required for rRNA binding and is highly protected against proteolytic digestion when bound to the RNA.

Ribosomal protein *Structural domain* *RNA-binding*

1. INTRODUCTION

Protein S8, sequenced M_r 13996 [2] is an important protein which binds to a 40 basepair region located in the middle of 16 S rRNA from the small subunit of the *Escherichia coli* ribosome [3].

This protein has been isolated under mild conditions avoiding the use of urea or acetic acid. It has been shown to have structure in solution as seen from circular dichroism [4] and nuclear magnetic resonance studies [1]. It is considered to be a globular protein from hydrodynamic and low angle X-ray and neutron scattering experiments (review [5]).

Limited proteolytic digestion has been used as a tool to investigate structural domains within this protein. The location within the primary sequence of a characteristic chymotryptic fragment is described here. Since most proteases tested give rise to a fragment representing approximately half of the protein molecule this was regarded as a structural domain within the protein. The importance of these fragments for rRNA binding and the protection conferred by rRNA on protein S8 against proteolytic cleavage are described below.

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2. MATERIALS AND METHODS

The protein S8 was prepared by a modification of the mild method in [6] avoiding the use of acetic acid, urea or lyophilization. During the preparation proteolytic degradation was minimized by using the proteolytic inhibitors, phenylmethylsulphonyl fluoride (2×10^{-5} M) and benzamidine (4×10^{-5} M) in all buffer solutions. Ribosomes and 30 S subunits were prepared by zonal centrifugation as in [6]. Before use the subunits were pelleted by centrifugation at $10000 \times g$ for 10–12 h in order to remove any polyethylene glycol remaining after the final precipitation. Protein S 8 was removed from a 'core' particle which had been pre-treated with 1 M NaCl in the presence of 1 mM EDTA to remove a group of ribosomal proteins not including S 8. These core particles were resuspended into 2 M NaCl, 0.01 M Tris–HCl (pH 7.5) 6 mM β-mercaptoethanol at $50 A_{260}$ units/ml. The concentration of the suspension was made 10 mM with respect to EDTA at pH 7 and stirred for 12 h at 4°C. The extracted proteins including S 8 were then separated from the rRNA core particles by cen-

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trifugation at 100000 × *g* for 12 h. S8 was fractionated from other ribosomal proteins by CM Sephadex chromatography and gel filtration on Sephadex G-75 superfine as in [6] but in the presence of NaCl instead of LiCl.

To determine the amino acid composition and the exact concentration of protein S8 and fragments, amino acid analysis in a Durrum D500 amino acid analyser was performed using norleucine as an internal control.

The protein was identified and checked for purity by two-dimensional gel electrophoresis [7] and slab-gel electrophoresis in the presence of SDS [8]. For the large-scale preparation of S8 fragment, 1–2 mg protein in isolation buffer containing 0.05 M sodium acetate at pH 5.6, 0.4 M NaCl, 0.6 mM 2-mercaptoethanol was brought to pH 7.0 by addition of 0.2 M Hepes buffer (pH 7.0). Protein digestion was carried out with a 10:1 protein: enzyme ratio (w/w) for chymotrypsin for 1 h at 0°C. The reaction was stopped by the addition of soya bean trypsin inhibitor in a weight ratio of 2:1, inhibitor to enzyme. The undigested protein was separated from the fragments produced by gel filtration on Sephadex G-75 or G-50 superfine (Pharmacia). The column was pre-equilibrated with isolation buffer and the profile followed by analysis of an aliquot of each fraction on SDS gel electrophoresis. The fragment peak was pooled and concentrated by dialysis against dry Sephadex G-150 or Ficoll 400 using Spectrapor 3 or 6 dialysis tubing (Spectrum Instruments, Los Angeles CA), M_r-cutoff 3500 and 1000, respectively.

Fingerprinting: 5–10 nmol fragment or intact protein were digested with trypsin (5:1 protein: enzyme ratio) at 37°C for 4 h and electrophoresed on microcrystalline cellulose plates (Polygram Gel 400 from Schleicher and Schell, Basel) for 2 h at 400 V in the first dimension in pyridine/acetic acid/acetone/water (50/75/15/60, by vol.) at pH 4.4. The dried plates were chromatographed in the second dimension in PBEW buffer (pyridine/*n*-butanol/acetic acid/water; 50/75/15/60, by vol.). The peptides were stained with ninhydrin. N-terminal sequence analysis of the fragments was performed by a manual micro-sequencing technique [9]. To determine the carboxyl end-groups of the fragment, digestion was performed with a mixture of carboxypeptidase A, B and Y; 10 nmol protein or fragment were dialysed against 2% acetic acid, lyophilized and dissolved in 0.2 M 4-Me-morpholinacetate buffer at pH 8.0. The C-terminal amino acids were split off with 5 μg carboxypeptidase after incubation for 2 h at 32°C. At the end of the digestion the sample was lyophilized, re-dissolved in citrate buffer at pH 2.2, and the free amino acids were determined in the analyser.

The 16S RNA was extracted from the 30S subunits of *E. coli*, MRE600, by two different methods. The first one was a modification of the standard method of phenol/SDS as in [10] and the second the acetic acid/urea procedure [11].

16S rRNA–protein complexes were formed by mixing the rRNA and the protein in buffer B, (0.01 M Tris–HCl (pH 7.5), 0.02 M $MgCl_2$, 0.35 M KCl, and 6 mM 2-mercaptoethanol) at a molar ratio of 3:1 excess of protein and incubating the mixture at 40°C for 1 h. The complex was cooled and the unbound protein removed by passage over a A-0.5 agarose column pre-equilibrated with buffer B. The complex peak was pooled and precipitated by the addition of trichloroacetic acid to 5% final conc. in the presence of 20 μl of 0.5% sodium deoxycholate.

For circular dichroism measurements protein samples at 0.1 mg/ml were dialyzed against buffer C (5 mM potassium phosphate (pH 7.0), 0.35 M KF). Measurement was made in a 0.101 cm pathlength cuvette in a dichrograph CD III (Roussel Jouan) at room temperature from 260–180 nm. The resulting spectra were curve-fitted as in [12].

3. RESULTS

The chymotryptic digestion of protein S8 produced two fragments estimated from gels with M_r 7000 and 9000. The total yield of the two fragments was ~70% (fig. 1). The separation of fragments from undigested protein is shown in fig. 2. The smaller fragment of S8 was always contaminated with a small amount of the larger fragment. The former was used for experiments to localise its position in the primary sequence of intact S8.

The tryptic fingerprints of the intact S8 and the S8 fragment revealed that the peptides TR9, TR11, TR12, TR14 and TR16 were missing in the fragment (fig. 3). The results indicated that the fragment was present in the N-terminal part of the intact protein. This was also supported by the amino

acid composition of the fragment. The N-terminus analysis of the fragment gave alanine–asparagine–leucine–threonine–arginine. This amino acid sequence could be traced in the protein sequence in only one place, namely residue 7 to residue 12 (fig. 4). Traces of other amino acids were also obtained which were derived from the large fragment which was present in the mixture as mentioned above. The C-terminal analysis showed a large amount of tyrosine and smaller amounts of lysine and leucine, suggesting a C-terminus: leucine–lysine–tyrosine. This sequence was traced in the intact protein sequence from residue 62–64. Therefore, the small chymotryptic fragment of S 8 contained 58 amino acids from residues 7–64 in the sequence and had M_r 6379.

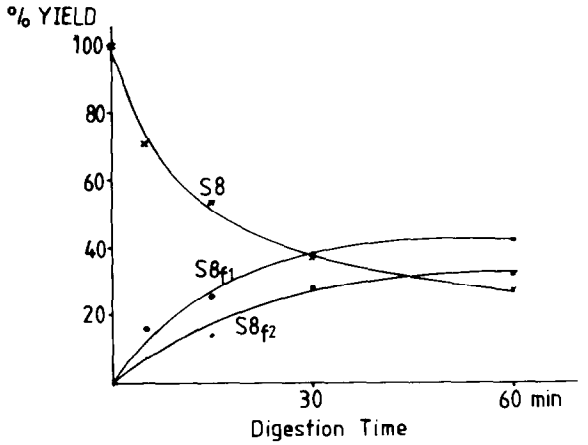

Fig. 1. Densitometry of SDS gel electrophoresis showing the time course for the digestion of protein S 8 with chymotrypsin.

The smaller chymotrypsin S 8 fragment described above had lost its ability to bind to 16 S RNA as had the larger chymotryptic fragment. This was shown by the standard method for rRNA binding as in section 2 and application of the precipitated RNA peak to an SDS–acrylamide gel. In the case of the chymotryptic fragments no protein was found associated with the rRNA. In view of this finding the approach was followed to digest the preformed 16 S RNA–protein complex in order to obtain a fragment protected by the rRNA. The 16 S RNA–S 8 complex was separated from unbound

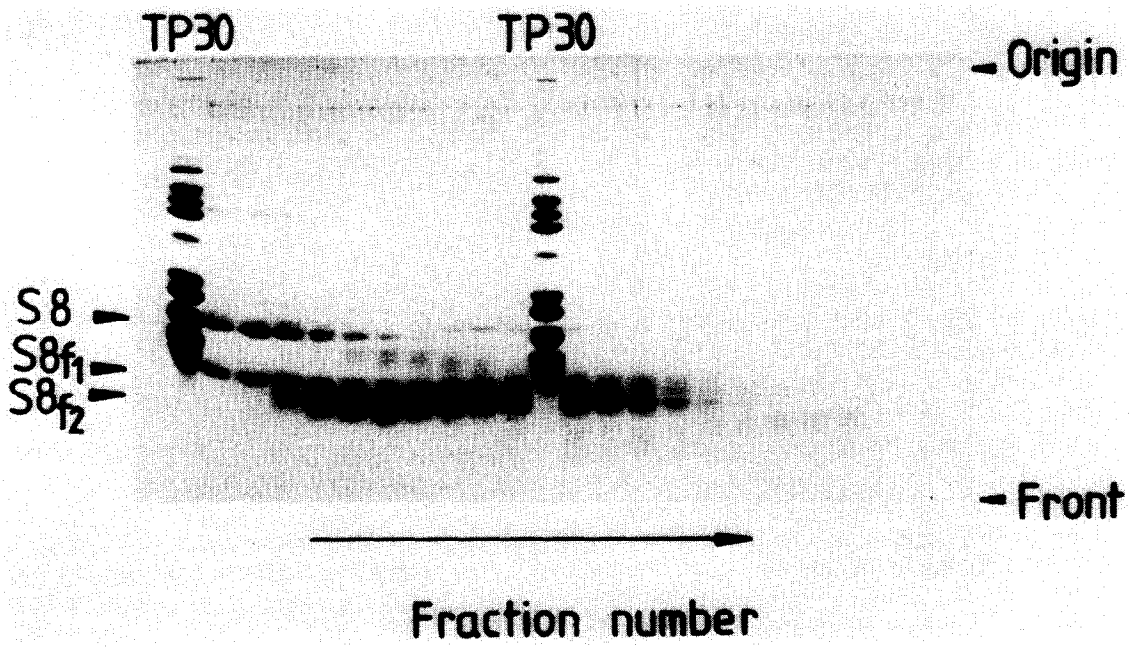

Fig. 2. SDS gel electrophoresis showing the separation of S 8 from the chymotryptic fragments by passage through Sephadex G-75 superfine; TP30, total 30 S protein.

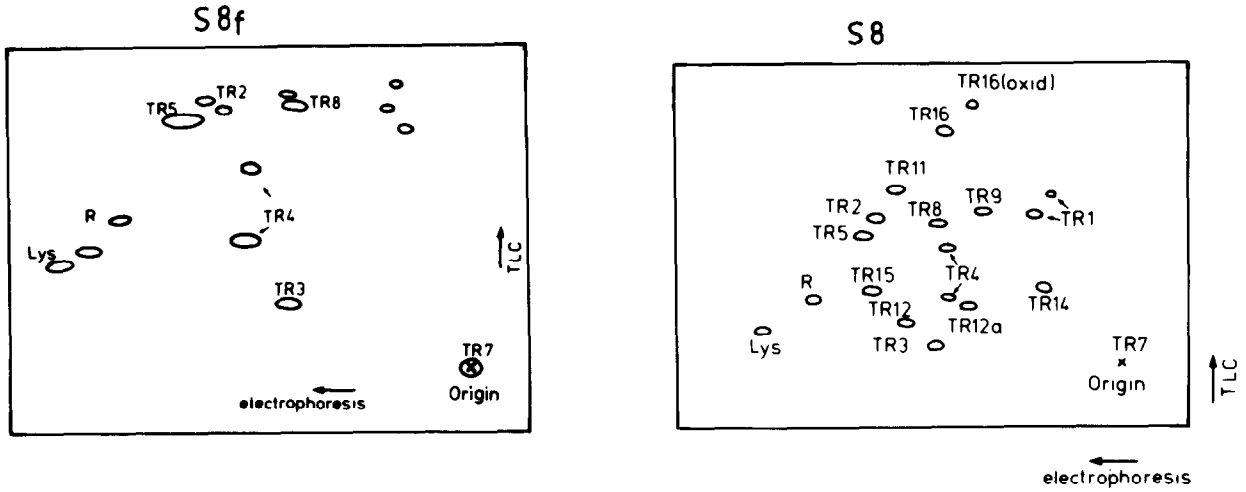

Fig. 3. Tryptic fingerprint of: (a) small chymotryptic fragment of S8; (b) S8 protein.

Amino Acid Composition

	Asx	Thr	Ser	Glx	Pro	Gly	Ala	Val	Cys	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg
S8	9	6	7	14	5	11	14	12	1	5	9	10	3	3	-	12	8
$S8_f$	5	4	3	8	2	5	6	5	-	1	3	4	-	1	-	6	4

Sequence of S8f

N-Terminal

NH_2-Ala-Asp-Met-Leu----

C-Terminal

---Leu-Lys-Tyr-COOH

Location in the Sequence of S8

NH_2-Ser-Met-Gln---Ile-Ala-Asp-Met----Leu-Lys-Tyr-Phe---Val-Ala-COOH

1 7 64 129

$S8_f$

Fig. 4. Amino acid analysis comparison for intact S8 and the small chymotryptic fragment of S8 (S8f). Location of this fragment within the amino acid sequence.

S 8 by gel filtration on a Bio-Gel column, and the resultant RNA complex peak was digested with chymotrypsin for 1 h at 1:1 protein:enzyme (w/w), at 15°C and 0°C, respectively. Even at 15°C and at this high ratio of enzyme only 5–10% of the S 8 was digested to a fragment with M_r 9000 as estimated from the gel. When this digestion mixture was passed over a further Bio-Gel column the fragment no longer migrated with the rRNA peak and had therefore lost its binding ability.

It was reported [13] that a fragment of S 8, produced by digestion of the 16 S RNA–S 8 complex with proteinase K, retained the binding ability of the intact protein. Therefore the same procedure as above was followed using instead of chymotrypsin, proteinase K. When the complex was incubated at 0°C for 15 min with proteinase K at 1:1 bound protein:enzyme (w/w) a fragment of M_r 9000–10000 was produced. Increase of the incubation time to 1 h gave a higher yield by a factor of two (20% of the intact protein). However, this fragment did not retain the ability to bind to the 16 S RNA even when exactly the same reconstitution buffer was used as reported.

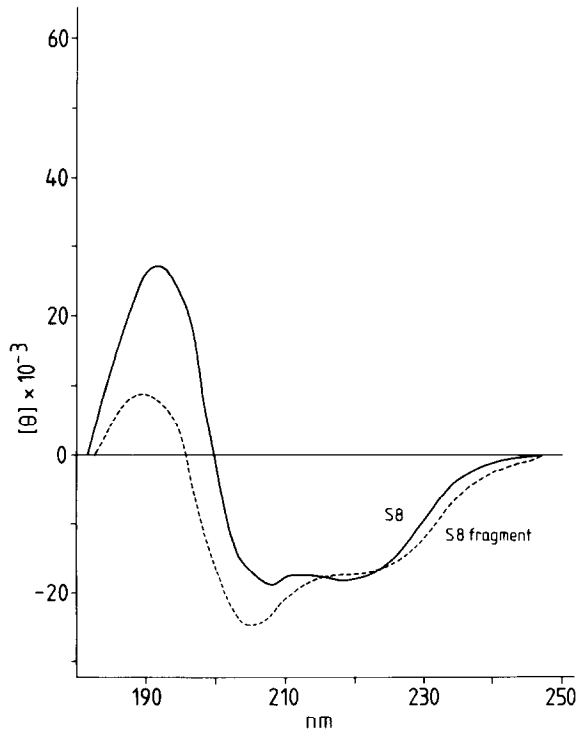

Fig. 5. Circular dichroism spectra of protein S 8 and the small chymotryptic fragment of S 8. The spectra are corrected for molar ellipticity.

The circular dichroism spectra of the protein showed the small chymotryptic fragment to have a higher α-helix and lower β-structure than the intact protein. A value of 52% α-helix and 3% β-structure was obtained for the fragment as opposed to 43% α-helix and 32% β-structure for the intact protein (fig. 5). Secondary structure prediction data for S 8 suggest that the N-terminal part of the protein is rich in α-helix structure [14] and that the C-terminal part is the only region predicted for β-structure. This is in agreement with the above values.

4. DISCUSSION

The protein S 8 from the *E. coli* ribosome is a globular protein but not totally resistant to proteolysis, as is the case with proteins S 15, S 16, S 17, L 30 [1]. It can, with most proteases, be digested to a more tightly folded domain which has no longer the ability to bind to rRNA. It appears that the bound protein is highly protected on the rRNA against any proteolytic digestion. The nature of this tight interaction and the fact that S 8 protects one of the smallest regions of rRNA against nuclease digestion (compared to other rRNA–protein complexes) makes this protein–RNA fragment complex highly favourable for further structural studies.

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