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PROTON MAGNETIC RESONANCE STUDIES OF *ESCHERICHIA COLI* RIBOSOMAL PROTEIN S4 AND A C-TERMINAL FRAGMENT OF THIS PROTEIN

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1. Introduction

The ribosomal protein S4 is one of the primary rRNA binding proteins from the small subunit of the *E. coli* ribosome. It has the ability to bind specifically to a large region at the 5′ end of 16 S RNA [1–4]. Physical studies with protein S4 have in most instances shown it to have an elongated shape [5–7] although a more recent neutron scattering study suggests that it is more compact [8]. The extended shape for S4 is in agreement with immunological studies [9] which found multiple binding sites on the surface of the ribosome for specific antibodies raised against this protein.

The protein used in this study has been isolated under conditions that avoid the use of urea, acetic acid or lyophilization [10]. Protein S4, isolated in the presence of acetic acid and urea, showed in a previous proton magnetic resonance (PMR) study, a tertiary structure involving a tyrosine residue and an apolar amino acid [11]. The existence of a perturbed tyrosine ring proton resonance in the low-field region and a ring-current shifted apolar methyl signal in the high-field region of the spectrum was taken as evidence for 'structure'. Protein S4 isolated under non-denaturing conditions and a C-terminal fragment of this protein [12–14] which retains its ability to bind to 16 S rRNA, both show more pronounced evidence of tertiary interactions. These results indicate that salt-extracted S4 contains a far higher proportion of structured molecules than the protein previously studied and that this structure is conserved in the fragment.

2. Materials and methods

Protein S4 was obtained by a stepwise LiCl extraction of the subunits followed by chromatography on CM–Sephadex C-25 using a LiCl gradient in 0.05 M sodium acetate (pH 5.6) [10] or on CM–Sephadex C-25 using a KCl gradient in 0.05 M potassium phosphate (pH 7.0) [15]. Further purification was obtained in both cases by gel filtration on Sephadex G-100 in the respective high ionic strength buffers. The protein was concentrated by dialysis against dry Sephadex G-150. The identity and purity of the S4 was established by two-dimensional gel electrophoresis [16–18] and one-dimensional slab-gel electrophoresis in the presence of dodecylsulphate [19].

The C-terminal fragment of S4 was obtained as a consequence of the isolation procedure due to endogenous proteolytic degradation, or could be generated by mild tryptic digestion at pH 7.0 for 20 min, at 0°C, at an enzyme:protein ratio of 1:100 (w/w). This fragment was further purified by passage through a Sephadex G-100 column in 0.05 M potassium phosphate (pH 7.0), 0.3 M KCl, 6 mM β-mercaptoethanol, 0.01 mM phenylmethylsulphonyl fluoride, 0.02 mM benzamidine hydrochloride.

The concentration of protein was determined by a nitrogen assay [20]. Ribosomal RNA was prepared by

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a phenol–dodecylsulphate procedure [21] with the addition of further deproteinization steps with phenol and bentonite to eliminate RNase contamination. Binding assays were performed as in [22] with the exception that the protein:RNA complex was separated from unbound protein on a Sephacryl 200 superfine column (Pharmacia). The complex was precipitated with 5% (w/v) trichloroacetic acid in the presence of sodium deoxycholate and electrophoresed on 15% polyacrylamide gels containing sodium dodecylsulphate [19]. Gels were stained and scanned as in [22]. Identification of the C-terminal fragment was carried out by comparison of the tryptic peptides of the fragment with the protein. Equivalent to 200 μg protein was incubated at 37°C for 4 h with 5 μg trypsin (TPCK Worthington). The digestion was stopped by rapid freezing and lyophilization. Peptides were separated by mapping on cellulose thin-layer plates [23], with the modification that 20 × 20 cm Polygram CEL 300 (Macherey and Nagel, Düren) plates were used. Electrophoresis was carried out at 480 V for 1.5 h, and chromatography was carried out for 5–6 h. The peptides were stained by spraying with 3% ninhydrin.

N-terminal sequence analysis was carried out by automated liquid-phase Edman degradation using a Beckman Sequenator (890C) and 600 μg of the S4 fragment. The C-terminus of the fragment of protein S4 was inspected by hydrolysis of 10 nmol protein with 5 μg carboxypeptidase A and 5 μg carboxypeptidase B, in a buffer containing 0.5% *N*-methylmorpholine acetate (pH 8.0) for 2 h at 37°C. The sample was lyophilized, dissolved into 80 μl of pH 2.2 buffer, incubated at 60°C for 10 min, centrifuged and the supernatant subjected to amino acid analysis.

For the PMR experiments the protein samples at 1–2 mg/ml were dialysed against 3 × 50 ml buffer 0.3 M KCl, 0.05 M potassium phosphate (pD 7.0), 5×10^{-4} M dithioerythritol in ^2H_2O. Protein solutions were then centrifuged and transferred into standard 5 mm NMR tubes. Spectra were recorded at 270 MHz on a Bruker WH 270 magnetic resonance spectrometer operating in Fourier Transform mode, using a pulselength of 12 μs and data collection over 0.5 s for each pulse. Spectra were obtained at 20°C over a period of 2–12 h. The free induction decay pattern was multiplied by an exponential function equivalent to line broadening of ~2 Hz. Chemical shifts were measured relative to sodium 2.2, dimethyl-2-silapentane sulphonate (DSS). After the PMR spectrum had been recorded the proteins were checked for proteolytic degradation by slab-gel electrophoresis in the presence of dodecylsulphate [19].

3. Results and discussion

The PMR spectra of acid–urea-extracted and salt-extracted S4 protein (fig.1b,c) are qualitatively similar

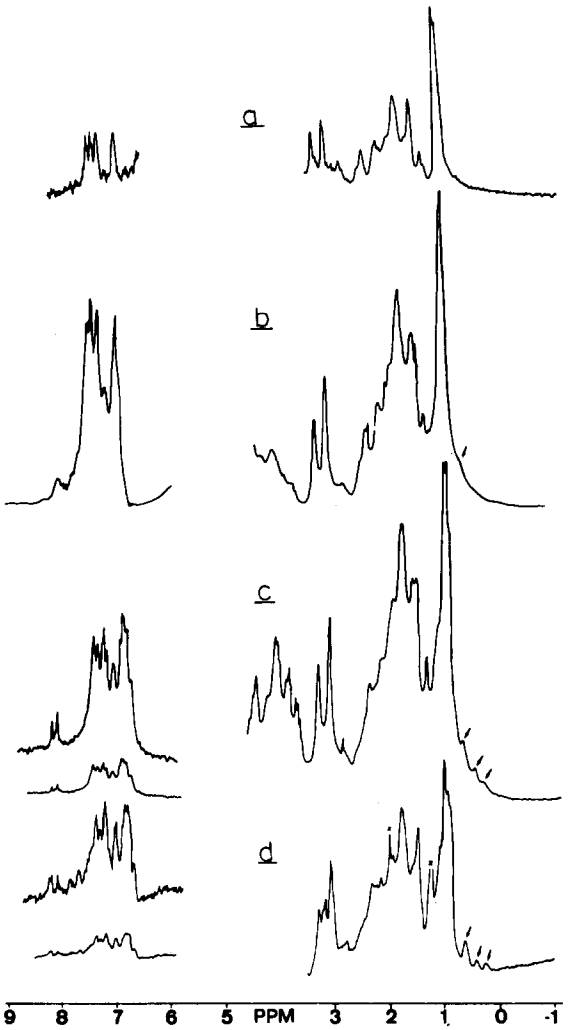

Fig.1. 270 MHz PMR spectra of protein S4: (a) in 6 M urea-denatured; (b) prepared with urea, acetic acid and lyophilization; (c) prepared by non-denaturing method; (d) fragment residues 47–203 of S4 prepared by non-denaturing methods. The symbol 'x' indicates an impurity in the protein PMR spectrum.

in their two main features, namely the ring-current shifted apolar methyl resonances at 0–0.8 ppm and the perturbations in the tyrosine ring proton signals around 6.7 and 7.5 ppm. However, in the spectrum of salt-extracted S4 these features are greatly enhanced such that their detailed resolution becomes very clear. Previously, convolution difference methods (at a loss in signal/noise) were necessary to resolve these resonance signals. Additionally, the spectrum of salt-extracted S4 exhibits greater dipolar broadening throughout.

These results suggest that salt-extracted S4 contains a far higher proportion of 'structured' protein than the earlier preparations. Indeed, spectrum 1b may be simulated by superimposing the spectrum of denatured S4 (fig.1a) upon that of 'structured' S4 (fig.1c). The latter sample contains very little or no denatured material whereas preparations of acid–urea-extracted S4 are extensively denatured. This is reflected by the efficiencies with which the two protein preparations specifically bind to 16 S rRNA, 3–4-times more acid–urea S4 being required for saturation than salt-extracted S4.

Similarly, production of the C-terminal proteolytic fragment described proceeds almost quantitatively from salt-extracted S4 whereas previously such high yields of fragment have only been achieved by first binding protein to the rRNA [12–14]. The latter procedure presumably selects for the 'structured' component of S4 as the denatured material does not bind to RNA. The possibility exists that some denatured S4 could renature under the conditions of the binding assay, i.e., incubation at 42°C, high salt, presence of rRNA.

The protein S4 used in this study was cut in successive steps from the N-terminal end down to a resistant fragment (fig.2). The fragment appeared identical, concerning the physical and chemical studies described here, whether it was produced from protein isolated at pH 5.6 or at pH 7.0 (see section 2). Confirmation that the fragment produced was identical to that in [12–14], i.e., residues 47–203, was demonstrated by comparison of the tryptic fingerprints (fig.3) and by N-terminal sequence determination as in section 2. The cleavage point of the protease was found to be between Arg 46 and Leu 47 in the sequence of the

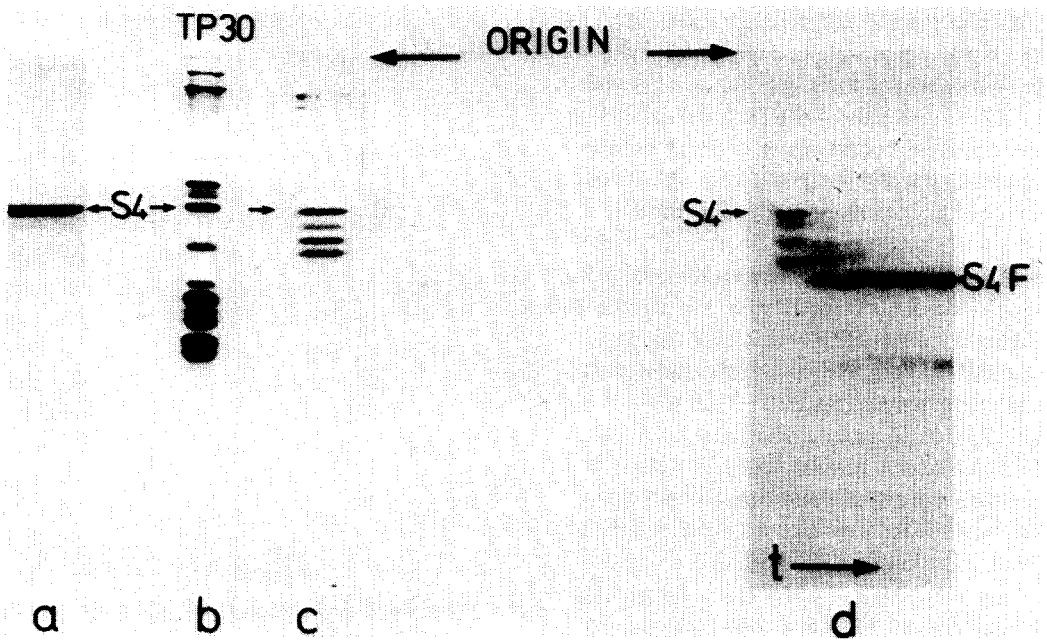

Fig.2. SDS gel electrophoresis: (a) S4 protein prepared at pH 5.6; (b) total protein from 30 S subunit; (c) S4 protein isolated at pH 7.0 showing fragments produced by endogeneous proteolytic activity; (d) digestion of S4 protein prepared at pH 7.0 with trypsin 1:100 w/w, 0, 5, 10, 15, 20, 30 min incubation time at 0°C.

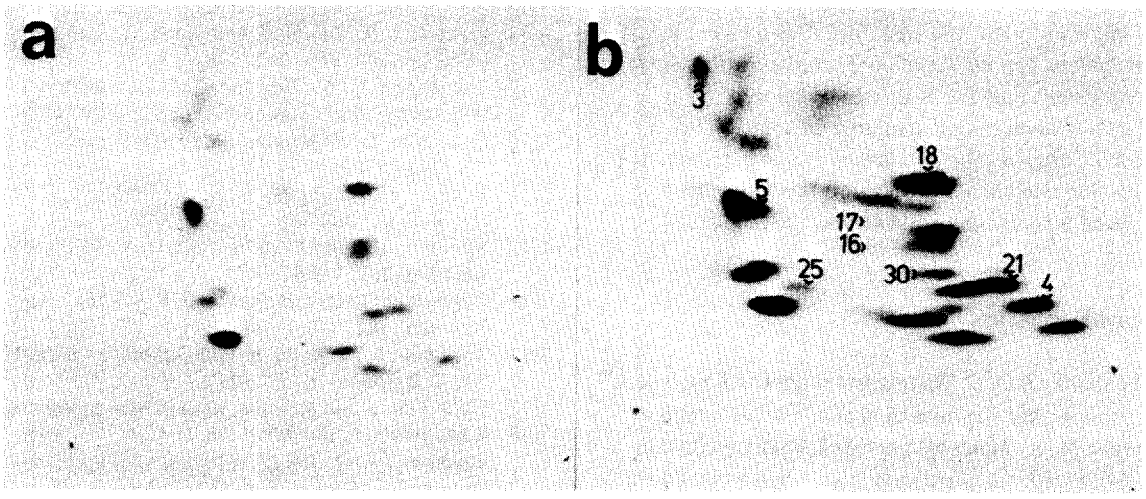

Fig.3. Tryptic fingerprint: (a) S4 fragment; (b) S4 protein with spots missing in the fragment indicated.

protein [24]. The fragment retained its ability to bind to 16 S RNA but smaller fragments produced upon prolonged tryptic digestion did not have this ability (fig.4). Hence it would appear that most amino acids between 47–203 are needed to maintain the structure for rRNA binding. Several mutants of protein S4 have been isolated that have an altered C-terminus [25]. In these proteins a reduced binding to 16 S rRNA was observed [26]. The C-terminus of the fragment described here was demonstrated to be intact as found in [14].

The PMR spectrum of fragment 47–203 of S4 protein (fig.1d) exhibits the same structural features as for the intact protein, namely the pattern of up-field methyl shifts and the tyrosine ring proton perturbations. These effects are more evident in the fragment than in the intact molecule and the dipolar broadening is greater. There is a loss of signal area in the apolar methyl region compatible with the removal of 25% of the total Leu and Ile residues and a comparison of the linewidths in this region suggests that these apolar residues are in an unstructured section in the intact protein. Likewise, reduction in resonance area but no structural changes may be attributed to the loss of Tyr_3 and Phe_{19} in the fragment. The loss of His_{40} may be seen as a reduction in the C_2–H signal at 8.12 ppm. Thus it appears that the structured region of S4 protein is located between Leu_{47} and the C-terminus.

This report demonstrates that:

1. Protein S4 isolated by the salt extraction method avoiding the use of urea, acetic acid or lyophilization is a more homogeneous and structured molecule as observed from the PMR spectra and proteolysis experiments, than protein isolated by denaturing methods (review [27]);
2. The C-terminal fragment of S4 from residues 47–203 which retains its ability to bind to rRNA,

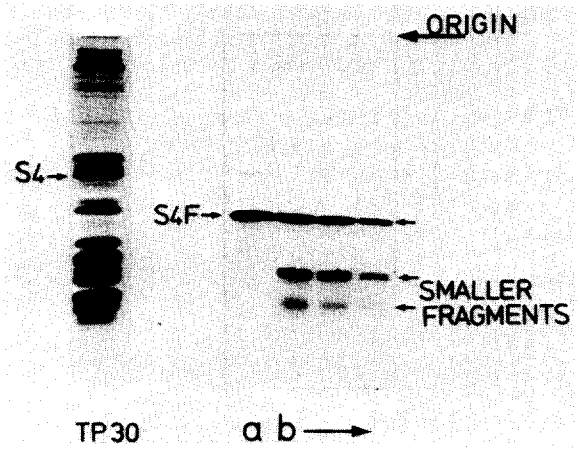

Fig.4. SDS gel electrophoresis showing total 30 S protein: (a) $S4_F$ bound to 16 S rRNA; (b) starting material in binding assays containing smaller fragments which do not bind to 16 S rRNA.

contains most of the structure found in the intact protein as can be observed from the PMR spectra. It is proposed that the N-terminal region of S4 is probably a random coil structure in solution being available for proteolytic cleavage and is necessary for interaction with other proteins in the ribosome as suggested by reconstitution experiments [13,28].

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References

[1] Zimmermann, R. A., Muto, A., Fellner, P., Ehresmann, C. and Branlant, C. (1972) Proc. Natl. Acad. Sci. USA 69, 1282–1286.
[2] Muto, A., Ehresmann, C., Fellner, P. and Zimmermann, R. A. (1974) J. Mol. Biol. 86, 411–432.
[3] Ungewickell, E., Garrett, R. A., Ehresmann, C., Stiegler, P. and Fellner, P. (1975) Eur. J. Biochem. 51, 165–180.
[4] Ehresmann, C., Stiegler, P., Carbon, P., Ungewickell, E. and Garrett, R. A. (1977) FEBS Lett. 81, 188–192.
[5] Paradies, H. H. and Franz, A. (1976) Eur. J. Biochem. 67, 23–29.
[6] Rohde, M. F., O'Brien, S., Cooper, S. and Aune, K. C. (1975) Biochemistry 14, 1079–1089.
[7] Österberg, R., Sjöberg, B., Garrett, R. A. and Littlechild, J. (1977) FEBS Lett. 73, 25–28.
[8] Serdyuk, I. N., Zaccai, G. and Spirin, A. S. (1978) FEBS Lett. 94, 349–352.
[9] Tischendorf, G. W., Zeichhardt, H. and Stöffler, G. (1975) Proc. Natl. Acad. Sci. USA 72, 4820–4824.
[10] Littlechild, J. and Malcolm, A. (1978) Biochemistry 17, 3363–3369.
[11] Morrison, C. A., Garrett, R. A. and Bradbury, E. M. (1977) Eur. J. Biochem. 78, 153–159.
[12] Schulte, C., Schiltz, E. and Garrett, R. A. (1975) Nucl. Acid Res. 2, 931–941.
[13] Changchien, L. M. and Craven, G. R. (1976) J. Mol. Biol. 108, 381–401.
[14] Newberry, V., Yaguchi, M. and Garrett, R. A. (1977) Eur. J. Biochem. 76, 51–61.
[15] Littlechild, J. and Malcolm, A. (1979) in preparation.
[16] Kaltschmidt, E. and Wittmann, H. G. (1970) Anal. Biochem. 36, 401–412.
[17] Mets, L. J. and Bogorad, L. (1974) Anal. Biochem. 57, 200–210.
[18] Kyriakopoulos, A. and Subramanian, A. R. (1977) Biochim. Biophys. Acta 474, 308–311.
[19] Laemmli, U. K. and Favre, M. (1973) J. Mol. Biol. 80, 575–599.
[20] Jaenicke, L. (1974) Anal. Biochem. 61, 623–627.
[21] Nirenberg, M. W. and Matthaei, J. H. (1961) Proc. Natl. Acad. Sci. USA 47, 1588–1602.
[22] Littlechild, J., Dijk, J. and Garrett, R. A. (1977) FEBS Lett. 74, 292–294.
[23] Yaguchi, M., Wittmann, H. G., Cabézon, R., DeWilde, M., Villarroel, R., Herzog, A. and Bollen, A. (1975) Mol. Gen. Genet. 142, 35–43.
[24] Schiltz, E. and Reinbolt, J. (1975) Eur. J. Biochem. 56, 467–481.
[25] Funatsu, G., Puls, W., Schiltz, E., Reinbolt, J. and Wittmann, H. G. (1972) Mol. Gen. Genet. 115, 131–139.
[26] Daya-Grosjean, L., Garrett, R. A., Pongs, O., Stöffler, G. and Wittmann, H. G. (1972) Mol. Gen. Genet. 119, 277–286.
[27] Wittmann, H. G. (1974) in: Ribosomes (Nomura, M. et al. eds) pp. 93–114, Cold Spring Harbor Labs, New York.
[28] Changchien, L. M. and Craven, G. R. (1978) J. Mol. Biol. 125, 43–56.