A STUDY OF THE REGIONS OF RIBOSOMAL PROTEINS S4, S8, S15 AND S20 THAT INTERACT WITH 16 S RNA OF *ESCHERICHIA COLI***

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1. Introduction

The capacity of ribosomal proteins S4, S8, S15 and S20 to bind specifically to 16 S RNA has been established [1–5]. Although the nucleotide sequences of the RNA binding sites have been characterized [5,6], there is no information available on the regions of the proteins that bind to the RNA, apart from circumstantial evidence for the involvement of the C-terminal end of protein S 4 [7–9]. In this work, we have investigated further the possible involvement of the C-termini of the proteins in the RNA interaction by determining the relative effect of carboxypeptidases on the proteins both unbound and complexed with RNA. Secondly, the effect of modification of tryptophan, cysteine, methionine and lysine residues on the RNA binding properties of the proteins was determined. It was concluded that the C-terminal regions of the proteins were much less accessible in the 16 S RNA complex and that, whereas modification of tryptophan and cysteine residues did not impair 16 S RNA binding, modification of methionine and lysine residues did.

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2. Experimental

2.1 *RNA and proteins and protein–RNA complexes*

16 S and 23 S RNA's were prepared [10]. 30 S subunit proteins were isolated [11] and checked for purity electrophoretically [12]. Protein concentrations were determined using the Folin reagent [13]. Protein–RNA complexes were prepared [14] and the amount of protein bound to 16 S RNA was determined electrophoretically [4,14]. Modified proteins were removed from 16 S RNA with 67% acetic acid [11].

2.2. *Carboxypeptidase digestion*

Digestions were performed as described [7], except that stronger enzyme conditions (1:20, enzyme:substrate ratio) were used.

2.3. *Chemical modification*

Cystine residues were carboxymethylated by dissolving 0.25 μmole of S4 and S8 in 1 ml 1 M Tris–HCl, pH 8.6, 8 M urea. 1 μmole of dithioerythreitol was added and the protein solution was stirred for 4 hr under nitrogen. 100 micromoles iodoacetic acid were added in three aliquots at 10 min intervals. The reaction was stopped by dialysis against water.

Tryptophan and cysteine residues were modified by 2-nitrophenylsulphenyl chloride [15] by adding a 3-fold excess of reagent in glacial acetic acid (calculated on the basis of the molarity of tryptophan and cysteine per mole of protein) to S4 and S8 dissolved in 40% acetic acid. After 10 min, ice-cold acetone:1 N HCl (39:1) was added to precipitate the proteins. They were centrifuged, dissolved in 2% acetic acid, and lyophilised.

Performic acid oxidation of methionine, tryptophan and cysteine residues was accomplished by dissolving 1 mg of each protein in a minimum volume of formic acid, cooling to −10°C, and adding 0.5 ml performic acid. The reaction was continued for 2 hr at −10°C and stopped by adding two volumes of cold water before lyophilising.

Cysteine and methionine modifications were checked by amino acid analysis [16]. The tryptophan modification was checked by Ehrlich reagent staining of peptide T 26 in paper chromatography [17].

The proteins (10^{-5} M) were trinitrophenylated [18] by treatment with trinitrobenzenesulphonate (3×10^{-3} M) in 0.3 ml 0.03 M dimethyl glutaric acid buffer, pH 7.2, 0.2 M NaCl, 10 mM $MgCl_2$ at 23°C. They were separated on a Sephadex G25 column (0.5 × 30 cm) that had been equilibrated with water. The modified proteins exhibited characteristic spectra [18]. A molar extinction coefficient of 2.8×10^{-4} M^{-1} was taken for N^{α}-trinitrophenylalanine and N^{ϵ}-trinitrophenyllysine [18]. The average number of lysines modified was estimated spectroscopically at 340 nm.

The relative protection of lysine residues by 16 S RNA was determined by modifying the proteins, and the portein: RNA complexes (both components 10^{-5} M), as described above, in TMK buffer (0.03 M Tris–HCl, pH 7.4, 0.02 M $MgCl_2$, 0.35 M KCl, and 6 mM 2-mercaptoethanol) at 37°C. The reaction was followed at 380 nm to prevent interference from RNA absorbance. No differences occurred in the degree of modification of the free proteins under the different buffer and temperature conditions.

3. Results

The amino acids released from the free protein and the protein–RNA complex are given in table 1. The C-terminal regions were much less accessible in the RNA complex than in the free proteins. None of the digested proteins bound to 16 S RNA. Partial precipitation of S8 occurred during the digestion and this probably accounts for the reproducible digestion of only about half of the S8 molecules (table 1).

The RNA binding properties of the proteins after complete modification of tryptophan, cysteine and methionine residues are summarised in table 2.

Table 1
Amino acids released by carboxypeptidase treatment of the proteins both free and complexed with 16 S RNA

Protein	Amino acids released	Number of moles of amino acids released per mole of protein: Protein alone	Protein–16 S RNA complex
S 4	lysine	1.0	0.25
	serine	1.0	0.3
	tyrosine	0.8	0.25
	leucine	1.3	0.25
	glutamic acid	0.5	< 0.1
	valine	0.5	< 0.1
	isoleucine	0.5	< 0.1
S 8	tyrosine	0.40	< 0.1
	leucine	0.20	< 0.1
	isoleucine	0.60	< 0.1
	valine	0.40	< 0.1
	alanine	0.45	< 0.1
S 15	arginine	4.0	1.0
	glycine	1.3	< 0.1
	alanine	1.3	< 0.1
	valine	1.3	< 0.1
	* threonine	1.0	< 0.1
S 20	alanine	9.0	0.45
	lysine	7.0	0.7
	* threonine	5.0	0.4
	isoleucine	3.75	0.2
	leucine	3.0	0.3
	arginine	3.0	0.2
	histidine	2.0	< 0.1
	aspartic acid	1.0	< 0.1
	glycine	1.0	0.2
	valine	1.0	< 0.1
	methionine	0.5	< 0.1

The number of moles of each amino acid released per mole of protein is given. Each experiment was performed in duplicate and the results were averaged. For protein S4 and S8 the order of amino acids given corresponds to the protein sequence ([17,19], H. STADLER, unpublished work). For S 4, the next amino acid in the sequence is leucine and this is probably also partly removed; this would account for the high molar yield of leucine. For S 15 and S 20 the C-terminal sequences are unknown and the amino acids are listed in order of the highest number of moles released.

* Indicates that this could also be asparagine.

Modification of the tryptophan residue in S4 [19,20] and the single cysteine residues in S4 and S8 [19–21] did not diminish RNA binding, whereas additional

Table 2
A summary of RNA binding results after chemical modification of tryptophan, cysteine, methionine and lysine residues

Protein	Tryptophan residues per protein [19–20]	Cysteine residues per protein [19–21]	Binding after tryptophan and cysteine treatment	Methionine residues per protein [16]	Binding after performic acid treatment	Lysine residues per protein [16]	Lysines modified in free protein (after 6 hr)	16 S RNA binding (± 15%)		Lysines modified in RNA complex (after 6 hr)
								Specific	Non-specific	
S4	1	1	100 (± 15)%	3	< 10 %	20	5	100 %	35 %	0.5
S8	0	1	100 (± 15)%	4	< 10 %	13	0.5	100 %	30 %	0.18
S15	0	0		2	< 15 %	10	1	100 %	35 %	0.2
S20	0	0		4	< 15 %	17	3	100 %	40 %	0.3

Protein–RNA binding was determined electrophoretically [4] in the presence of the excess protein that was necessary to achieve saturation of binding [14].

modification of methionine residues resulted in non-binding.

The large trinitrobenzenesulphonate reagent was selected for lysine modification, first, to react mainly with those lysines that were accessible for RNA binding, after it was shown that reductive methylation resulted in modification of each of the lysine residues in the free protein [20,23] and, secondly, because the reagent only reacts with those lysine residues with the lowest pK values, which have been shown to be important in ribonuclease–RNA interactions [22]. The time courses of the reactions were followed for 6–8 hr, after which there was a marked falling off in the reaction rate (fig. 1). After 6 hr modification each of the proteins bound strongly to the 16 S RNA, but there was additional non-specific 23 S RNA binding (table 2).

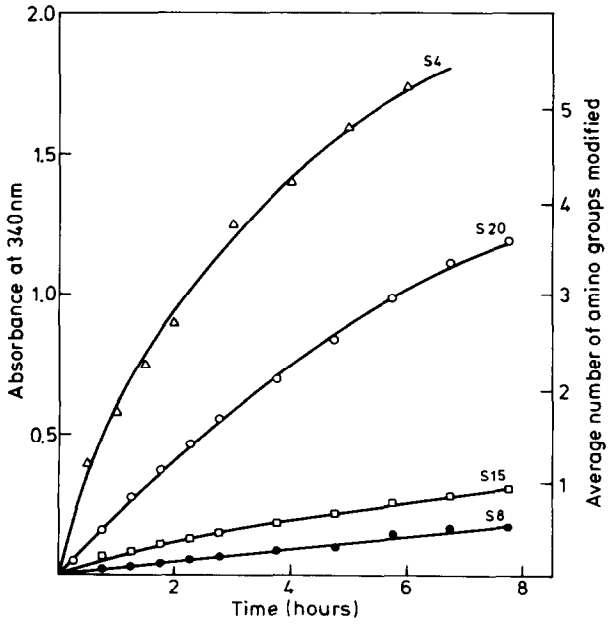

Fig. 1. The time course of trinitrophenylation of ribosomal proteins S4, S8, S15 and S20 in 0.03 M dimethylglutaric acid buffer, pH 7.2, 0.2 M NaCl and 10 mM $MgCl_2$.

The relative degree of modification of the lysine residues in the free protein and in the complex was determined. The results (table 2) indicate that for each protein a significantly lower level of modification occurred in the complex after 6 hr. In a control experiment, a non-RNA binding protein, S19, was modified to the same extent in the presence and absence of 16 S RNA.

4. Discussion

Further evidence was found for the involvement of the C-termini of each of the proteins in 16 S RNA binding in that they were all much more resistant to carboxypeptidase digestion in the complex than in the free state. These results supplemented the earlier finding that S4 proteins isolated from *E. coli* mutants, which were altered in their C-terminal amino acid sequences [7], bound less strongly to 16 S RNA than the wild type S4 [8,9].

The modification of the tryptophan residue of S4 and the cysteine residues of S4 and S8 did not affect RNA binding and these residues are therefore unlikely to be involved in binding. Non-binding after additional modification of methionine residues may indicate involvement of the latter in RNA binding. However, it is difficult to eliminate the possibility that an irreversible conformational change in the protein has occurred. This is the subject of further conformational studies.

The results for the lysine modification indicate that a very small number of lysine residues in the protein–RNA complex are important for 16 S RNA binding. First, because they are almost completely inaccessible in the protein–RNA complex and, secondly, because their modification results in a high level of additional non-specific binding (table 2). A similar conclusion has been reached for S4, on the basis of other modification experiments; evidence was found for the involvement of three lysine residues [23].

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References

[1] Mizushima, S. and Nomura, M. (1970) Nature 226, 1214–1218.
[2] Schaup, H. W., Green, M. and Kurland, C. G. (1970) Molec. Gen. Genet. 109, 193–205.
[3] Schaup, H. W., Green, M. and Kurland, C. G. (1971) Molec. Gen. Genet. 112, 1–8.
[4] Garrett, R. A., Rak, K. H., Daya, L. and Stöffler, G. (1971) Molec. Gen. Genet. 114, 112–124.
[5] Zimmermann, R. A., Muto, A., Fellner, P., Ehresmann, C. and Branlant, C. (1972) Proc. Natl. Acad. Sci. U. S. 69, 1282–1286.
[6] Zimmermann, R. A., Mackie, G., Muto, A., Garrett, R. A. Ungewickell, E., Ehresmann, C., Ebel, J. P. and Fellner, P. (1974) submitted for publication.
[7] Funatsu, G., Puls, W., Schiltz, E., Reinbolt, J. and Wittmann, H. G. (1972) Molec. Gen. Genet. 115, 131–139.
[8] Daya-Grosjean, L., Garrett, R. A., Pongs, O., Stöffler, G. and Wittmann, H. G. (1972) Molec. Gen. Genet. 119, 277–286.
[9] Green, M. and Kurland, C. G. (1971) Nature 234, 273–274.
[10] Robinson, H. K. and Wade, H. E. (1968) Biochem. J. 106, 897–903.
[11] Hindennach, I., Stöffler, G. and Wittmann, H. G. (1971) Eur. J. Biochem. 23, 7–11.
[12] Kaltschmidt, E. and Wittmann, H. G. (1970) Anal. Biochem. 36, 401–412.
[13] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[14] Schulte, C., Morrison, C. A. and Garrett, R. A. (1974) Biochemistry 13, 1032–1037.
[15] Scoffone, E., Fontana, A. and Rocchi, R. (1968) Biochemistry 7, 971–979.
[16] Kaltschmidt, E., Dzionara, M. and Wittmann, H. G. (1970) Molec. Gen. Genet. 109, 292–297.
[17] Schiltz, E. and Reinbolt, J. (1974) Eur. J. Biochem., ters, submitted for publication.
[18] Kasai, H., Takahashi, K. and Ando, T. (1969) J. Biochem. Japan 66, 591–601.
[19] Reinbolt, J. and Schiltz, E. (1973) FEBS Letters 36, 250–252.
[20] Daya-Grosjean, L. (1974) Doctrate Thesis, University of Paris.
[21] Bakardjieva, A. and Crichton, R. R. (1974) FEBS Letter, submitted for publication.
[22] Murdoch, A. L., Grist, K. L. and Hirs, C. H. W. (1966) Arch. Biochem. Biophys. 114, 375–385.
[23] Amons, R., Möller, W., Schiltz, E. and Reinbolt, J. (1974) FEBS Letters, submitted for publication.