STUDIES ON THE BINDING SITES OF PROTEIN S4 TO 16S RNA IN *ESCHERICHIA COLI* RIBOSOMES

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1. Introduction

Chemical modification with bifunctional protein reagents, as well as limited digestion with nucleases, yielding specific ribonucleoprotein complexes, have been important tools to elucidate the neighbour relations in ribosomes. Based on these methods, recently a tentative three-dimensional arrangement of the proteins in the 30S subunit has been presented [1]. However, the nature of the interactions between these proteins and between a particular protein and its rRNA binding site(s) is still obscure.

We have focussed on the neighbour interactions of the rRNA binding protein S4. This protein is perhaps the best studied 30S protein for the following reasons: i) S4 protein plays an essential role in Nomura's assembly map [2]; ii) the primary structure of S4 protein has been elucidated recently [3]; iii) many mutant S4 proteins are known having shorter or longer polypeptide chains and altered 16S rRNA binding properties [4–6]; iv) the binding sites on 16S rRNA of S4 protein have been isolated and partially characterized [7–9].

In order to determine whether specific lysine residues of S4 protein are involved in interactions with 16 S rRNA and neighbouring proteins, the protein was reductively methylated in three different states: 'free' in solution, complexed to 16S rRNA and in situ, i.e. in the intact 30S subunit. As a result, the lysine residues involved in these interactions could be tentatively identified.

2. Experimental procedure

30S ribosomes from *Escherichia coli* MRE 600 were prepared as described [10]. S4 protein was separated chromatographically from a mixture of 30S proteins according to Hardy et al. [11], and, if necessary, further purified by the procedure of Hindennach et al. [12]. 16S rRNA was prepared from 30S subunits by phenol extraction. The protein S4–16S rRNA complex was prepared as described by Traub et al. [13]. A slight modification of the described procedure for reductive methylation, using $H^{14}CHO$ [14] was applied: in order to avoid precipitation of 'free' S4 protein during modification, it was found to be necessary to add first the borohydride solution and subsequently the formaldehyde solution (cf. also ref. [15]). (This precipitation is very probably

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related to the lowering of the isoelectric point of S4 protein, due to the formation of *N*-hydroxymethylamino groups, to a value near the pH of the reaction buffer.) Solutions of protein S4 alone, protein S4–16S rRNA complex and 30S ribosomes, each in a concentration of 2 mg/ml, were dialyzed into 10 mM sodium tetraborate and 10 mM magnesium acetate (pH 9.0). To these solutions, 0.1 vol. of 1% $NaBH_4$ in the same buffer was added at 0°C, followed by adding dropwise, during 30 min, 0.167 vol of 1% HCHO (treated as described [14]) in the same buffer. In case of foaming, octan-1-ol (2–5 μl/ml reaction mixture) was added. After 30 min stirring at 0°C, the solutions were dialyzed overnight against the same buffer. The solution, containing free modified S4 protein, was subsequently dialyzed against 0.1% HCOOH and lyophilized. Both the methylated protein S4–16S rRNA complex and 30S ribosomes were isolated by centrifugation and extracted with acetic acid [11]. The latter protein preparation was subsequently chromatographed on phosphocellulose [11]. (Reductive methylation of the proteins did not influence their chromatographic behaviour.) After tryptic digestion of the modified S4 proteins, autoradiography of the peptide maps was performed to locate the junction peptides [14]. Identification of the radioactive and non-radioactive peptides was based on amino acid analysis after acid hydrolysis, and comparison with the known sequence [3].

3. Results and discussion

Reductive methylation of lysine residues renders the peptide bonds involving their carboxyl groups resistant to trypsin. With exception of proteins having a carboxyterminal lysine, peptides which are

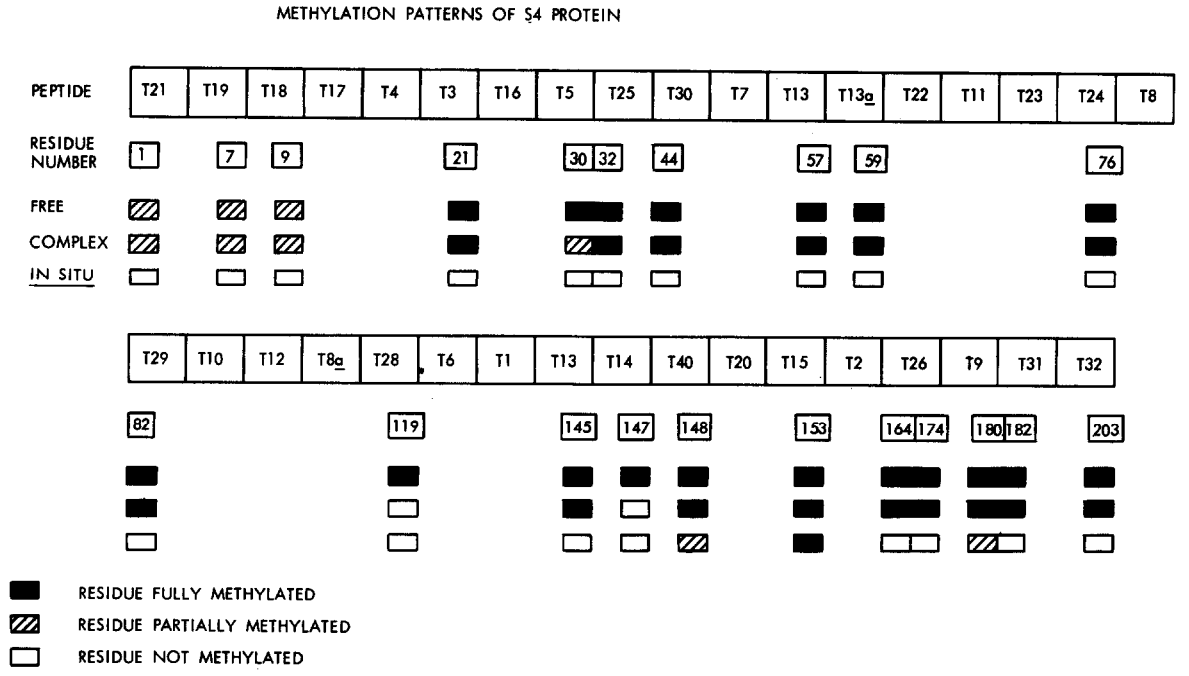

Fig. 1. On reductive methylation the N-terminus of the protein, and the ε-amino groups of its lysine residues can be mono- or dimethylated. The reactivity was calculated from the incorporated radioactivity as the number of methyl groups per residue recovered, according to the second method of ref. [14], except that the numbers were averages over lysine, ε-*N*-monomethyl-lysine and ε-*N*-dimethyl-lysine, since here no separate estimations of ε-*N*-monomethyl-lysine and ε-*N*-dimethyl-lysine were made. A fully methylated residue contained 0.9–2.0 methyl groups; a partially methylated residue contained 0.4–0.9 methyl groups. A residue, containing less than 0.4 methyl groups was considered not to be methylated.

found after this modification, have a carboxyterminus which is either unmodified lysine or arginine [14], apart from the peptide derived from the carboxyterminus of the protein. Lysines involved in salt-bridges or hydrogen bonds are supposed not to be accessible to reductive methylation [14].

From an analysis of the different tryptic peptides originating from S4 protein methylated in isolation, bound to 16S rRNA and in situ, it was possible to compare the reactivity of each of the different lysine residues in the three cases, see fig. 1. The results allow some interesting conclusions to be drawn. In the 'free' state all lysine residues of S4 protein are subjected to modification; this observation suggests that the association of S4 protein in solution [16] does not involve amino groups except, perhaps, those of Ala-1, Lys-7 and Lys-9. As a consequence of complex formation between S4 protein and 16S rRNA, two lysine residues (Lys-119 and Lys-147) have lost their reactivity towards reductive methylation, while one other residue (Lys-30) becomes only partially methylated. The most simple interpretation is that Lys-119 and Lys-147, and possibly also Lys-30, are part of 16S rRNA binding sites. A more complex interpretation is that conformational changes of the protein upon its association with 16S rRNA could render protein sites not directly related to rRNA binding, inaccessible to modification.

Multiple rRNA binding sites for S4 protein have been inferred from ribonuclease digestion studies of S4 protein–16S rRNA complexes [7–9]. The ribonuclease resistant parts are rather large and it has been proposed that they encompass non-contiguous nucleotide sequences brought together into the domain of the protein by the mode of folding of the different loops in the rRNA. In the absence of information on the three-dimensional structure of the rRNA chain around S4 protein, it may be necessary to try to prepare smaller binding fragments before the relative contribution of single- or double-stranded rRNA regions to the actual binding sites to S4 protein can be determined. Here we confine ourselves to the remark that no obvious homologies in the amino acid sequence on either side of the two lysine residues involved in the 16S rRNA binding are detectable (fig. 2).

When present in the 30S particle, only three lysine residues of S4 protein can be partially or fully methylated (Lys-148, Lys-180 and Lys-153). Interestingly, these residues are found in the carboxyterminal part of the polypeptide chain, suggesting that this part of the molecule is relatively accessible to modification. (Significantly labeled, but less than 0.4 methyl groups per lysine residue, is Lys-182.) The small dimensions of the modifying agents, HCHO and BH_4^-, argue against a burial of S4 protein within the ribosome structure. Since there are no indications for major conformational changes of proteins upon reductive methylation [15], we infer that Lys-21, Lys-32, Lys-44, Lys-57, Lys-59, Lys-76, Lys-82, Lys-145, Lys-164, Lys-174, Lys-182 and Lys-203 may play a role in interactions of S4 protein with its neighbour proteins or with additional parts of 16S rRNA, once the 30S proteins are grouped around S4 protein. Finally, the protective action of especially the surrounding proteins on S4 protein is opposite to the situation of the 50S proteins L7 and L12, where in situ the reactivity of the lysine residues is larger than in isolation [14].

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-Arg-Ala-Glu-Ala-Arg-Gln-Leu-Val-Ser-His-Lys*-Ala-Ile-Met-Val-Asn-Gly-Arg-Val-Val-Asn-
110 120

-Pro-Asn-Ser-Val-Val-Ile-Arg-Glu-Lys-Ala-Lys*-Lys-Glu-Ser-Arg-Val-Lys-Ala-Ala-Leu-Glu-
140 150

* protected lysine

Fig. 2. Amino acid sequences around the proposed rRNA binding sites.

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