5S rRNA sugar-phosphate backbone protection in complexes with specific ribosomal proteins

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Abstract 5S ribosomal RNA forms stable specific complexes with ribosomal proteins L18, L25 and L5. In this work, interaction of phosphate residues of *E. coli* 5S rRNA within 5S rRNA-protein complexes has been studied. For this purpose 5S rRNA with statistically distributed phosphorothioate residues has been used for complex formation and the accessibility of phosphorothioates to iodine cleavage in the complex and in the free state has been studied. In free 5S rRNA, the phosphate residue at A73 was partially protected, probably due to being involved in the organization of the spatial structure of 5S rRNA. This protection is stronger in the complex with three proteins when the 5S rRNA structure is stabilized. In the 5S rRNA-L18 complex only two phosphate groups, G7 and A34, were protected. L25 in a complex with 5S rRNA protects large numbers of phosphorothioate groups concentrating in two clusters, indicating the possibility of two binding sites for this protein on 5S rRNA. The protection pattern differs from that for individual proteins because of the possible rearrangement of the structure.

Key words: 5S Ribosomal RNA; Ribosomal protein; Footprinting; Phosphorothioate; Iodine cleavage; RNA structure; RNA-protein interaction

1. Introduction

5S rRNA is strongly bound to at least three ribosomal proteins and forms a separate domain of the large ribosomal subunit (for reviews see [1,2]). We have recently shown that the 5S rRNA-protein domain is located in close proximity to both the peptidyl transferase and the L11-binding domain of the *E. coli* ribosome [3]. 5S rRNA-protein complexes are formed and can exist independently of large and small ribosomal subunits, at least in eukaryotic cells [1]. Therefore, the structure of 5S rRNA-protein complexes in the free state and in the ribosome is of great interest. Different chemical and physical approaches have been used to identify protein binding sites in 5S rRNA and to assess 5S rRNA-protein interactions (reviews [1,2,4]). However, the spatial organization of 5S rRNA in complexes with specific ribosomal proteins remains unknown. In this study, we have used the method of Eckstein et al. [5] to characterize 5S rRNA phosphate groups which are involved in strong RNA-protein and RNA-RNA interactions in complexes of 5S rRNA with proteins L5, L18 and L25 from the *E. coli* ribosome. The method is based on the statistical substitution of phosphate residues for phosphorothioate groups in RNA. Modified inter-nucleotide bonds in RNA complexed with a specific protein can be cleaved with iodine unless the corresponding phosphorothioate groups are masked by a ribosomal component. This approach was successfully applied in studies concerning interactions of sugar-phosphate backbones of tRNAs with aminoacyl-tRNA synthetases [5,6] and of tRNA as well as of mRNA with ribosomes [7,8].

2. Materials and methods

Ultrapure rNTPs were obtained from Pharmacia, phosphorothio-NTPs from DuPont, alkaline phosphatase and T4 polynucleotide kinase from Boehringer Mannheim, and [γ-^{32}P]ATP from Amersham. The T7 RNA polymerase was isolated from *E. coli* strain BL21 containing the plasmid pAR 1219 according to a published procedure [9]. Individual ribosomal proteins L5, L18 and L25 were obtained as described in [10].

2.1. Preparation of modified 5S rRNA

The DNA template containing the *E. coli* 5S rDNA sequence linked to a T7 promoter was prepared as described [11]. Transcription reactions contained 240 pmol/ml 5S rDNA, 3.8 mM of each NTP (20% of one of the NTP in each probe was substituted with the corresponding phosphorothioated NTP), 5 mM DTT, 104 μg/ml BSA, 40 mM Tris-HCl, pH 8.8, 22 mM $MgCl_2$, 10 mM spermidine, 504 μg/ml pyrophosphatase and 11 μg/ml T7 polymerase. The reaction volume was 150 μl; reaction mixtures were incubated at 37°C for 14 h.

5S rRNA was purified by electrophoresis in 4% PAAG with 7 M urea [8]. Radioactive labeling at the 5′ end was carried out using [γ-^{32}P]ATP and T4 polynucleotide kinase [8]. After the second purification by electrophoresis in 4% PAAG with 7 M urea 5S rRNA was used for complex formation.

2.2. 5S rRNA-protein complex formation

A 25 μl mixture contained 10 pmol 5S rRNA and 30 pmol protein L25 or protein L18; when three proteins were added 15 pmol of each of the proteins L5, L18 and L25 were present. The mixture further contained 20 mM Tris-HCl, pH 7.8 (0°C), 20 mM $MgCl_2$, 400 mM NH_4Cl and 4 mM 2-mercaptoethanol; incubation was carried out at 30°C for 30 min. After cooling to 0°C the reaction mixture was adjusted to 10 mM $MgCl_2$, 100 mM NH_4Cl and 4 mM 2-mercaptoethanol in 20 mM Tris-HCl, pH 7.8 (0°C), and used for iodine cleavage (see below). Complex formation was checked by non-denaturing 6% PAAG (38:2) in 25 mM Tris-acetic acid, pH 7.8, 5 mM $Mg(CH_3COO)_2$. Pre-electrophoresis was carried out for 1 h at 15 mA. Loading buffer consisted of 25 mM Tris-acetic acid, pH 7.8, 5 mM $Mg(CH_3COO)_2$, 0.2% (w/w) bromophenol blue, 0.2% (w/w) xylene cyanol and 30% (w/w) glycerol and was added to the sample in a 3:1 ratio.

2.3. Iodine cleavage

5 pmol of 5S rRNA or its complex with proteins was incubated in 50 μl 20 mM Tris-HCl, pH 7.8 (0°C), 10 mM $MgCl_2$, 100 mM NH_4Cl with 1 μl of 50 mM iodine solution in ethanol at 4°C for 2 min. The reaction was stopped by the addition of an equal volume of water-saturated phenol. RNA fragments were precipitated with 100% ethanol, washed with 80% ethanol and separated in a 12% PAAG [8].

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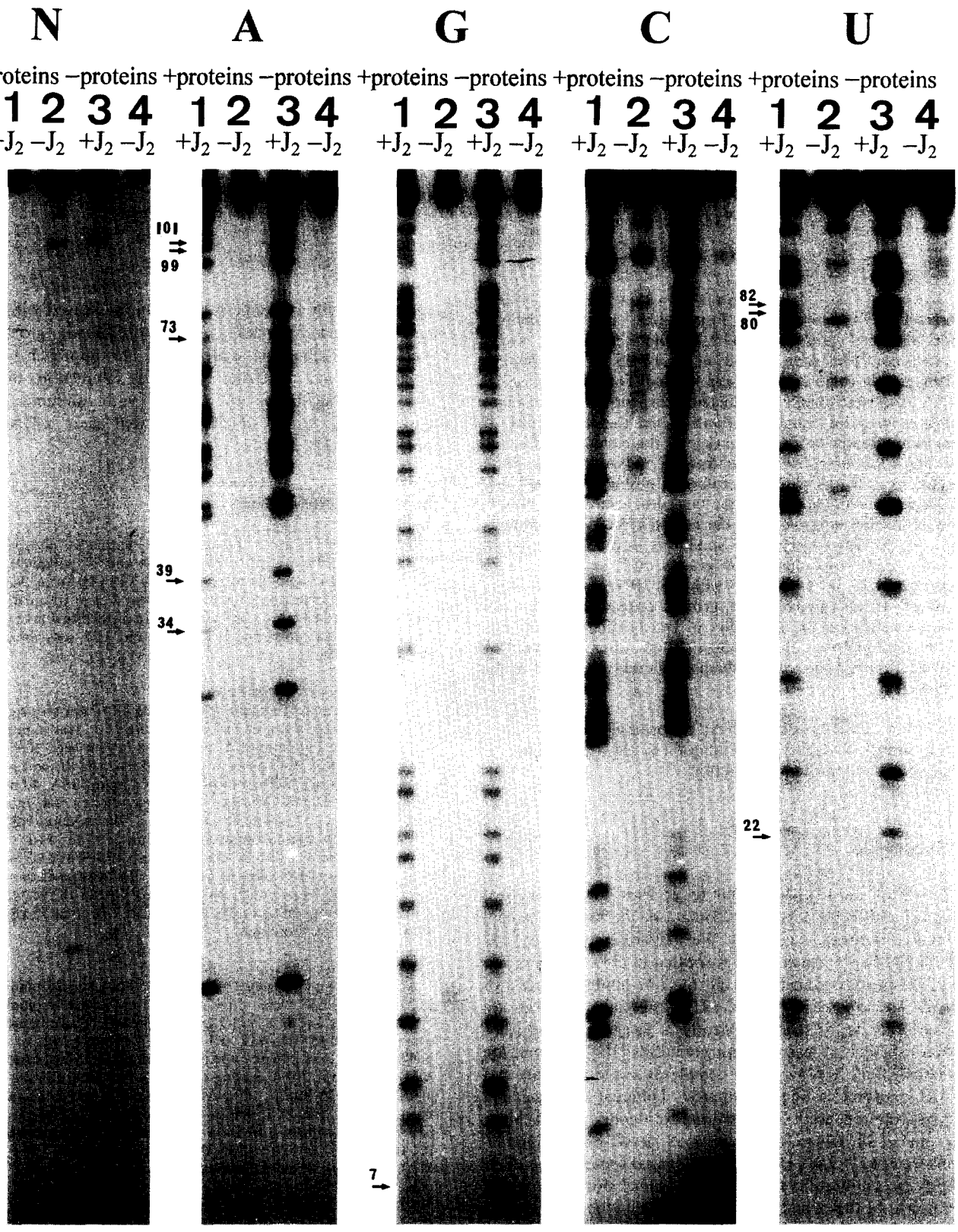

Fig. 1. Contact patterns of 5S rRNA complexed with all three proteins. The sequence blocks A, G, C and U represent cleavage patterns derived from 5S rRNA which contain the respective thioated nucleotide. N, 5S rRNA without thioated nucleotides; $-J_2$, without iodine treatment; 1,2, complex with three proteins with or without iodine treatment: 3,4, free 5S rRNA with or without iodine treatment. The arrows indicate protected nucleotides.

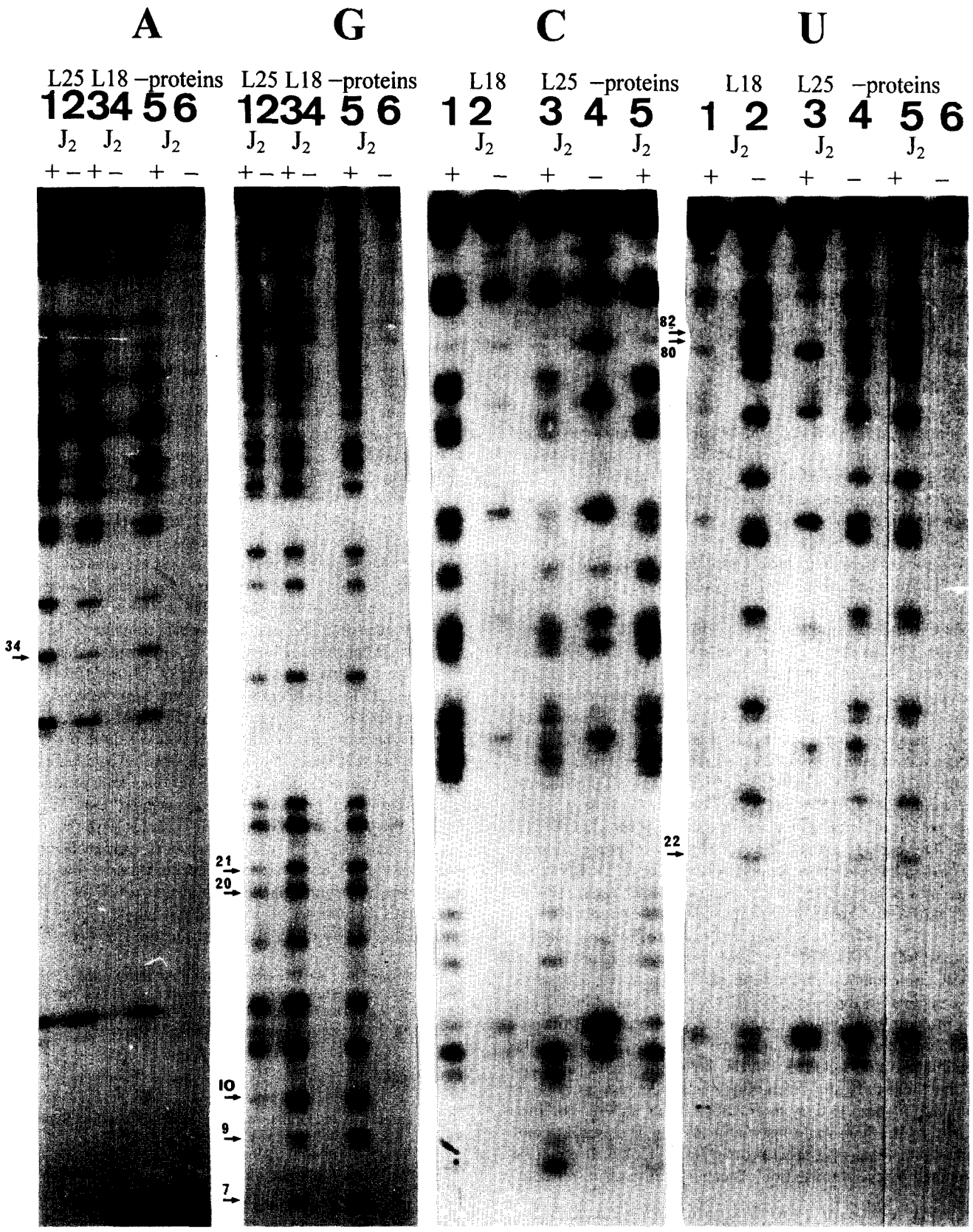

Fig. 2. Contact patterns of 5S rRNA complexed with either L18 or L25. Other explanations as in the legend to Fig. 1. 1,2, complexes with L25 with or without iodine treatment; 3,4, complexes with L18 with or without iodine treatment; 5,6, free 5S rRNA with or without iodine treatment.

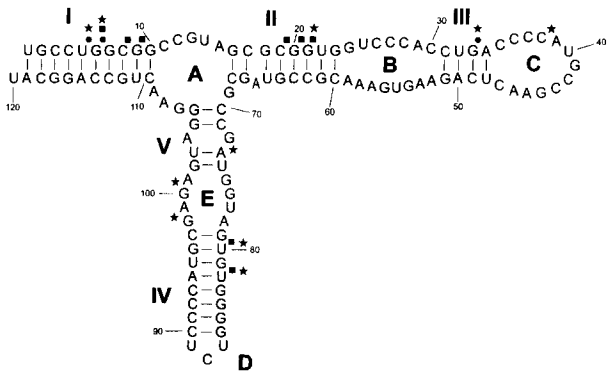

Fig. 3. Secondary structure of the 5S rRNA containing the protection sites. (●) Protection caused by L18; (■) protection caused by L25; (★) protection observed when all three proteins are present.

3. Results and discussion

Transcribed RNA containing phosphorothioates has about the same biological activity as its unmodified counterpart; this could be demonstrated with tRNAs and mRNAs [5–7,12]. Here 5S rRNA was used containing six or less phosphorothioate residues per molecule. This level of modification of 5S rRNA did not affect the ability of 5S rRNA to participate in ribosome assembly in vitro and did not reduce the activity of reconstituted ribosomes in polyU-dependent polyPhe synthesis (not shown).

Stoichiometric complexes of 5S rRNA with the proteins L18 or L25 or with all three proteins L5, L18, and L25 were obtained under standard conditions, complex formation was proven by electrophoresis under non-denaturing conditions [13]. Unmodified 5S rRNA was stable during incubation under cleavage conditions in the presence of iodine (Fig. 1, N4) as was modified 5S rRNA without proteins in the absence of iodine (Fig. 2, A6, G6, C6, U6). In contrast, all positions of free modified 5S rRNA could be cleaved by iodine with the only exception being A73 which is partially protected (Fig. 1, A3). The general accessibility of phosphorothioate residues supports a previous conclusion [14] that free 5S rRNA molecules lack a defined tertiary structure in contrast to tRNA. The partial protection of the inter-nucleotide bond on the 5′ side of A73 is hard to explain according to the generally excepted Y-like model of 5S rRNA tertiary structure [14] or NMR conformation of the loop E [15]. However, several ribose residues in the loop E 5S rRNA region were found to be less reactive with hydroxyl radicals in the presence of magnesium ions than in magnesium-free solution [16]. Thus, the possibility is not excluded that the sugar-phosphate backbone of the loop E region (including nucleotide residue A73) is involved in some tertiary interactions of 5S rRNA. The protection of the phosphate residue at A73 is increased in complexes with three ribosomal proteins (Fig. 1, A1). It could be explained by the stabilization of tertiary contacts in this complex.

In the 5S rRNA-L18 complex two phosphorothioate groups – G7 and A34 – are appreciably protected (Fig. 3, A3, G3). G7 belongs to helix I in the secondary structure of 5S rRNA and A34 to helix III. The protection of G7 is in excellent agreement with the observation that L18 prevents the hydrolysis of the phosphodiester bond linking G6 and G7 by the RNase CV [4] probably reflecting an interaction of the basic N-terminal part of L18 with helix I [13]. The protection of A34 is a surprise. Although helix III contains one of the major binding sites of protein L18 the phosphodiester bond between G33 and A34 can be attacked by the RNase alfa-sarcin in the 5S rRNA-L18 complex [17]. At least two explanations are possible. This particular phosphorothioate group can interact more strongly with the protein or, alternatively, our complex allows stronger interactions between rRNA and protein than that used in the alfa-sarcin experiment.

L25 in a complex with 5S rRNA protects a large number of phosphorothioate groups (Fig. 3): G7, G9, G10 (helix I), G20, G21, U22 (helix II), U80 and U82 (helix IV). Evidence has been presented that the L25 binding site comprises the region helix IV-loop-helix V [18]. Our results demonstrate that other parts of 5S rRNA in addition participate in the binding of protein L25. Indeed, it has been shown that the stability of helix II – directly or indirectly – influences the association of protein L25 with 5S rRNA [19]. One may expect, however, that in the ribosome the contacts of protein L25 with helices I and II would be rearranged due to the interaction of 5S rRNA with proteins L18 and L5.

In agreement with this expectation, we have observed that positions G9, G10, G20 and G21 are accessible for iodine cleavage in the complex of 5S rRNA with proteins L5, L18 and L25, whereas the adjacent positions G7 and G6 are protected. In addition to G6, four new protected positions A39, A73, A99 and A101 were discovered in this complex (Fig. 3). It is not surprising [20] that some weak contacts of 5S rRNA with a single protein L18 or L25 are stabilized in the complex with three proteins, since the binding of all three proteins L5, L18 and L25 is characterized by strong cooperativity.

Only nine out of 120 phosphate groups of 5S rRNA were found to be protected in a complex with all three proteins. In spite of the fact that our approach only reveals very strong contacts between sugar-phosphate residues and proteins, the small number of protected residues suggest that 5S rRNA retains its open conformation in the complex with the proteins L5, L18 and L25. Our preliminary data (Shpanchenko, unpublished) show, however, that incorporation of this complex into active ribosomal particles is accompanied by a drastic increase of the number of protected phosphate groups indicating a tight folding of 5S rRNA within the ribosome.

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