Crystallization of RNA/protein complexes

Maria Garber,[a*] George Gongadze,[a] Vladimir Meshcheryakov,[a] Oleg Nikonov,[a] Alexey Nikulin,[a] Anna Perederina,[a] Wolfgang Piendl,[b] Alexander Serganov[a 1] and Svetlana Tishchenko[a]

[a]Institute of Protein Research Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia, and [b]Institute of Medical Chemistry and Biochemistry, University of Innsbruck, Austria. E-mail: garber@vega.protres.ru

Different complexes of ribosomal proteins with specific rRNA fragments have been crystallized and studied by our group during the last six years. There are several factors important for successful crystallization of RNA/protein complexes, among them: length and content of RNA fragments, homogeneity of RNA and protein preparations, stability of the complexes, conditions for mixing RNA and protein components before crystallization, effect of Se-Met on RNA/protein complex crystal quality. In this paper we describe findings and methodical details, which helped us to succeed in obtaining X-ray quality crystals of several RNA/protein complexes.

Keywords: RNA/protein complexes; homogeneity of RNA fragments; hybrid complexes; crystallization; additives

1. Introduction

In the recent years, there were many publications on successful crystallization and structural studies of different specific RNA/protein complexes. The first prerequisite for success in these studies was the development of the methods for *in vitro* transcription and purification of RNA fragments (Uhlenbeck, 1987; Taira *et al*., 1991). Very useful methodical publications are available on large scale preparation of RNA suitable for crystallographic studies, on a general module for RNA crystallization and on application of protein engineering for protein/RNA complex crystallization (Price *et al*., 1995; Oubridge *et al*., 1995; Ferre-D'Amare *et al*., 1998).

Table 1

Crystals of ribosomal RNA/protein complexes.

Complex	Final resolution	References
S15/rRNA from Thermus thermophilus	2.8 Å	Nikulin et al. (2000)
S8/rRNA from Methanococcus jannaschii	2.6 Å	Tishchenko et al., (2001)
CTC/rRNA, the protein from T. thermophilus, 5S rRNA from Escherichia coli	2.3 Å	Fedorov et al. (2001)
L5/rRNA, the protein from T. thermophilus, 5S rRNA from E. coli	2.5 Å	Perederina et al., unpublished

Our group during the last six years focused efforts on studies of ribosomal RNA/protein complexes. Four crystallized binary complexes between ribosomal proteins and fragments of ribosomal RNAs are listed in Table 1. In every concrete case it was necessary to identify minimal specific binding site for the protein on its target RNA molecule and to design suitable RNA fragments for complex preparation and crystallization (Serganov *et al*., 1996; Gongadze *et al*., 1999; 2001; Tishchenko *et al*., 2001). Three complexes (among the four listed in the table) were prepared with RNA fragments transcribed *in vitro*. In the case of CTC/RNA complex, we used stable fragments of 5S ribosomal RNA, obtained by limited hydrolysis with ribonuclease A (RNase A). In each case we tried several RNA fragments of different content and length to succeed in obtaining perfect crystals. We also tried to crystallize hybrid complexes containing homologue RNA and protein components from different organisms.

2. Experimental

2.1. Preparation and crystallization of S15/rRNA complex

The 57-nt RNA fragment was obtained by *in vitro* transcription with T7 RNA polymerase from linearized plasmid DNA followed by self–excision of hammerhead ribozyme from the 3' end of synthesized RNA. The reaction mixture was concentrated using a Centricon, and then the RNA fragment was purified by gel-electrophoresis in the presence of 8 M urea (Sambrook *et al*., 1989). Wild type and mutant S15 were produced in a B834(DE3) strain and purified by ion-exchange chromatography (Serganov *et al*., 1997). Selenium (Se) was incorporated into the protein during the growth on minimal media containing selenomethionine (Se-Met). RNA and protein were mixed in equimolar amounts and crystals were grown by hanging drop technique from 1.0 M $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 50 mM KCl and 50 mM sodium cacodylate (pH 6.2) at 24°C during 3-5 days. For the MAD phasing, a two-wavelength data set was obtained from a crystal of the complex containing S15 with two Se-Met residues replacing Met57 and Met58. In the second step, a three-wavelength MAD experiment was performed with a crystal of the complex containing S15 protein mutated at Ile11 and Ala79 for two additional Se-Met. Before freezing in liquid ethane, the crystals were transferred to 25% glucose with 2.0 M $(NH_4)_2SO_4$ and 100 mM sodium cacodylate at pH 6.2, and soaked in this solution for 3 h at room temperature.

2.2. Preparation and crystallization of S8/rRNA complex

All RNA fragments were obtained by transcription from synthetic DNA templates using T7 RNA polymerase. RNA was purified on denaturing polyacrylamide slab gel (Wyatt et al., 1991), eluted, precipitated by ethanol and dissolved in 15 mM sodium cacodylate buffer, pH 6.5, with 5 mM $MgCl_2$. The gene for the *M. jannaschii* ribosomal protein S8 was cloned and overexpressed in the *E.coli* strain BL21(DE3) as a host. To avoid potential misincorporation of amino acids (e.g. Lys instead of Arg as in Calderone et al., 1996), the host strain was cotransformed with pUBS520, a plasmid carrying the gene for $tRNA^{ARG}_{AGA/AGG}$ (Brinkmann et al., 1989). For MAD phasing, Se-MetS8 was produced by essentially the same procedure, but with the *E.coli* strain B834(DE3) and minimal media containing Se-Met (Leahy et al., 1992). The protein was purified by cation-exchange chromatography and dialyzed against 40 mM sodium cacodylate buffer at pH 6.5, containing 40 mM NaCl and 2 mM DTT. The RNA fragment and protein S8 were mixed in equimolar amounts (3mg/ml for RNA and 3.5 mg/ml for S8). Crystals were grown by the vapor diffusion method at 4°C. Hanging drops (3 µl) were made by mixing the RNA/S8 complex with 2.8 M $(NH_4)_2SO_4$ in 100 mM sodium cacodylate buffer, pH 6.5, in a 2:1 volume ratio, respectively. The well solution was made from 2.2 M $(NH_4)_2SO_4$ in 100 mM sodium cacodylate buffer at pH 6.5. Crystals appeared within three days. Before freezing in liquid nitrogen, the crystals were soaked in the next cryo-protective solution: 30% glucose with 2.5 M $(NH_4)_2SO_4$ and 100 mM sodium cacodylate at pH 6.5. A

[1] Present address: Laboratory of Nucleic Acid and Protein Structures, Memorial Sloan-Kettering Cancer Centre, 1275 York Avenue, Box 557, New York, NY 10021, USA.

 © 2002 International Union of Crystallography • Printed in Denmark – all rights reserved

mixture of ammonium sulphate with glucose was added to the crystallization solution, step by step, and when the final concentration of glucose was reached, the crystals were kept in the cryoprotective solution for 1 h at room temperature.

2.3. Preparation and crystallization of L5/rRNA complex

L5 from *T. thermophilus* (TthL5) was cloned and overproduced in *E. coli* cells as described by Gongadze *et al.* (2001). The protein was purified by combination of hydrophobic and cation-exchange chromatography as previously reported for other *T. thermophilus* ribosomal proteins (Vysotskaya *et al.*, 1994; Meshcheryakov *et al.*, 1997). The RNA fragment corresponding to the binding site for L5 on *E. coli* 5S rRNA (nucleotides 28-56) was synthesized by *in vitro* transcription from linearized plasmid using T7 RNA polymerase. Extra G and C nucleotides were added at 3' and 5' ends of the RNA to stabilize its double helix. The plasmid contained the following elements: a T7 promoter, a template for the target RNA sequence, and Sma I site used for plasmid linearization. The RNA fragment was isolated as described by Gongadze *et al.* (2001). The purified components, L5 and fragment of 5S rRNA, were mixed in equimolar amounts in 10 mM sodium cacodylate at pH 7.0, 10 mM $MgCl_2$ and 200 mM KCl. The concentration of the complex in the crystallization solution was 2 mg/ml. The complex solution was mixed with an equal volume of the precipitant solution: 50 mM sodium cacodylate, pH 6.5, 100 mM $Mg(CH_3COO)_2$, 50 mM KF and 10% (w/v) polyethylene-glycol 8000 (PEG_{8000}). For crystallization, handing drops of the 2-5 µl of the mixture were placed on siliconized cover slips over 0.25-0.5 ml reservoir solution containing 50 mM sodium cacodilate pH 6.5, 100 mM $Mg(CH_3COO)_2$, 200 mM KCl and 10% (w/v) PEG_{8000}. Crystals were grown within a few days to a size of up to $0.05 \times 0.05 \times 0.1$ mm at room temperature. Subsequently, a Se-Met derivative for L5 was prepared, its complex with the fragment of 5S rRNA was obtained, and crystals of this complex were produced under conditions identical to those described for the native L5/rRNA complex. For diffraction data collection, the following cryo-protective solution was used: 25 mM sodium cacodylate at pH 6.5, 50 mM $Mg(CH_3COO)_2$, 100 mM KCl, 25 mM KF, 5% (w/v) PEG_{8000} and 20% (v/v) 2-methylpentane-2,4-diol (MPD). The crystals were soaked for 2 h and flash-frozen in liquid nitrogen.

2.4. Preparation and crystallization of CTC/rRNA complex

The protein was prepared from an *E. coli* strain-overproducer BL21(DE3)pLysS-pET11c-CTC (Gryaznova *et al.*, 1996) and purified as described by Meshcheryakov et al. (1997). 39 nt. fragment *E. coli* 5S rRNA protected by CTC from RNase A activity was prepared and purified as described in Gongadze *et al.* (1999). The purified components, CTC and 5S rRNA fragment, were mixed in equimolar amounts to a final complex concentration of 6 mg/ml in 10 mM sodium cacodylate at pH 7.0, 50 mM $MgCl_2$ and 50 mM KCl. The complex solution was mixed with an equal volume of the precipitant solution: 50 mM sodium cacodylate at pH 7.0, 50 mM $MgCl_2$, 50 mM KCl, 4 mM $CdCl_2$ and 7% (v/v) MPD. For crystallization, hanging drops of the 2-5 µl of the mixture were placed on siliconized cover slips over 0.25-0.5 ml reservoir solution containing 50 mM sodium cacodylate at pH 7.0, 200 mM KCl and 15% (v/v) MPD. Crystals were grown within a few days at room temperature. Subsequently, a Se-Met derivative for CTC was prepared, its complex with the fragment of 5S rRNA was obtained, and crystals of this complex were produced under conditions identical to those described for the native CTC/rRNA complex. For diffraction data collection, the next cryoprotective solution was used: 10 mM sodium cacodylate at pH 7.0, 100 mM $Mg(CH_3COO)_2$, 100 mM KCl, 4 mM $CdCl_2$, 15% (v/v) MPD and 10% (w/v) PEG_{400}. The crystals were soaked for 1 h and flash-frozen in liquid nitrogen.

3. Results

3.1. Length and homogeneity of RNA fragments

Size and homogeneity of RNA fragments are highly important for successful crystallization and for crystal quality. Below we describe our experience in selecting RNA fragments and on their purification for crystallization of several RNA/protein complexes.

3.1.1. S15/rRNA complex. S15 is one of the most important proteins of the small ribosomal subunit. It binds specifically and strongly to 16S rRNA independently of the presence of other ribosomal proteins. The minimal rRNA fragment binding S15 protein from *Thermus thermophilus* was determined by chemical and biochemical approaches (Serganov et al., 1996). It consists of 56 nt and contains three shortened helices H20, H21 and H22 of 16S rRNA, the latter two being closed by UUCG tetraloops (Fig. 1).

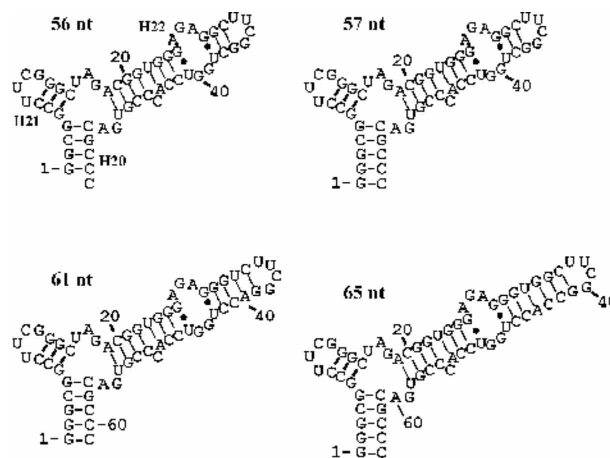

Figure 1

T. thermophilus 16S rRNA fragments binding ribosomal protein S15.

However, S15 protein complexed with this minimal fragment was not crystallizable. Therefore we tried several RNA fragments of different length: 57, 61 and 65 nt. (Fig. 1) for the complex preparation and crystallization. Crystals were obtained only for the complex containing the 57 nt. fragment. Larger fragments were not suitable for crystallization. A difference of only one nucleotide at the 5'end of RNA molecule was crucial for crystallization. When the structure of the complex was determined, we found that two symmetry-related molecules of RNA in the unit cell within the crystal interact by the ends of helices 20. Therefore one unpaired nucleotide at the 3' end could prevent crystallization (Fig. 2*a, b*).

First the 57-nt RNA fragment was obtained by run-off *in vitro* transcription from the linearized plasmid DNA (scheme in Fig. 3a), and the reaction mixture was fractionated by gel-filtration on Prep BioSil SEC-250. However, as a result of the T7 RNA-polymerase non-specificity, the transcription product besides the 57 nt. fragment contained also in rather large amount 58 and 59 nt. fragments, which could not be removed by gel-filtration (Fig. 4*a* and line 10 in Fig. 4*b*). These fragments could be separated by gel-electrophoresis in denaturing conditions as in Sambrook *et al.* (1989), but it leads to a great loss of RNA. To overcome this problem, we decided to use DNA construction with a 3' *cis*-acting hammerhead ribozyme (Fig. 3*b*). However, the transcription product was again non-homogeneous and could not be purified by gel-filtration only. Then we prepared a

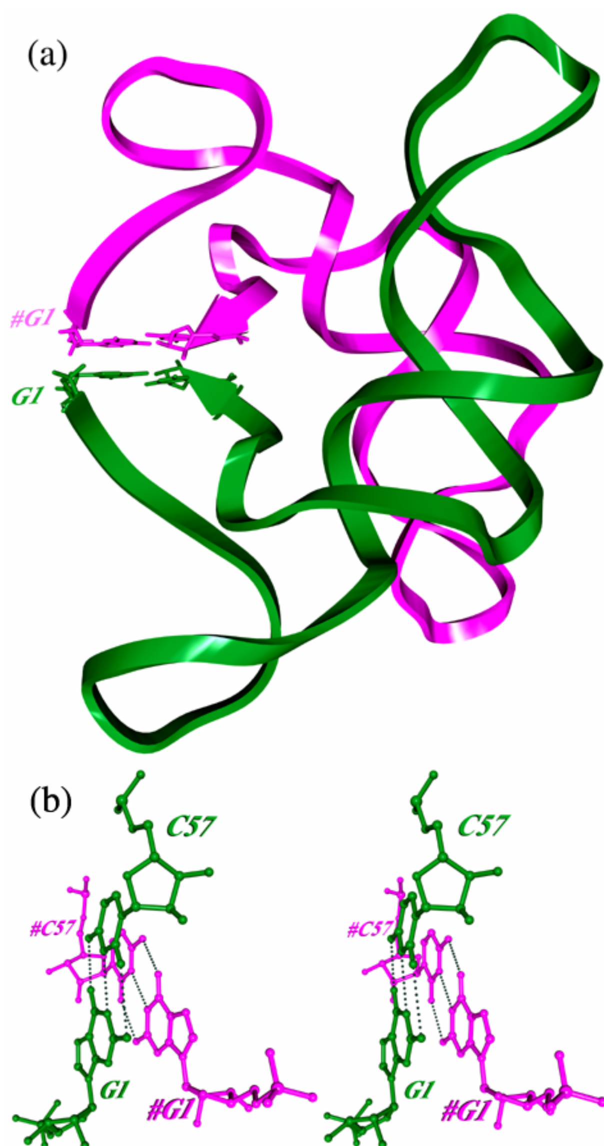

Figure 2

The ends of symmetry-related RNA molecules form a stacking interaction in the S15/rRNA crystal. *(a)* Overall view. (b) Stereo view of the stacking interaction between two G-C pairs at the ends of two symmetry-related RNA molecules.

construction with two *cis*-acting hammerhead ribozymes (Fig. 3c) as in Price *et al*. (1995). In this case the product of transcription was homogeneous and could be purified by gel-filtration only, but the yield of this transcript was rather low (Fig. 4b lines 4-9). Moreover, it turned out that this transcript was not as good for crystallization as the transcript prepared from the construct with only the 3‘ *cis*-acting ribozyme. The ends of RNA transcripts were different: the cleavage by a 5’ *cis*-acting ribozyme generates hydroxyl group at the 5’ end of the RNA transcript instead of three-phosphate group (see Fig. 3*c*). Probably, this can influence the interaction between the ends of the two RNA molecules in the crystal. Finally, the best crystals of the S15/rRNA complex were obtained using the transcript from the construct with the 3‘ *cis*-acting ribozyme. This RNA fragment was purified by gel-electrophoresis in the presence of 8 M urea (Fig. 4*c*, *d*).

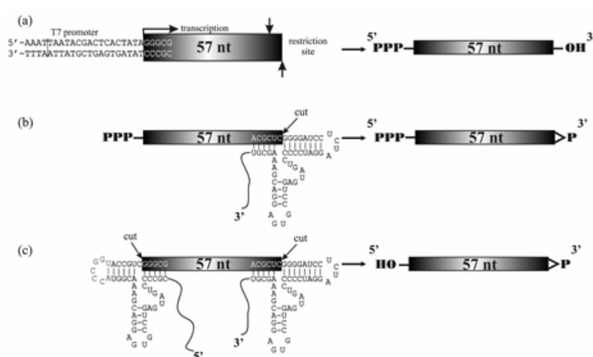

Figure 3

Scheme for *in vitro* transcription of the 16S rRNA 57 nt. fragment binding S15 protein: *(a)* run-off transcription from the linearized plazmid; *(b)* transcript from the DNA construct flanked by 3’ *cis*-acting hammerhead ribozyme; *(c)* transcript from the DNA construct flanked by 5’ and 3’ *cis*-acting hammerhead ribozymes.

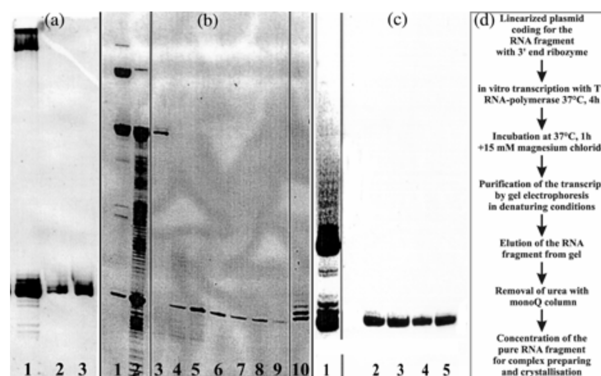

Figure 4

Purification of the 16S rRNA 57 nt. fragment binding S15 protein (analysis by polyacrylamide gel electrophoresis): *(a)* standard run-off transcription: lane 1 - transcription mixture, lanes 2 and 3 – the 57 RNA fragment and RNA impurities of similar size after gel-filtration; *(b)* transcription from the DNA construct flanked by 5’ and 3’ *cis*-acting hammerhead ribozymes: lane 1 – initial transcription mixture, lane 2 – transcription mixture after incubation at 50°C in the presence of 30 mM $MgCl_2$, lane 3 – ribozyme fraction after gel-filtration, lanes 4-9 – fractions of the 57 nt RNA fragment after gel-filtration, lane 10 – the 57 nt RNA fragment obtained by standard run-off transcription after gel-filtration; *(c)* transcription from the DNA construct flanked by 3’ *cis*-acting hammerhead ribozyme: lane 1 –transcription mixture after incubation at 37°C in the presence of 30 mM $MgCl_2$, lanes 2-5 – fractions of the 57 nt. fragment after purification by gel-electrophoresis followed by chromatography on mono-Q column; *(d)* Scheme of purification of the 16S rRNA 57 nt. fragment binding S15 protein.

3.1.2. S8/rRNA complex. Binding site for ribosomal protein S8 on helix H21 of 16S rRNA is located near the binding site for S15. Similarly to S15, S8 protein binds to 16S rRNA specifically, strongly and independently on the presence of other ribosomal proteins. The nucleotide sequence of the binding site for S8 is conserved among Bacteria and Archaea. Nucleotides, essential for S8 recognition, are located in a very irregular region, which interrupts the helix. For crystallization trials we used bacterial S8 protein from *T. thermophilus* (TthS8) and its archaeal homologue from Methanococcus *jannaschii* (MjaS8). Several fragments of bacterial 16S rRNA from *T. thermophilus* and archaeal 16S rRNA from *M. jannaschii* were prepared by *in vitro* transcription, and four of them were suitable for crystallization in complex with S8 protein (Fig. 5). We found, that the minimal fragment binding S8 should contain not less than 35 nt. Such fragment of bacterial rRNA binds

S8 from both *M. jannaschii* and *T.thermophilus*, but shorter RNA fragments do not possess the binding capacity. All RNA fragments were synthesized by *in vitro* transcription with T7 RNA-polymerase. First we tried to crystallize the fragments purified by gel-filtration, but it turned out that the quality of crystals was much better after purification by gel-electrophoresis at denaturing conditions (2.8 Å resolution in comparison with 4.0 Å resolution).

Figure 5

16S rRNA fragments used for crystallization and binding ribosomal protein S8: *(a)* fragments of bacterial 16S rRNA from *T. thermophilus*; *(b)* fragments of archaeal 16S rRNA from *M. jannaschii*.

3.1.3. CTC/rRNA complex. CTC is a 5S rRNA-binding protein from the large ribosomal subunit in Bacteria. Homologous proteins are absent in Archaea and Eukarya. We worked with CTC protein from T. thermophilus (TthCTC). It was shown that CTC binds specifically to the so-called loop E region of 5S rRNA (Fig. 6). This region of 5S rRNA containing loop E is rather stable to RNase A activity, therefore fragments for crystallization could be prepared by limited RNase A digestion. Limited hydrolysis of 5S rRNA at mild conditions allows preparation of a 62 nt. fragment comprising the binding site for CTC protein. Large bi-pyramidal crystals were obtained for the complex of 62 nt. fragment with CTC protein, but they diffracted to 10 Å resolution. At more aggressive conditions of hydrolysis, but in the presence of the protein, a shorter 39 nt. RNA fragment (Fig. 6) binding CTC was prepared as in Gongadze et al. (1999). Hexagonal crystals of CTC complexed with the shorter RNA fragment diffracted much better, to about 3.5 Å resolution. $CdCl_2$ added to the crystallization solution improved the crystal quality essentially (see below).

3.1.4. TthL5/rRNA complex. The specific binding site for another 5S rRNA-binding protein, L5 from *T. thermophilus,* was identified from limited RNase hydrolysis of 5S rRNA in the presence of TthL5 (Gongadze *et al*., 2001). Minimal binding site in *E. coli* 5S rRNA for this protein consists of 29 nt. (Fig. 6). This fragment with two extra nucleotides at the ends (G at the 5'-end and C at the 3'-end) was synthesized by *in vitro* transcription and used for the complex preparation and crystallization. Some crystals of this complex were obtained, but their quality was not high enough to use them for X-ray studies. To stabilize the helical part of the 29 nt. fragment, two additional G nucleotides were attached to its 5'-end and three C - to the 3'-end (Fig. 6). The resulting 34 nt. RNA fragment complexed with TthL5 was much more suitable for crystallization. The fragment was synthesized by *in vitro* transcription and purified by gel-electro phoresis in denaturing conditions. The pure RNA preparation was used for the complexing with TthL5 (Fig. 6).

Figure 6

Secondary structure of *E. coli* 5S rRNA. Binding sites for CTC and TthL5 proteins are boxed. TthL5-binding RNA fragment with additional nucleotides (bold) is shown. Pictures of gel-shift analysis are given.

3.2. Hybrid rRNA/protein complexes

It is known that many ribosomal proteins are structurally and functionally interchangeable within the ribosomes from different species (Gourse *et al*., 1981). This assumes that RNA-binding sites on the proteins and protein-binding sites on the corresponding RNA molecules are structurally highly conserved, therefore hybrid complexes are suitable for studying specific RNA/protein recognition. In our experiments hybrid complexes between ribosomal proteins and specific RNA fragments from different organisms were sometimes better for crystallization than homologous non-hybrid complexes.

3.2.1. TthCTC/EcoRNA. CTC protein from an extreme thermophilic bacterium *T.thermophilus* was crystallized in complex with the RNA fragment from 5S rRNA of *E. coli* (Fig. 6). It is interesting to note that, in contrast to our success in crystallization of the hybrid complex, the non-hybrid complex of CTC with the similar small RNA fragment of *T. thermophilus* 5S rRNA was not crystallizable in our trials. The fragments of *E. coli* and *T. thermophilus* 5S rRNA are identical in the region of the protein-binding site, but there are some differences in the flanking regions. The flanking regions are involved in RNA-RNA interactions within the crystal, and probably interfered with crystallization in case of the non-hybrid complex.

3.2.2. TthL5/EcoRNA. L5 from *T. thermophilus* also binds as efficiently to *T. thermophilus* as to the *E. coli* 5S rRNA. The specific protein-binding sites in 5S rRNAs of both organisms are practically identical. It was shown earlier that TthL5 binds to *E. coli* 5S rRNA much stronger than the homological EcoL5 (Gongadze et al., 1993). Suitable crystals were grown for TthL5 complexed with a 34 nt. RNA fragment comprising L5-binding site of 5S rRNA from *E. coli*. The recently solved crystal structure of this hybrid TthL5/rRNA complex shows that Arg96 in TthL5 (instead of Gly in EcoL5) forms three hydrogen bonds with 5S rRNA. This explains the increased stability of the hybrid complex between TthL5 and 5S rRNA from *E.coli* in comparison with the native *E.coli* L5/5S rRNA complex.

3.2.3. Hybrid complexes of archaeal and bacterial S8 proteins. First, some crystals have been obtained for TthS8 in complex with 41nt fragment of *T. thermophilus* 16S rRNA. These crystals

diffracted only to 8 Å resolution. A hybrid complex of the same RNA fragment with archaeal MjaS8 gave crystals diffracting to 3.6 Å resolution. The second hybrid complex was prepared for archaeal MjaS8 with 37 nt. *T. thermophilus* 16S rRNA fragment, the crystals diffracted to 4.0 Å resolution. Noteworthy the complexes of archaeal MjaS8 were extremely stable even in the absence of Mg^{2+} ions (Fig. 7). Probably it was the reason why the hybrid complexes of MjaS8 with *T. thermophilus* RNA fragments were better for crystallization than the homologous complexes of TthS8. However the best crystals of S8/rRNA were obtained for non-hybrid fully archaeal complex containing MjaS8 and 37 nt. fragment of *M. jannaschii* 16S rRNA. Crystals of this complex diffracted to 2.8 Å resolution. We believe that the reason of this success was in the extremely high stability of the crystallized complex.

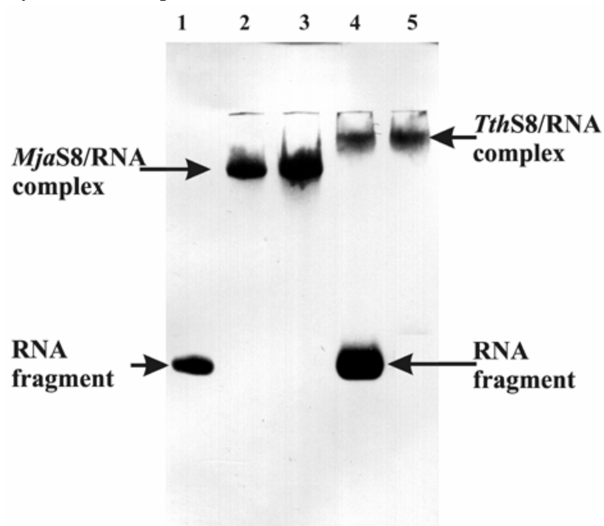

Figure 7

Gel-shift analysis of S8/rRNA complexes (Batey et al., 1996): lane 1 – 41 nt. fragment of 16S rRNA, lane 2 - MjaS8/rRNA complex formed in the absence of $MgCl_2$, lane 3 – MjaS8/rRNA complex formed in the presence of 5 mM $MgCl_2$, lane 4 – TthS8/rRNA complex formed in the absence of $MgCl_2$, lane 5 - TthS8/rRNA complex formed in the presence of 5 mM $MgCl_2$.

3.3. Effect of incubation time and temperature on RNA/protein complexing

Conditions for complex preparation also influence crystal growth. Usually before mixing we heated RNA preparations at 40°C (sometimes even at 60°C) for 15 min, then incubated the mixture at the room temperature for 5-15 min and after that put it in the refrigerator for about 1 h. Such a procedure for RNA/protein complex preparing was needed to avoid an aggregation observed in many cases at fast mixing of the ingredients.

For S15/rRNA complex we found, that 95% of the RNA and protein molecules were complexed 15 min after the mixing, however, time of the incubation should not be less than 30 min, and increasing incubation time to 3 h leads to better crystals.

3.4. Additives stabilizing crystal packing and decreasing mosaicity in crystals of RNA/protein complexes

Different additives are often used in protein crystallography to improve the crystal quality. Our experience in improving of crystal quality using additives for two RNA/protein complex crystals is described below.

3.4.1. KF in crystallization of ThtL5/rRNA complex. Crystals of TthL5/rRNA complex were obtained first without KF but with 200 mM KCl in the crystallization solution. They diffracted well, but possessed of rather high disorder. To improve their quality we used KF, because earlier this reagent had helped to avoid high degree of mosaicity for the crystals of ribosomal protein S6 (Lindhal *et al.*, 1994). In the case of the TthL5/rRNA complex, replacement of 100 mM KCl for 25 mM KF in the crystallization solution led to decreasing mosaicity of the crystals from more than 1° to about 0.4°.

3.4.2. CdCl2 in crystallization of CTC/rRNA complex. To improve the quality of CTC/rRNA complex crystals, which diffracted to 3.5 Å resolution, we tried different concentrations of bi- and mono-valent cations. First of all, we varied $MgCl_2$ concentration between 10 to 100 mM, and found that the number of crystals in the drop and time of crystal growth depended very much on the $MgCl_2$ concentration. However, it did not affect the diffraction quality. We could improve the resolution essentially only by adding $CdCl_2$ to crystallization mixture. Crystals prepared with 2-4 mM $CdCl_2$ diffracted to 2.3 Å resolution and were used for data collection. Moreover the crystals were obtained much faster and reproduced without problems. When the structure of the complex was determined, we were surprised that all Cd^{2+} ions were bound to the protein, not to RNA. The Cd^{2+} ions were coordinated by solvent and protein groups, usually with six ligands (Fedorov et al., 2001). The ligands are nitrogens of histidines, oxygens of acidic residues and water molecules. Two Cd^{2+} ions were found to interact with each other through water molecule. Cd^{2+} ions were involved in the stabilization of interactions between the protein molecules in the crystals. It explains their effect on the crystal quality.

3.5. Derivatives

Usually, it is rather hard to obtain heavy-atom isomorphous derivatives for RNA/protein complexes because of high molecular flexibility and not tight crystal packing. In the case of S15/rRNA complex, we tried to use synthetic RNA with four bromouridines, but no crystals appeared in drops at the appropriate crystallization conditions. To incorporate sufficient amount of selenium, we should prepare mutated S15 with four Met residues instead of the two ones existing in the native protein. It turned out that the quality of the crystals containing S15 mutant with four Se-Met residues was remarkably better, than the quality of the original crystals.

In the case of S8 protein, where four Met residues exist in the native protein, we observed a similar positive effect of Se-Met on the crystal quality without a mutation. The crystals of the native S8/rRNA complex diffracted to 2.8 Å resolution, but those with Se-MetS8 protein diffracted to 2.6 Å resolution.

3.6. Cryoprotective solutions

Crystals of S15/rRNA and S8/rRNA complexes were grown with ammonium sulphate as precipitant. A mixture of precipitant solution with ammonium sulphate and 25% - 30% (w/v) of glucose was selected as cryoprotective solution for these crystals. All other known reagents (ethanol, MPD, PEG, ethylene glycol, sucrose) used as cryoprotectants were not suitable for mixing with concentrated ammonium sulphate.

In the case of crystals grown with PEG (L5/rRNA complex), a mixture of the same PEG with 20% (v/v) MPD was suitable. And for crystals grown with MPD (CTC/rRNA complex), low-molecular PEG_{400} added to the precipitant solution was helpful.

4. Discussion

Summarizing our experience in crystallization of binary RNA/protein complexes, we can say that the most important step in this work was the design and the selection of suitable RNA

fragments. Specific RNA fragments of ribosomal RNAs binding corresponding ribosomal proteins were identified by different chemical and biochemical methods and prepared by *in vitro* transcription or by limited hydrolysis. The length of the fragments we used in our experiments was about 35-60 nt. in average. Tetraloops and extra nucleotides were inserted to close the ends of RNA moieties or/and to stabilize helices. Variations in the content of RNA ends were used to select fragments most suitable for crystallization. It is noteworthy that RNA fragments suitable for crystallization in complex with specifically bound proteins were often crystallizable in the unbound state also. It means that they possessed stable secondary and tertiary structure in the unbound state. Selection of the protein component for preparing RNA/protein complexes suitable for crystallization was not so crucial. We did not apply protein engineering, but using homologue proteins from different organisms we could prepare stable hybrid complexes, which were sometimes more suitable for crystallization than the native ones. Protein engineering was applied only to insert additional Met or Cys residues needed for preparing heavy-atom derivatives. Interestingly such mutations and replacement of Met for Se-Met influenced positively on the crystal quality of RNA/protein complex crystals.

The quality of RNA/protein com*plex* crystals depended very much on the homogeneity of RNA fragments. *In vitro* transcription with T7 RNA-polymerase is a very convenient method for obtaining RNA fragments at preparative scale. However this enzyme adds some nucleotides non-specifically to the ends of RNA transcripts, and as a result, generates a mixture of similar size RNA fragments. It was possible to obtain some crystals of RNA/protein complexes using non-homogeneous RNA preparations isolated after transcription by gel-filtration only, but the quality of crystals was rather low in such cases. The quality of crystals could be improved efficiently if homogeneous RNA fragments after purification by gel electrophoresis in the presence of 8 M urea were used. Application of *cis*-acting ribozymes shows that different forms of the ends of RNA transcripts can also influence crystallization.

This work was supported by the Russian Academy of Sciences and the Russian Foundation for Basic Research. The research of MG was supported in part by International Research Scholar's award from the Howard Hughes Medical Institute. The research of WP was supported by the Austrian Science Fund (FWF; grant P14550-GEN) and the Austrian-Russian Collaboration Program (project I.5).

References

Batey, R. T. & Williamson, J. R. (1996). *J. Mol. Biol.* **261**, 536-549.

Brinkmann, U., Mattes, R. E. & Buckel, P. (1989). *Gene,* **85**, 109-114.

Calderone, T. L., Stevens, R. D. & Oas, T. G. (1996). *J. Mol. Biol.* **262,** 407-412.

Fedorov, R., Meshcheryakov, V., Gongadze, G., Fomenkova, N., Nevskaya, N., Selmer, M., Laurberg, M., Kristensen, O., Al-Karadaghi, S., Liljas, A., Garber, M. & Nikonov, S. (2001). *Acta Cryst.* D**57**, 968-976.

Ferre D'Amare, A. R., Zhou, K. & Doudna, J. A. (1998). *J. Mol. Biol.* **279**, 621-631.

Gongadze, G. M., Tishchenko, S. V., Sedelnikova, S. E. & Garber, M. B. (1993). *FEBS Lett*. **330**, 46-48.

Gongadze, G. M., Meshcheryakov, V., Serganov, A., Fomenkova, N., Mudrik, E., Jonsson, B.-H., Liljas, A., Nikonov, S. & Garber, M. (1999). *FEBS Lett*. **451**, 51-55.

Gongadze, G. M., Perederina, A., Meshcheryakov, V., Fedorov, R., Moskalenko, S., Rak, A., Serganov, A., Shcherbakov, D., Nikonov, S. & Garber, M. (2001). *Mol. Biol.* **35**, 521-526.

Gourse, R., Thurlow, D., Gerbi, S. & Zimmermann, R. (1981). *Proc. Natl Acad. Sci. USA*, **78** 2722-2726.

Gryaznova, O., Davydova, N., Gongadze, G., Jonsson, B., Garber, M. & Liljas, A. (1996). *Biochimie*, **78**, 915-919.

Leahy, D. S., Hendrickson, W. A, Aukhil, I. & Erickson, H. P. (1992). *Science*, **258**, 987-991.

Lindahl, M., Svensson, L. A., Liljas, A., Sedelnikova, S., Eliseikina, I. A., Fomenkova, N. P., Nevskaya, N., Nikonov, S. V., Garber, M. B., Muranova, T. A., Rykunova, A. I. & Amons, R. (1994). *EMBO J*. **13**, 1249-1254.

Meshcheryakov, V., Gryaznova, O., Davydova, N., Mudrik, E., Perederina, A., Vasilenko, K., Gongadze, G. and Garber,_M. (1997). *Biochemistry (Moscow)*, **62**, 537-542.

Nikulin, A., Serganov, A., Ennifar, E., Tishchenko, S., Nevskaya, N., Shepardà, W., Portier, C., Garber, M., Ehresmann, B., Ehresmann, C., Nikonov, S. & Dumas, P. (2000). *Nature Struct. Biol*. **7,** 273-277.

Oubridge, C., Ito, N., Teo, C.-H., Fearnley, I. & Nagai, K. (1995). *J. Mol. Biol.* **249**, 398-408.

Price, S. R., Ito, N., Oubridge, C., Avis, J. M. & Nagai, K. (1995). *J. Mol. Biol.* **249**, 409-423.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning – A laboratory manual,* 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, USA.

Serganov, A., Masquida, B., Westhof, E., Cachia, C., Portier, C., Garber, M., Ehresmann, B. & Ehresmann, C. (1996). *RNA*, **2**, 1124-1138.

Serganov, A., Rak, A., Garber, M., Reinbolt, J., Ehresmann, B., Ehresmann, C., Grunberg-Manago, M. & Portier, C. (1997). *Eur. J. Biochem*. **246**, 291-300.

Taira, K., Nakagawa, K., Nishikawa, S. & Furukawa, K. (1991). *Nucleic Acids Res.* **19**, 5125-5130.

Tishchenko, S., Nikulin, A., Fomenkova, N., Nevskaya, N., Nikonov, O., Dumas, P., Moine, H., Ehresmann, B., Ehresmann, C., Piendl, W., Lamzin, V., Garber, M. & Nikonov, S. (2001). *J. Mol. Biol*. **311,** 331-334.

Tishchenko, S., Vassilieva, I., Platonova, O., Serganov, A., Fomenkova, N., Mudrik, E., Piendl, W., Ehresmann, C., Ehresmann, B. & Garber, M. (2001). *Biochemistry (Moscow)*, **66,** 948-953.

Uhlenbeck, O. C. (1987). *Nature*, **328**, 596-600.

Vysotskaya, V., Tishchenko, S., Garber, M., Reinbolt, J., Kern, D., Ehresmann, C. & Ehresmann, B. (1994). *Eur. J. Biochem*, **223**, 437-445.

Wyatt, J. R., Chastain, M. & Puglishi, J. D. (1991). *BioTechniques*, **11**, 764-769.

Received 25 March 2002 • Accepted 7 August 2002