Biochemistry (Moscow), Vol. 69, No. 12, 2004, pp. 1319-1323. Translated from Biokhimiya, Vol. 69, No. 12, 2004, pp. 1619-1624.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-105, June 27, 2004.

Role of N-Terminal Helix in Interaction of Ribosomal Protein S15 with 16S rRNA

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Received March 19, 2004
Revision received April 20, 2004

Abstract—The position and conformation of the N-terminal helix of free ribosomal protein S15 was earlier found to be modified under various conditions. This variability was supposed to provide the recognition by the protein of its specific site on 16S rRNA. To test this hypothesis, we substituted some amino acid residues in this helix and assessed effects of these substitutions on the affinity of the protein for 16S rRNA. The crystal structure of the complex of one of these mutants (Thr3Cys S15) with the 16S rRNA fragment was determined, and a computer model of the complex containing another mutant (Gln8Met S15) was designed. The available and new information was analyzed in detail, and the N-terminal helix was concluded to play no significant role in the specific binding of the S15 protein to its target on 16S rRNA.

Key words: RNA–protein interactions, ribosomal proteins, crystal structure, S15 protein, 16S ribosomal RNA

Many fundamental processes in biology depend on interactions of proteins with ribo- and deoxyribonucleic acids. Determination of spatial structures of ribosomal subunits with atomic resolution was a great step forward in studies on the interaction of ribosomal proteins with ribosomal RNAs. At present, complete models are known of the large subunit at 2.4 Å resolution [1] and of the small subunit at 3.05 Å resolution [2]. These models present valuable information on the tertiary structure of ribosomal proteins and ribosomal RNAs, their mutual positions, and intermolecular contacts. However, the recognition by a protein of its target on a ribosome and the specific binding to rRNA on the recognized site are to be studied additionally. The detection of amino acid residues and nucleotides responsible for the recognition may be significantly facilitated if the structures of free partners are known and/or some mutations in the region of RNA–protein interface are produced, and their effects on the complex formation are estimated.

In the present work the structure of RNA–protein complex of ribosomal protein S15 with some mutations in the region of the RNA–protein interface is described, and all known structures of S15 protein both free and complexed with 16S rRNA are analyzed. Based on the findings, amino acid residues of the N-terminal helix contacting with 16S rRNA are concluded to be insignificant for the specific binding.

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MATERIALS AND METHODS

The following reagents were used: a system for isolation of plasmid DNA and Taq DNA polymerase (Boehringer Mannheim, Germany); restriction enzymes *BamH*I, *Nde*I (New England Biolabs, USA); isopropyl-β-D-thiogalactoside (IPTG), ATP, GTP, UTP, CTP (Pharmacia Biotech, Sweden); a TaqTrack Sequencing System kit for DNA sequencing (Promega, USA); nutrient media (Difco, USA).

Directed mutagenesis. Substitutions in the nucleotide sequence of the previously cloned gene of protein S15 from *Thermus thermophilus* pES151-1 [3] were introduced by polymerase chain reaction (PCR) using oligonucleotides complementary to the protein gene sequence containing point nucleotide substitutions to obtain adequate mutations of the amino acid sequence. The following primer sequences were used:

THR3CYS, 5′-GAGATATACATATGCCCATCTGCAAGGAAGAGAAG-3′ (sense)

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K4C, 5′-GAGATATACATATGCCCATCACGTGC-GAAGAGAAGCAG-3′ (sense)

Q8M, 5′-GAGATATACATATGCCCATCACGAAGG-AAGAGAAGATGAAGGTCATCCA-3′ (sense)

V10M, 5′-GAGATATACATATGCCCATCACGAAGG-AAGAGAAGCAGAAGATGATCCAGGAGTTC-3′ (sense)

Antisense: 5′-GCCGGATCCCCAGTTTAACCCCGG-ATGCCCAG-3′

After PCR, the fragments were cloned into vector pET-11c as described in [3]. Mutations were checked by determination of nucleotide sequences of DNA. Mutant vectors were transformed to be expressed in *Escherichia coli* strain B834 (DE3).

Expression and purification of the mutant forms of ribosomal protein S15. The protein was isolated and purified as described in [3]. An overnight culture (30 ml) was added to 300 ml of medium LB containing ampicillin (100 μg/ml) and grown in a 1-liter flask at 37°C. When the absorption at 600 nm reached 0.6, expression was induced by addition of IPTG to the final concentration of 0.5 mM. Then the culture was incubated at 37°C for 4-5 h. The cells were separated by centrifugation at 4°C, suspended in buffer containing 100 mM Tris-HCl (pH 8.0), 150 mM $MgCl_2$, 0.1 mM EDTA, and 0.8 M NaCl, and broken by ultrasonication. The broken cells were removed by centrifugation (13,000*g*, 4°C, 10 min) and then centrifuged at 45,000*g* for 1 h at 4°C to remove ribosomes. The supernatant was diluted with 50 mM sodium acetate buffer (pH 5.5) to the final concentration of 0.3 M NaCl. Then the protein solution was loaded onto a column with CM-Sepharose equilibrated with buffer containing 0.3 M NaCl in 50 mM sodium acetate (pH 5.5). After loading the sample, the column was washed with the same buffer. The bound proteins were eluted with buffer containing 50 mM sodium acetate (pH 5.5) with a NaCl concentration gradient from 0.3 to 1 M. The fractions were analyzed by SDS-PAGE. Purified samples were concentrated by ultrafiltration on Centricon (Amicon, USA) to about 30-40 mg/ml and stored at 4°C in solution containing 0.8 M NaCl.

Preparation and purification of the 16S rRNA fragment. The 16S rRNA fragment of 57 nucleotides which specifically bound with protein S15 was prepared by *in vitro* transcription from linearized plasmid DNA containing the T7 promoter followed by excision from the transcript of the self-excision hammerhead-ribozyme on the 3′-end of the resulting RNA as described in [4, 5].

Crystallization of mutant protein complexes with the 16S rRNA fragment. Aliquots of RNA in buffer containing 2.6 mM $MgCl_2$, 50 mM KCl, and 10 mM sodium cacodylate (pH 6.2) were renatured for 15 min at 42°C, cooled on ice, and on continuous stirring were stepwise supplemented with protein aliquots in buffer containing 50 mM KCl and 10 mM sodium cacodylate (pH 6.2). Quantities of the rRNA fragment and S15 protein in the corresponding aliquots were equimolar. The mixture was incubated at 4°C for 1-2 h. The resulting complexes were used for crystallization.

The complexes were crystallized by a technique of "vapor diffusion in hanging drop" [6]. Crystallization was performed under the following conditions: the drops contained the rRNA fragment (6 mg/ml), the S15 protein (3 mg/ml), 2.6 mM $MgCl_2$, 50 mM KCl, and 10 mM sodium cacodylate (pH 6.2) were placed on siliconized glasses over a reservoir solution containing 2.4 M ammonium sulfate, 100 mM sodium cacodylate (pH 6.2). Crystals were grown at 22°C within 3-5 days to maximum dimensions of 0.1-0.2 mm.

Collection and processing of diffraction data. The data collection experiments were performed at the beamline ID14 of the European Synchrotron Radiation Facility (Grenoble, France). To increase the lifetime in the X-ray beam, the crystals were cooled to 110 K using a cryostat with liquid nitrogen (Oxford Cryosystems, UK). The diffraction data were processed using the DENZO and SCALEPACK programs [7].

Determination of spatial structure of the complex. The structure of the Thr3Cys S15 complex with the 16S rRNA fragment was determined by molecular replacement method using the CNS program [8]. The known structure of the ribosomal protein S15 complex with the 16S rRNA fragment (PDB bank code 1DK1 [4]) was used as a model to calculate the initial set of phases. The final model refined to the R factor of 23.1% (R_{free} = 32.2%) at resolution of 2.84 Å included 83 amino acid residues and 57 nucleotides. The final model showed good quality and had no residues in the disallowed regions of the Ramachandran plot. Coordinates of the model of the Thr3Cys S15–16S rRNA complex have been deposited in the Protein Data Bank (code 1KUQ).

RESULTS AND DISCUSSION

The structure of protein S15 is known in both the free [9, 10] and bound with 16S rRNA [2, 4, 11] states. It is a completely helical protein consisting of four α-helices. It binds to the central domain of 16S rRNA interacting with two regions on its surface. The first and second sites are located, respectively, in the upper part of helix H22 and in the region of junction of helices H20, H21, and H22 (Fig. 1). The loop between the helices α2 and α3 interacts with the first site, whereas amino acid residues of the helices α1, α2, and α3 and interconnecting loops interact with the second region. The role of each helix in the specific binding is still unclear. From this viewpoint, the N-terminal helix α1 is of special interest

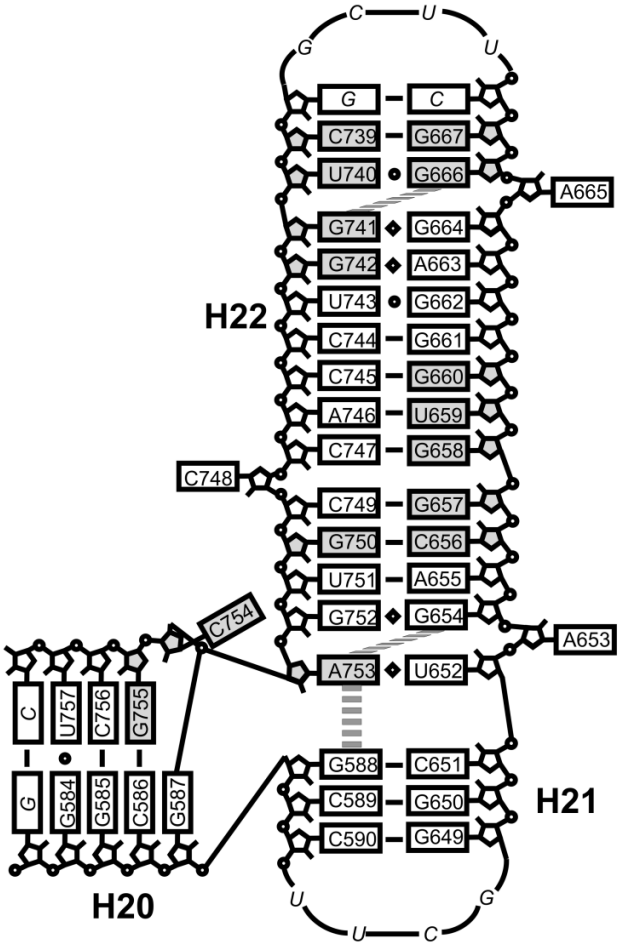

Fig. 1. Scheme of secondary structure of the 57-nucleotide 16S rRNA fragment from *T. thermophilus*. Nucleotides interacting with protein S15 are shown in gray color (numbering corresponds to 16S rRNA from *E. coli*).

because it is rather flexible in structures of the free protein, and its location varied in models of the protein from *Thermus thermophilus* and *Bacillus subtilis* obtained by NMR and X-ray methods, respectively. Therefore, it was suggested that the conformational variability of the N-terminal helix should be sufficient for protein–RNA binding [2].

The crystal structure of mutant protein S15 complexed with the 57-nucleotide fragment 16S rRNA from *T. thermophilus* was determined earlier in our group [4]. The structure was solved by multiwave anomalous diffraction method (MAD) using the derivative containing four selenomethionines (4SeMet S15). To obtain the protein with four methionines instead of two existing in a wild type protein, two mutations were made, Ile11Met and Ala79Met, and all methionines were replaced by selenomethionines.

Considering the structure of the selenomethionine complex, the mutation in position 79 should have no influence either on the protein structure (because this residue is located on the surface) or on the complex structure (because it is not in the RNA–protein interface). The second mutation, Ile11Met, was located in the N-terminal helix. The partially hidden residue in position 11 was involved in formation of hydrophobic core of the protein. However, this residue belongs to the helix, which is involved in formation of the RNA–protein interface, and mutation in this position could affect the RNA–protein affinity. The structure of this complex was compared to the structure of the corresponding region of the small ribosomal subunit, and no significant changes were found, i.e., the mutation in position 11 in the N-terminal helix should not affect the binding.

To better elucidate the role of the N-terminal helix in recognition and specific binding with 16S rRNA, a number of proteins were prepared with mutations in this helix (Thr3Cys, Lys4Cys, Gln8Met, and Val10Met). These proteins bound to the 57-nucleotide fragment of 16S rRNA with virtually the same binding constant as that of the selenomethionine-containing protein, and the resulting complexes crystallized under the same conditions as the initial complex. This could occur either in the absence of significant influence of N-terminal helix on the specific binding or when the mutations did not act on the RNA–protein interface or in the case of mutual compensation of the induced changes. To make clear this situation, it was necessary to determine the structure of at least one more complex of mutant ribosomal protein S15 with 16S rRNA.

Crystals suitable for X-ray diffraction experiment were obtained for the complex containing the Thr3Cys

Data obtained for crystals of the S15–16S rRNA complex

	Thr3Cys S15–16S rRNA	4SeMet S15–16S rRNA
Space group	$P6_422$	$P6_422$
Unit-cell parameters, Å	$a = 128.23$; $b = 128.23$; $c = 64.95$	$a = 128.80$; $b = 128.80$; $c = 65.10$
Resolution, Å	25-2.85	20-2.80
Completeness, %	98.8	98.1
R_{sym}, %	3.1	3.8
Number of unique reflections	7724	7679
Relative difference of data sets, %	19.5	

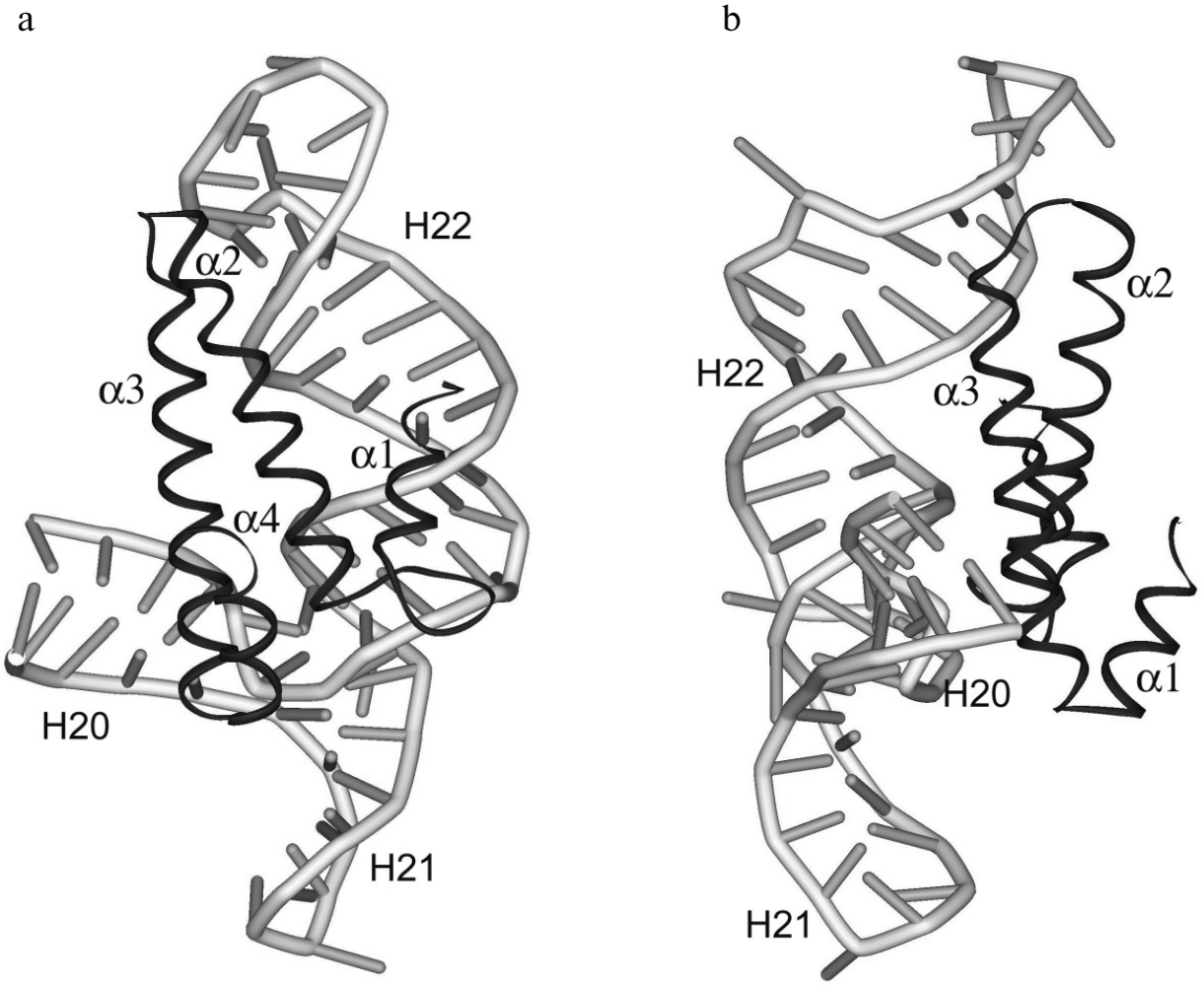

Fig. 2. Structure of the protein Thr3Cys S15 from *Thermus thermophilus* complexed with the 16S rRNA fragment. The protein helices α1, α2, α3, and α4 and helices of the fragment 16S rRNA H20, H21, and H22 are shown. a) View from the side of protein S15; b) the complex is turned relatively (a) by approximately 90° around the vertical axis.

S15 protein. The diffraction data measured from these crystals were compared with the data set used to determine the structure of the complex with the selenomethionine-containing protein. The difference in structure amplitudes was 19.5% at virtually the same parameters of the unit cell (table). This suggested that the structures of the two complexes should be slightly different.

The structure of the mutant protein Thr3Cys S15–rRNA complex was solved (Fig. 2) and compared with previously determined homologous structures.

By detailed comparative analysis, the main chains were detected to noticeably differ only in the N- and C-terminal regions of the molecules. Nevertheless, in all four α-helices conformational changes occurred in the side chains of some amino acid residues, which resulted in redistribution of electrostatic interactions and hydrogen bonds (Fig. 3). Thus, the Thr3Cys mutation abolished the electrostatic interaction between the side chain of the amino acid residue Lys4 and phosphate of nucleotide G660. Instead, the side chain of Gln8 formed hydrogen bonds with the sugar-phosphate backbone of nucleotides U658 and G659 (Fig. 3b), and this slightly changed the orientation of the N-terminal helix. Thus, interactions in the RNA–protein interface were redistributed, but these changes failed to influence the binding with 16S rRNA.

Using the available structures of the S15 protein from *T. thermophilus* complexed with 16S rRNA, we additionally modeled the structure of the mutant protein Gln8Met S15–16S rRNA complex. The resulting structure was analyzed, and the network of hydrogen bonds between Gln8 and 16S rRNA was found to be destroyed. However, these changes also failed to significantly affect the binding to 16S rRNA.

Thus, mutations in helix α1 of the ribosomal protein S15, which modify the protein–RNA interactions but are

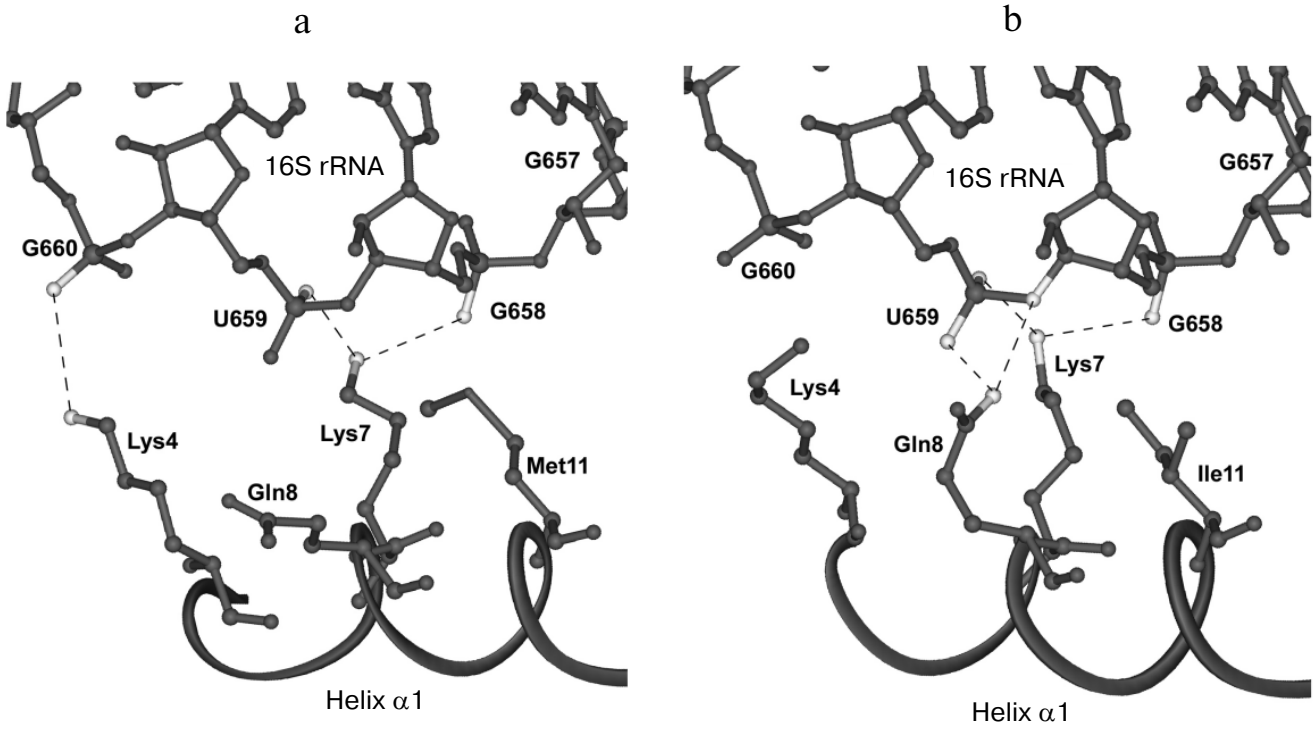

Fig. 3. The contact region of the N-terminal helix of protein S15 with 16S rRNA: a) in the 4SeMet S15–16S rRNA complex; b) in the Thr3Cys S15–16S rRNA complex. The numeration of nucleotides corresponds to 16S rRNA from *E. coli*. Dotted lines show hydrogen bonds.

unaffected by the protein–RNA binding suggested that the role of the N-terminal helix in the RNA–protein recognition should be insignificant and residues of this helix should not be involved in the recognition by S15 protein of its site on 16S rRNA. This conclusion is consistent with data on mutagenesis of 16S rRNA from *E. coli* [12].

This work was supported by the Russian Foundation for Basic Research (project No. 04-04-49634), Council for Grants of the President of the Russian Federation (NSh No. 1969.2003.4), and the program of fundamental studies of the Presidium of the Russian Academy of Sciences "Molecular and Cell Biology".

REFERENCES

1. Ban, N., Nissen, P., Hansen, J., Moore, P. B., and Steitz, T. A. (2000) *Science*, **289**, 905-920.
2. Wimberly, B. T., Brodersen, D. E., Clemons, W. M., Jr., Morgan-Warren, R., Carter, A. P., Vonrhein, C., Hartsch, T., and Ramakrishnan, V. (2000) *Nature*, **407**, 327-339.
3. Serganov, A., Rak, A., Garber, M., Reinbolt, J., Ehresmann, B., Ehresmann, C., Grunberg-Manago, M., and Portier, C. (1997) *Eur. J. Biochem.*, **246**, 291-300.
4. Nikulin, A., Serganov, A., Ennifar, E., Tishchenko, S., Nevskaya, N., Shepard, W., Portier, C., Garber, M., Ehresmann, B., Ehresmann, C., Nikonov, N., and Dumas, P. (2000) *Nature Struct. Biol.*, **7**, 273-277.
5. Garber, M., Gongadze, G., Meshcheryakov, V., Nikonov, O., Nikulin, A., Perederina, A., Piendl, W., Serganov, A., and Tishchenko, S. (2002) *Acta Crystallogr. D Biol. Crystallogr.*, **58**, 1664-1669.
6. Ducruix, A., and Giege, R. (1997) *Crystallization of Nucleic Acids and Proteins. A Practical Approach*, Oxford University Press.
7. Otwinowski, Z., and Minor, W. (1997) *Meth. Enzymol.*, Vol. 276, *Macromolecular Crystallography* (Carter, C. W., Jr., and Sweet, R. M., eds.) Pt. A, Academic Press, pp. 307-326.
8. Brunger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, N., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T., and Warren, G. L. (1998) *Acta Crystallogr.*, **D54**, 905-921.
9. Berglund, H., Rak, A., Serganov, A., Garber, M., and Hard, T. (1997) *Nature Struct. Biol.*, **4**, 20-23.
10. Clemons, W. M., Jr., Davies, C., White, S. W., and Ramakrishnan, V. (1998) *Structure*, **6**, 429-438.
11. Agalarov, S. V., Prasad, G. S., Funke, P. V., Stout, C. D., and Williamson, J. R. (2000) *Science*, **288**, 107-112.
12. Serganov, A., Ennifar, E., Portier, C., Ehresmann, B., and Ehresmann, C. (2002) *J. Mol. Biol.*, **320**, 963-978.