Crystallization and preliminary diffraction studies of the structural domain E of *Thermus flavus* 5S rRNA

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Abstract The ribosomal 5S RNA is an essential constituent of the large ribosomal subunit. To overcome the difficulties of crystallizing large RNA molecules such as 5S rRNAs, we decided to divide the 5S rRNA in five domains A through E to determine their structure. Recently we determined the crystal structural of the helical domain A. Here we report the crystallization of the chemically synthesized domain E of the *Thermus flavus* 5S rRNA. The crystal form is trigonal with unit cell dimensions: $a = b = 42.80$ Å and $c = 162.20$ Å. Diffraction-data to 2.8 Å have been recorded and the structure solution is currently underway by means of MIR and MAD techniques.

Key words: 5S rRNA; RNA hairpin; Crystal

1. Introduction

The ribosomal 5S RNA is a constituent of the large ribosomal subunit of ribosomes occurring in prokaryotes and eukaryotes. Ribosome reconstitution experiments demonstrated that 5S rRNA is essential for ribosomal function [1–6]. Nevertheless, its precise role in protein synthesis remains unclear. Better understanding of the 5S rRNA function will certainly arise from structural investigations. Chemical probing, enzymatic modification studies and sequence alignments permitted to build general models for the 5S rRNA secondary structure [7]. These studies give also hints about possible tertiary structure interactions.

The different models, however, are so far supported only by limited physical data. The size of 5S rRNA ($M_w \sim 40,000$) makes structural investigations by NMR difficult and attempts to obtain 5S rRNA crystals diffracting to high resolution have not been successful up to now [8,9]. To overcome these difficulties, we decided to consider the 5S rRNA molecule as an assembly of smaller structural elements or domains. This approach is basically making the assumption that the smaller fragments will retain the conformation they have in the intact molecule. Based upon the secondary structure the 5S rRNA can be divided in five domains A through E (nomenclature varies between authors) constituted of helical regions interrupted by loops and single-stranded sequences (Fig. 1) [9,10]. White et al. [11] determined the solution structure of the domain A from *E. coli*. More recently we reported the crystal structure of the domain A from *Th. flavus* [10]. The structures of the other domains have not been investigated yet.

Among these fragments the loop domain E is of major interest. (i) Crosslinks between 5S rRNA and 23S rRNA demonstrated that the location of the hairpin loop in domain E is near the peptidyltransferase centre of the ribosome [12]. (ii) The hairpin of domain E belongs to the highly conserved class of very stable 5′-GNRA tetraloops occurring in ribosomal RNA (N is any nucleotide, R is G or A) [13]. The exact function of these loops is not known. The solution structures of the GCAA and GAAA tetraloops have been determined by NMR [14] and recently H.W. Pley et al. [15] determined the crystal structure of the GAAA tetraloop occurring in the hammerhead ribozyme. Nevertheless, physical data available on GNRA tetraloops remain sparse.

Here we report the crystallization and first diffraction data of the hairpin loop domain E from nucleotides 79–97 of the *Th. flavus* 5S rRNA with the sequence 5′-CUGGGCGG(GCGA)-CCGCCUGG (nucleotides in the loop are in brackets).

2. Materials and methods

2.1. Synthesis and purification of oligoribonucleotides

The solid phase synthesis of oligoribonucleotides was performed on a PCR-MATE EP model 391 DNA Synthesizer (Applied Biosystems) with 2′-*O*-triisopropylsilyl-protected phosphoramidite synthons. Typical phosphoramidite concentration was 0.15 M. To achieve high coupling efficiencies, the coupling step was extended to 900 s. After synthesis, the dimethoxytrityl protecting group at the 5′-hydroxyl moiety was cleaved with 3% trichloracetic acid in methylene chloride using the synthesizer's automatic procedure. Incubation of the controlled pore glass beads in 1 ml of 32% ammonia/ethanol (3:1, v/v) at 55°C for 24 h removed the protecting groups on the phosphates and on the exocyclic amino groups of the bases. Subsequent incubation at room temperature for 72 h in 1.1 M tetrabutylammonium fluoride (TBAF) in tetrahydrofurane:ethanol (10:1, v/v) removed the 2′-hydroxyl protecting group (TBAF was purchased from Aldrich). The deprotected oligoribonucleotides were desalted by ion exchange chromatography on a Quiagen Tip 500 column (Diagen) and the RNA was eluted with 2 M triethylammonium acetate (TEAAc), pH 7.0. Samples were evaporated to dryness and resuspended in distilled water. The full-length product was purified by reversed phase HPLC on an ODS C18 Beckman Ultrasphere column (4.6 × 250 mm) heated at 45°C. The system was operated using a linear gradient of 1–15% buffer B over a period of 60 min at a flow rate of 1 ml/min (buffer A: 0.1 M TEAAC, pH 7.0; buffer B: 80% acetonitrile in 0.1 M TEAAc, pH 7.0).

2.2. Preincubation

Pure RNA (150–200 μg) was resuspended in 1 ml of 0.1 M TEAAc, pH 7.0, heated to 95°C for 2 min in a water bath and slowly cooled to 4°C. The buffer was removed by evaporation in a speed vac concentrator. At this step care was taken not to evaporate the samples to dryness.

2.3. Crystallization

Preincubated oligoribonucleotides corresponding to the domain E
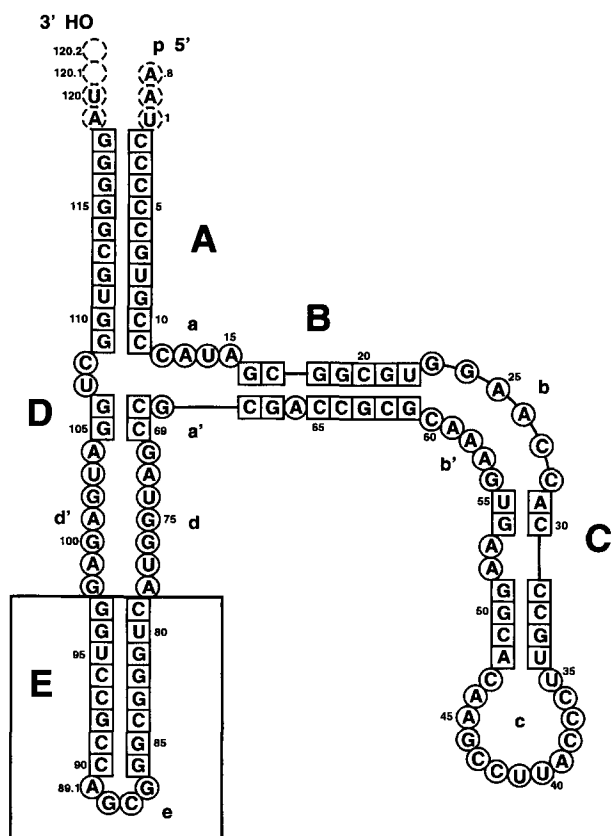
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Fig. 1. Secondary structure of *Thermus flavus* 5S rRNA. The individual domains are marked A through E. The chemically synthesized domain E is boxed.

were crystallized by the vapour diffusion method. In a typical crystallization trial 0.5 μl of RNA solution (0.5 mM) was mixed with 0.5 μl crystallization buffer, layered on a 22 × 22 mm plastic coverslip, and equilibrated against 1 ml reservoir solution dispensed in the well of a 24-well tissue culture tray (Linbro). The crystallization set ups were incubated at temperatures ranging from 4 to 50°C.

3. Results and discussion

The full-length 20-mer product was purified by reversed phase HPLC (Fig. 2). This purification method permitted to separate truncated products, resulting from incomplete coupling during synthesis, and incompletely deprotected oligoribonucleotides from the full-length deprotected 20-mer product. The purity and homogeneity of the purified oligoribonucleotides were checked by analytical HPLC and 20% denaturing (7 M urea) polyacrylamide gel electrophoresis (PAGE) (data not shown). The absence of 5'-2' linkages, nucleoside modifications, and the complete deprotection of the RNA were assessed by nucleoside analysis. For this purpose the purified oligoribonucleotide (1 nmol) was digested with 2 units RNase T2 (BRL Life Technologies) and 2 units alkaline phosphatase (Boehringer) by incubation at 37°C for 60 min. After incubation, the digestion products were analyzed by reversed phase HPLC as described above except that the gradient was run from 1% to 20% buffer B in 60 min. Under these conditions the undigested oligomer eluted at 38.4 min. The digestion of the oligoribonucleotide resulted in the four nucleosides cytidine (C), uridine

Table 1
Data collection and processing

Space group	P3$_1$21
Cell constants	a = b = 42.80 Å, c = 162.20 Å
Crystal volume/Dalton; (V$_M$)	3.2 Å^3/Dalton
X-ray source	Storage ring DORIS, EMBL, beam line X11
Wavelength used	0.92 Å
Detector	Image plate scanner
Crystal to detector distance	250 mm
Maximum resolution	2.8 Å
Rotation for each exposure	2.0°
Time for each exposure	300 sec.
Total rotation for a data set	30°
Number of observed reflections	10,753
Number of unique reflections in the range 20.0–2.8 Å	4,702
Overall merging R(I) factor	7.2%
last resolution shell	34.0%
Completeness	93.0%
last resolution shell	96.0%

(U), guanosine (G), and adenosine (A) with retention times 4.1 min, 5.2 min, 8.5 min, and 13.9 min, respectively. The identity of the peaks was assessed by comparison with the retention times of standards. As can be inferred from the chromatogram of the digested oligoribonucleotide (Fig. 3), the purified product was free of modified or incompletely deprotected nucleosides. The straight base line after the peaks corresponding to the four nucleosides reveals the absence of oligoribonucleotides containing 2'-5' linkages. Indeed, 2'-5' phosphodiester bonds are not cut by the RNase T2 and undigested 2'-5' linked oligomers would elute after adenosine (data not shown). Oligoribonucleotides containing 2'-5' linkages can result from the solid phase synthesis and are a major problem when totally pure material is needed since they cannot be separated from the normally occurring 3'-5' linked products by either HPLC or PAGE. Because of the similarity with 3'-5' oligoribonucleotides, RNAs containing 2'-5' phosphodiester bonds are especially deleterious for crystallization. The total absence of 2'-5' linkages in our oligoribonucleotides is likely due to the extra-
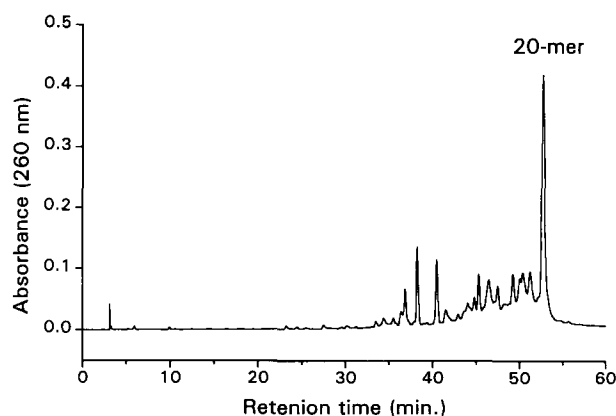

Fig. 2. Reversed phase HPLC purification of the chemically synthesized domain E of *Th. flavus* 5S rRNA. Solid phase: ODS C18, Beckman Ultrasphere (4.6 × 250 mm). Mobile phase: 1% to 15% buffer B in 60 min (buffer A: 0.1 M TEAAc, pH 7.0; buffer B: 80% acetonitrile in 0.1 M TEAAc, pH 7.0). Flow rate, 1 ml/min. Column temperature, 45°C. Under these conditions the full-length deprotected 20-mer eluted at 52.7 min. Peaks with shorter retention times are truncated oligoribonucleotides present in the raw product after synthesis and deprotection.

stability of the 2′-hydroxyl-protecting groups used in our synthons. While 2′-O-triisopropylsilyl-protecting groups are more bulky than other protecting groups used in RNA chemistry and need longer deprotection times, they are more stable during the alkaline deprotection step.

For the first crystallization experiments we used 12 standard conditions from a screening protocol established in our laboratory (manuscript in preparation). In a second step fine screening of sensitive parameters such as pH, temperature and crystallizing agent concentration permitted to obtain crystals of reasonable size. Finally the effects of various additives on crystal growth were tested. The best crystals were grown when a drop of 0.25 mM RNA, 200 mM $MgCl_2$, 20 mM sodium cacodylate, pH 7.0 and 7.5% PEG 400 were equilibrated against 1 ml of reservoir solution consisting of 400 mM $MgCl_2$, 40 mM sodium cacodylate, pH 7.0, and 10% PEG 400. The crystallization was performed at 32°C. Under these conditions crystals grow usually in 5 days. The RNA crystals with hexagonal morphology had a maximum size of $140 \times 70 \times 70$ µm (Fig. 4). Further addition of divalent cations (Co^{2+}, Ni^{2+}, Mn^{2+} and Ca^{2+}), sodium chloride or polyanions (spermine, spermidine) did not improve the crystal size.

X-Ray diffractions were recorded from a crystal mounted in a thin-walled glass capillary with some mother liquor. A data set was collected up to 2.8 Å resolution with synchroton radiation using a MAR 300 mm image plate detector at the EMBL beam line X11 (Table 1 for summary). The storage ring was operated at 4.7 GeV and 60–80 mA. The wavelength was 0.92 Å. The images of this data set were processed using the program DENZO [16]. The space group was found to be $P3_121$ with unit cell parameters of $a = b = 42.80$ Å and $c = 162.20$ Å. The packing parameter V_m is 3.2 Å^3/Da [17] for two fragments per asymmetric unit. The reduced data set contains 4,702 reflections and shows a completeness of 93%. The R-merge, defined as $R(I) = \Sigma I - <I>/\Sigma I$, is 7.2%. This data set was in use for the MR (molecular replacement) calculations using the coordinates of the synthetic RNA helix $[U(UA)_6A]_2$ [18] as starting model and coordinates of the domain A of Th. flavus 5S rRNA [10]. As it was so far not possible to solve the structure by applying straightforward MR calculations, we assume a major

Fig. 4. Crystal of the domain E from Th. flavus 5S rRNA. Crystal size is $140 \times 70 \times 70$ µm.

change from the helical conformation of the model structures. Therefore a combination of MIR (Multiple Isomorphous Replacement) and MAD (Multiwavelength Anomalous Dispersion) techniques will be used to solve the phase problem.

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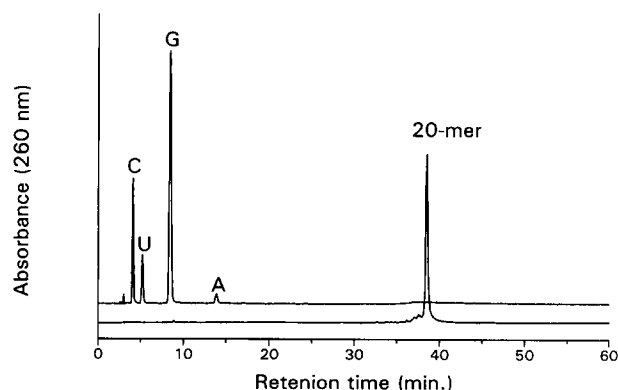
Fig. 3. Nucleoside analysis of the purified oligoribonucleotide. The figure represents superimposed chromatograms of the purified 20-mer (lower curve) and of its degradation products after enzymatic digestion (upper curve). The chromatograms were obtained by reversed phase HPLC as described in the text. Peaks C, U, G and A are the four nucleosides cytidine, uridine, guanosine, and adenosine. The full-length product eluted at 38.5 min.