Detection of multiple conformations of the E-domain of 5S rRNA from *Escherichia coli* in solution and in crystals by NMR spectroscopy

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**ABSTRACT**

NMR spectroscopy of the E-domain fragment of *Escherichia coli* 5S rRNA indicates that this molecule exists in solution as either a stem–loop or as a duplex with two U–U base pairs in the bulge region. At temperatures below 27°C, interconversion between the monomeric and dimeric forms in solution occurs on a time scale of weeks and allows the preparation of samples on which NMR structure determinations can be carried out on predominantly monomeric or dimeric species. The NMR results obtained provide comparison data for the distinction between A- and B-form *E.coli* 5S rRNA and for the possible kinetics of conversion between these forms. NMR evidence is presented that the duplex form also exists in crystals and suggestions are made for means to obtain stem–loop conformations of E-domain and other small RNA stem–loop sequences in crystals.

**INTRODUCTION**

The determination of structures for larger RNA molecules such as 5S rRNA has so far been limited by difficulties in obtaining crystals of adequate size and diffractive resolution (1,2) or by the lack of appropriate NMR methods for dealing with RNA molecules of this size. This has led to attempts to obtain high resolution structures for smaller fragments of the large RNA molecule and in our case has led to X-ray studies of the A- and E-domains of 5S rRNA (3,4). An important precondition is that the fragments assume the same conformation in the crystal as in the complete RNA molecule, e.g. a duplex for the A-domain (3). In the case of the E-domain of *Escherichia coli* 5S rRNA, alternative structures involving either a stem–loop or the formation of a duplex with the C-domain have been proposed (5) and it is therefore of interest to identify whether the E-domain has multiple structural possibilities and to define conditions under which specific structures can be obtained for detailed structural determinations. We are also interested in obtaining structural data for the E-domain by both NMR and crystallography in order to compare the structures and hydration seen by the two methods. As with structural studies of proteins, use of structural data obtained by both methods can also be important to provide calibration (Karplus curves) for interpretation of spin–spin coupling information derived from NMR spectra.

Stem–loops represent a fragment type of particular interest because they frequently exist in more complex RNA secondary structures. However, small RNA hairpins may exist in equilibrium between a monomeric stem–loop and its anti-symmetric dimer and/or even higher agglomerates. If stem–loops embedded in more complex structures [as in tRNA (6–8) or the hammerhead ribozyme (9–11) or in complexes with proteins (see for example 12,13)] are disregarded, then so far small RNA fragments capable of forming a stem–loop structure have largely been crystallized as the anti-symmetric dimers instead (14–19). Similarly, it is often observed by NMR methods that small stem–loops can exist in solution in equilibrium with duplex forms. It is therefore very desirable to establish procedures for obtaining defined stem–loops and to have a method for rapid testing of whether such a form has been obtained prior to an extensive X-ray or NMR study. In this paper we assess the question of distinguishing a stem–loop from an anti-symmetric duplex in both the liquid and crystalline states of an RNA fragment by NMR spectroscopy. This study provides data for assessing the structure adopted by the E-domain in the full 5S rRNA molecule, as well as kinetic data relevant to whether conversion between the two proposed forms of 5S rRNA is likely to be of functional importance. From the results of the present study, conclusions are also drawn regarding procedures which may help in obtaining stem–loop forms in crystals.

**MATERIALS AND METHODS**

¹H NMR spectra of the exchangeable protons were acquired on a Varian 600 MHz Unityplus and on a Bruker AMX600 spectrometer using either the jump–return (20) or the 1331 (21,22) water suppression sequences with the carrier frequency at the water resonance and the excitation maximum at 12.5 p.p.m. Preparatory experiments (not using crystallized material) were performed to monitor the dependence of the ¹H spectrum of the *E.coli* 5S rRNA E-domain on RNA concentration, NaCl concentration, temperature, pH and on the cooling rate used for the folding procedure. This allowed observation of the spectra with structural studies of proteins, use of structural data obtained by both methods can also be important to provide calibration (Karplus curves) for interpretation of spin–spin coupling information derived from NMR spectra.

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Figure 1. Dependence of the imino proton spectrum of the E-domain of *E. coli* 5S rRNA on RNA concentration and on the cooling rate used during the folding procedure. M and D indicate the signals of the monomer and the dimer subspectra. Non-overlapping resonances are shown in italic. (a) 0.85 mM E-domain, slowly cooled. (b) 0.85 mM E-domain, quickly cooled. (c) 0.17 mM E-domain, slowly cooled. (d) 0.17 mM E-domain, quickly cooled. The sequence of the E-domain fragment and the form of the monomer–dimer equilibrium are indicated in the scheme at the top of the figure. The spectra were acquired at 27°C using the NMR buffer described in Materials and Methods including 150 mM NaCl.

from the stem–loop and duplex species possible for this fragment. In the following discussion, ‘slowly cooled’ and ‘quickly cooled’ during the folding represent a temperature change from 75 to 25°C by slow cooling of a water bath over 4 h and a fast quench from 75 to 0°C by placing the sample in an ice bath, respectively. A 2D NOE spectrum of nearly exclusively the monomer form was acquired with a quickly cooled 0.7 mM sample in 90% H2O, 10% D2O, pH 6.5, 10 mM phosphate buffer and 50 μM EDTA on a Bruker AMX600 spectrometer at 2°C. Water suppression was achieved by replacing every 1 H pulse of the standard NOESY pulse sequence by a jump–return unit. Phase-sensitive detection in t1 was done via the TPPI method. A corresponding experiment for a quickly cooled 0.85 mM monomer/dimer mixture was performed at 27°C in the same NMR buffer, except for the addition of NaCl to a concentration of 150 mM.

RNA synthesis, purification and crystallization were performed as described (4). The E-domain of *E. coli* 5S rRNA (5′-rGUGUGGGGUCUCCCAUGC-3′, molecular wt 6.04568 kDa), corresponding to nt 79–97, was synthesized chemically on an Applied Biosystems PCR-MATE EP model 391 DNA Synthesizer using 2′-O-trisopropylsilyl-protected phosphoramidite synths and standard DNA synthesis reagents. After alkaline deprotection with ammonia/ethanol (3:1) for 24 h at 55°C and deprotection with tetrabutylammonium fluoride (1.0 M in tetrahydrofuran) for 72 h at room temperature, the oligonucleotide was desalted on a Qiagen tip-500 column. The final purification was achieved by reversed phase chromatography on an ODS C18 Beckman Ultrasphere® column (4.6 x 250 mm). Purified RNA (480 μg, 1 A260 = 42 μg) was evaporated to dryness in a Speedvac concentrator, redissolved in 1 ml 20 mM triethylammonium acetate, pH 7.0, heated to 90°C for 5 min and slowly cooled to 4°C. Crystals of the E-domain were obtained by the vapor diffusion method at 32°C. The following steps for the NMR measurements were performed at 4°C. Samples of 50 drops (each 1 μl, containing 2.1 μg RNA) from hanging drop crystallization experiments were combined, using only drops without unwanted amorphous precipitate. After centrifugation at 320 g and removal of the solution, the crystals were washed with reservoir solution (8% PEG 4000, 40 mM calcium acetate, 40 mM sodium cacodylate and 1 mM spermine), which was also removed after a second centrifugation.

For the NMR experiments the crystals were dissolved in 60 μl NMR buffer (10 mM sodium phosphate buffer, pH 6.5, 150 mM NaCl, 50 μM EDTA) and the solution was transferred into a coaxial microtube insert (Wilmaid WGS-5BL) which fits into the usual 5 mm NMR tube filled with 99.9% D2O. The E-domain concentration in this solution was determined to be 0.12 mM by UV absorbance spectroscopy.

RESULTS AND DISCUSSION

In NMR studies of short oligoribonucleotides it is commonly observed that both monomeric stem–loops as well as dimeric duplexes can exist in solution. Under appropriate experimental conditions (buffer and temperature) it is often possible to resolve resonances, especially from imino hydrogens of guanine and uridine, which are characteristic of these two species. The goals of the present paper were to use these NMR characteristics to test whether monomorphic or dimeric species exist in both the liquid and crystalline states of the E-domain fragment of *E. coli* 5S rRNA.
and to obtain NMR data which can be used to test whether a stem–loop conformation exists in the full 5S rRNA molecule. For the latter test, chemical shifts and NOEs for the imino hydrogens of G and U provide an appropriate comparison. For the crystals this involves dissolution in an appropriate NMR buffer, immediately acquiring an NMR spectrum and comparing this spectrum with predefined monomer and dimer spectra in solution. A further requirement of this procedure is that the kinetics of conversion between the monomer and dimer species are slow compared with the time required to dissolve the crystals and record the NMR spectrum.

The distribution between monomeric and dimeric forms of the E-domain in solution is strongly influenced by both total concentration and the thermal procedure used to produce the NMR sample. This can be used to identify NMR resonances arising from each species. For example, a slowly cooled 0.85 mM sample of the E-domain (Fig. 1a) shows a substantial intensity for two sets of resonances which can be shown to arise from monomer and dimer species. The proportion of resonances arising from the monomeric stem–loop can be increased by rapid cooling of the sample (Fig. 1b) or by slow cooling of a more dilute sample (Fig. 1c). For rapid cooling of a 0.17 mM sample, the NMR spectrum shows almost exclusively resonances from the monomeric stem–loop (Fig. 1d).

This interpretation of the observed spectra has been confirmed by means of NOE results. A NOESY spectrum (Fig. 2b) recorded at 2°C for a sample almost exclusively in the putative monomeric form allowed all 10 expected imino resonances (Fig. 2a) of the hairpin stem to be observed and assigned. As is usual in NMR spectra of stem–loops, the imino hydrogens of the loop uridines (Fig. 1) were not observed, presumably as a consequence of rapid exchange with solvent water. A schematic summary of the NOE connectivities involving the exchangeable protons of this monomer form is given in Figure 2c and indicates the formation of a typical double helix in the stem. A comparison of the chemical shifts of the putative monomer and dimer imino resonances (Fig. 1a) shows that corresponding imino signals have very similar chemical shifts between the termini of the molecules and the G84–C92 base pair, whereas pronounced differences, together with the appearance of additional resonances for the duplex form, occur for the remaining imino signals in nt 85–91. The NOESY spectrum of the sample in Figure 1b recorded at 27°C (data not shown) shows additional cross-peaks which link the two dimer resonances at 10.9 and 11.3 p.p.m. and also connect each of them to the dimer line at 13.4 p.p.m. The signals at 10.9 and 11.3 p.p.m. can be attributed to formation of a U87–U89 base pair in the dimeric form (Fig. 1), with both of the U87, U89 imino hydrogens showing a sequential cross-peak to the G86 imino hydrogen at 13.4 p.p.m. A U87–U89 base pair would have a very short imino–imino hydrogen distance of <3 Å and, concomitant with this, the U87–U89 NOE cross-peaks were amongst the strongest in the NOE spectrum (as indicated in Figure 2, this cross-peak can even be observed for the residual dimer present in the largely monomeric sample used to record Figure 2). It is of interest that
the palindromic duplex appears to be able to accommodate two U–U base pairs in the bulge region (insert to Fig. 1) with only very minor alterations to the flanking stems. This latter NOE spectrum was acquired at 27°C and quickly cooling to 0°C. The NMR buffer described in Materials and Methods including 150 mM NaCl was used for all spectra. We attribute the very small differences in the observed chemical shifts between spectra (a) and (b), as well as between spectra (c) and (d), to the presence of residual crystallization buffer in samples (b) and (d). 26 624 scans with a relaxation delay of 1.3 s were accumulated for the spectra of (b) and (d). Figure 3. Imino proton NMR spectra at 27°C of the dissolved crystals and of test samples for comparison. (a) 2.5 mM test sample slowly cooled from 75 to 25°C and nearly exclusively showing dimer resonances. D indicates dimer lines at chemical shifts where no monomer signals appear. (b) Solution of the E-domain crystals in NMR buffer, immediately after dissolution of the crystals. (c) 0.05 mM test sample quickly quenched from 75 to 0°C and exclusively showing monomer signals. M indicates monomer lines at chemical shifts where no dimer resonances appear. (d) Solution of the E-domain crystals of (b) after heating to 75°C and quickly cooling to 0°C. The NMR buffer described in Materials and Methods including 150 mM NaCl was used for all spectra. We attribute the very small differences in the observed chemical shifts between spectra (a) and (b), as well as between spectra (c) and (d), to the presence of residual crystallization buffer in samples (b) and (d). Non-equilibrium transitions between so-called A-form (E-domain in a stem–loop) and B-form (E-domain forming a duplex with the C-domain) under the influence of proteins which bind to the RNA molecule (23). This would involve a third conformational form for the E-domain sequence. The present evidence for the very slow interconversion of monomers and dimers for this RNA sequence suggests that if such a process occurs, catalysis of the interconversion between A- and B-forms, presumably as a consequence of bound proteins, would be required. By comparison of the data contained in the NOE spectrum of the monomeric form of the isolated E-domain (Fig. 2) with a 3D 15N-edited NOE spectrum of [15N]G,U-labelled E.coli 5S rRNA, we have recently been able to obtain strong evidence that the stem–loop observed for the monomeric E-domain is also present in the A-form of the complete E.coli 5S rRNA (24).

To test for the form of the E-domain present in crystals, an imino proton spectrum of the E-domain crystals recorded immediately after rapidly dissolving them in NMR buffer is presented in Figure 3b. Comparison with the signals of nearly exclusively the dimeric (slowly cooled 2.5 mM sample; Fig. 3a) and the monomeric (quickly cooled 0.05 mM sample; Fig. 3c) forms clearly demonstrates the existence of dimers in the crystalline state. The spectrum of Figure 3b is the sum of five individual experiments which did not show any time-dependent changes during the time covered by acquisition of the NMR data (9.6 h). After storing the sample of the dissolved crystals at 27°C for 6 days, a spectrum identical to Figure 3b was again obtained. This indicates that residual amounts of crystallization buffer contained in this sample did not alter the previously observed slow dimer–monomer interconversion. It may be noted that because of the downfield chemical shift of the imino hydrogen resonances in the NMR spectrum, the presence of residual crystallization buffer did not interfere with recording of the imino hydrogen spectra and that it is in principle possible to also determine the state of the oligonucleotide when dissolved in the crystallization buffer. Figure 3d shows the spectrum of the dissolved crystal sample after heating to 75°C and rapid cooling to 0°C. As expected for the RNA concentration of 0.12 mM, this procedure results in the appearance of nearly pure monomer resonances.

CONCLUSIONS

We have shown that the E-domain of E.coli 5S rRNA can exist as both a stem–loop and as a duplex with two U–U base pairs in the bulge region. Furthermore, by manipulation of folding conditions, it was possible to obtain predominantly monomeric or dimeric forms in solution and to show that interconversion between these forms was very slow. The spectra recorded for the monomeric stem–loop provide NMR data which have been used to obtain evidence for a stem–loop in the full 5S rRNA molecule (24) and the slow kinetic interconversion indicates that if transitions between A-form and B-form 5S rRNA are functionally important, this will need to be a catalyzed process.

The present results also indicate that NMR spectroscopy can be used to decide whether oligonucleotides crystallize as monomeric stem–loops or dimeric duplexes. Besides the identification of a stem–loop and a duplex subspectrum in preparatory experiments, the only condition for this method to be applicable is a slow interconversion between the two species. Our experience with the E.coli E-domain and also with similar oligoribonucleotides is that a slow interchange with a time scale of weeks to months exists for
stem–loops typically consisting of about eight or more base pairs. Whereas there has been a tendency to regard slow cooling of samples as appropriate for obtaining native RNA structures during crystallization attempts, if one is interested in the stem–loop form, it may be advantageous to rapidly cool the crystallization batches to favor the monomeric form before starting the crystallization. If necessary, the stem of the hairpin species could be extended by some base pairs in order to further slow down the monomer–dimer conversion. Unlike X-ray investigations, the NMR method does not require a minimal size or high diffractive resolution of the crystals. Instead, small or even imperfect crystals, which will be produced anyhow during screening for appropriate crystallization conditions, can be used. Furthermore, very little material is required and the NMR experiment is simple to perform. In our case, only 7 nmol of oligonucleotide, corresponding to 42 \( \mu \)g of E-domain crystals, was sufficient to acquire the decisive spectrum. In order to obtain a crystal structure for the stem–loop form of the E-domain, we are currently using NMR spectroscopy to check whether various folding protocols and crystallization buffers lead to predominantly stem–loop conformations of the E-domain in the crystallization buffer and in the resulting crystals.

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