Physical evidence for a domain structure in *Escherichia coli* 5 S RNA

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The downfield (9.5–15 ppm) proton spectra of a RNase A-resistant fragment of 5 S RNA and of its parent molecule are compared. The data show that the hydrogen-bonded structure of the fragment is the same as that of the corresponding sequences of A form 5 S RNA. The fragment must constitute a stable structural domain of 5 S RNA.

5 S RNA *NMR spectroscopy* *Imino proton* *Ribonuclease A* *Domain structure* *Fragment*

1. INTRODUCTION

In 1979 an extensive study was made of a RNase-resistant fragment of *Escherichia coli* 5 S RNA by Douthwaite, Garrett and Feunteun [1]. It consists of 61 bases comprising the terminal stem and the procaryotic loop of 5 S RNA (bases 1–11, 69–87, 89–120). Under proper conditions, the yield of the fragment in a digestion mixture approaches 100%. The fragment retains the L25 binding activity, but has lost the L18 binding property of intact 5 S RNA.

We present 500 MHz proton NMR spectra of the downfield (9.5–15 ppm) region of the fragment and of intact 5 S RNA in several of its conformational states [2]. This part of the proton spectrum of a nucleic acid reports the environments of hydrogen bonded ring NH protons [3]. Thus it reflects the interactions responsible for secondary and tertiary structure, which in the case of 5 S RNA are still only partly understood [4]. These spectra presented demonstrate a striking similarity of structure on the part of the fragment and A form 5 S RNA.

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2. MATERIALS AND METHODS

2.1. *5 S RNA*

This was purified from *E. coli* MRE 600 as in [2]. For several of the experiments done on 5 S fragment, the starting material was purified from whole-cell, low-M_r RNA (*E. coli* B) purchased from Plenum Scientific Research (Hackensack NJ). 5 S RNA was purified from this mixture by chromatography on Sephacryl S200 in 0.15 M NaCl, 1% methanol, 0.1 M sodium acetate (pH 5.0).

2.2. *5 S RNA fragment*

5 S RNA was adjusted to 20 A_{260}/ml in 0.1 M KCl, 5 mM $MgCl_2$, 50 mM Tris–borate (pH 7.8). RNase A (Worthington) was added at 10 μg/ml. After 45 min at 0°C, the reaction was stopped by addition of SDS at 0.5% followed by phenol extraction. The ethanol precipitated product was dissolved in 0.1 M NaCl, 5 mM $MgCl_2$, 10 mM cacodylate (pH 6.0) and purified by chromatography on Sephadex G-75 in the same buffer at 30°C. Fig.1 shows an acrylamide gel of a typical purified fragment preparation run in the presence and absence of L25 to document its binding capacity for this protein.

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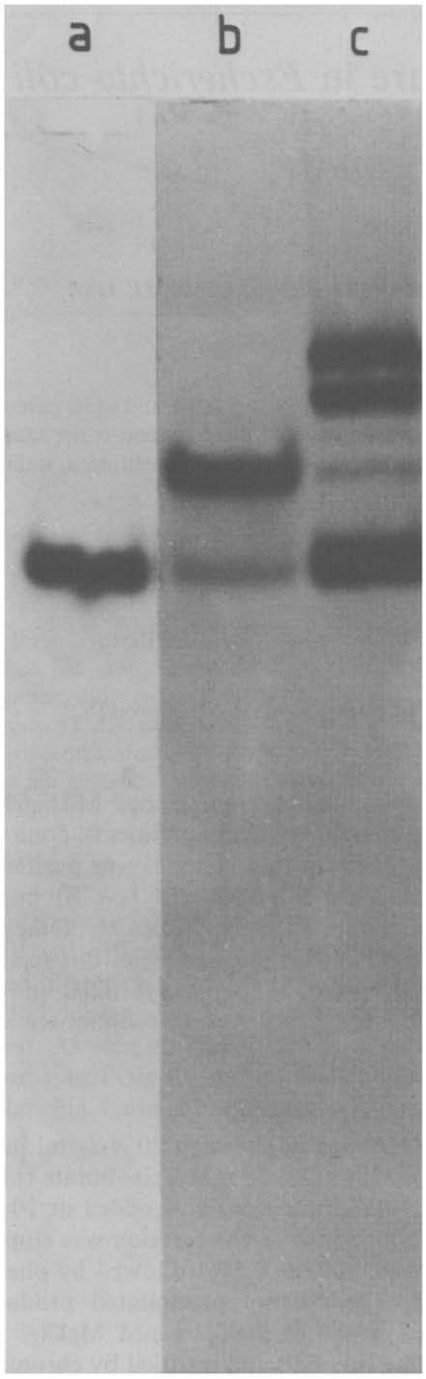

Fig.1. Electrophoretic properties of the RNase-resistant fragment of 5 S RNA. Fragment was purified as in section 2 and examined by acrylamide gel electrophoresis as in [2]. The gels are stained for RNA using methylene blue: (a) 3 μg purified fragment in a 50 μl sample volume; (b) 3 μg purified fragment mixed with 6.0 μg protein L25 in 50 μl; (c) A mixture of 3 μg fragment, 6.4 μg intact 5 S RNA and 3 μg L25 in 50 μl. In all cases the samples were suspended in the same buffer the gel was run in, i.e., 0.1 M KCl, 5 mM $MgCl_2$, 50 mM Tris–borate (pH 7.8). Reading from the bottom of the gel upwards, the bands seen correspond to free fragment, fragment with L25 bound, free 5 S RNA and 5 S RNA bound to L25. (These assignments have been confirmed using protein stained gels and gels including free 5 S RNA only (not shown); see also [1,2].)

2.3. *Conformational variants of 5 S RNA*

5 S RNA, at the time its purification is completed, is in the A form [2,5]. The high Mg^{2+} form (H form) is prepared by dialysis of A form RNA into buffers with > 2 mM Mg^{2+}. The low Mg^{2+} form (L form) is obtained when A form RNA is dissolved in buffers lacking Mg^{2+} [2]. B form RNA was prepared as in [5].

2.4. *NMR samples*

Dilute aliquots of RNA were dialyzed against 0.25 l of the appropriate buffer made up in 5% D_2O/95% H_2O. These samples were concentrated to 0.5 ml by ultrafiltration using YM5 membranes (Amicon). About 2 μl of 1 M aqueous dioxane were added to each sample as a chemical shift reference. The buffer used for fragment was 4 mM $MgCl_2$, 0.1 M KCl, 5 mM cacodylate (pH 7.2). [The fragment is not stable in the absence of Mg^{2+} (unpublished).] For H form 5 S RNA the same buffer was used except that [Mg^{2+}] was set at 2 mM. L form 5 S RNA was suspended in 0.1 M KCl, 5 mM cacodylate (pH 7.2). B form 5 S RNA was dissolved in 50 mM KCl, 10 mM cacodylate (pH 7.2).

2.5. *NMR spectroscopy*

All spectra were accumulated on a Bruker WM 500 NMR spectrometer, operating in the Fourier transform mode. A 45°–t–45° observe pulse train was used to permit observation of exchangeable proton resonances in the presence of H_2O. The total time for the (45°–t–45°) was 110 μs and the frequency was offset by ~5560 Hz from the H_2O resonance. 45° pulses were delivered in 10 μs (τ). The power spectrum for such a sequence has a null at roughly $\Delta\nu.(t + \tau) = 0.5$, where $\Delta\nu$ is the dif-

ference in frequency between the irradiation frequency and the frequency of observation. By adjusting the offset and t, this null can be made to coincide with the frequency of the H_2O resonance in the sample. The H_2O resonance can be suppressed 1000-fold or more in this way bringing the 1 mM resonances of the sample well within the dynamic range of the 16 bit digitizer on the WM-500 spectrometer with respect to the residual H_2O signal.

Difference spectra and all absolute intensity spectra presented were accumulated using a total pulse cycle time of 5 s and data were collected in 32 K memory blocks. Nuclear Overhauser effect (NOE) automatic, interleaved difference spectra, however, were acquired using a presaturation pulse length of 200 ms and 8 K of memory to give a total pulse cycle time of ~400 ms. Spectra were obtained on samples held at 303 K. The proton resonance of added dioxane was taken as having a chemical shift of 3.741 ppm relative to the methyl resonance of 3-(trimethylsilyl)-propane sulfonic acid.

3. RESULTS

3.1. *The spectra of the RNase-resistant fragment and intact 5 S RNA*

Fig.2 compares the downfield spectrum of the nuclease-resistant fragment in Mg^{2+} (a) with those of 3 configurational forms of intact 5 S RNA. Spectrum (b) is that of A form 5 S RNA in the presence of Mg^{2+} (H form). Spectrum (c) is from A form 5 S RNA in the same buffer as (b) but in the absence of Mg^{2+} (L form). (The large difference between spectra (b) and (c) confirms reports indicating that the configuration of A form (native) 5 S RNA is Mg^{2+}-dependent [2,6,7].) Spectrum (d) is that of 5 S RNA in the B form (denatured). It resembles the 400 MHz B form spectrum in [8]. Acrylamide gels were run confirming that the samples in question were A or B form as required.

3.2. *The number of base pairs*

Rough estimates of the number of hydrogen-bonded bases in these different structures can be obtained by integration of the downfield region. A series of spectra were obtained with 5 s delays, to ensure full relaxation of all resonances, and the total intensity detected in the 9.5–15 ppm region

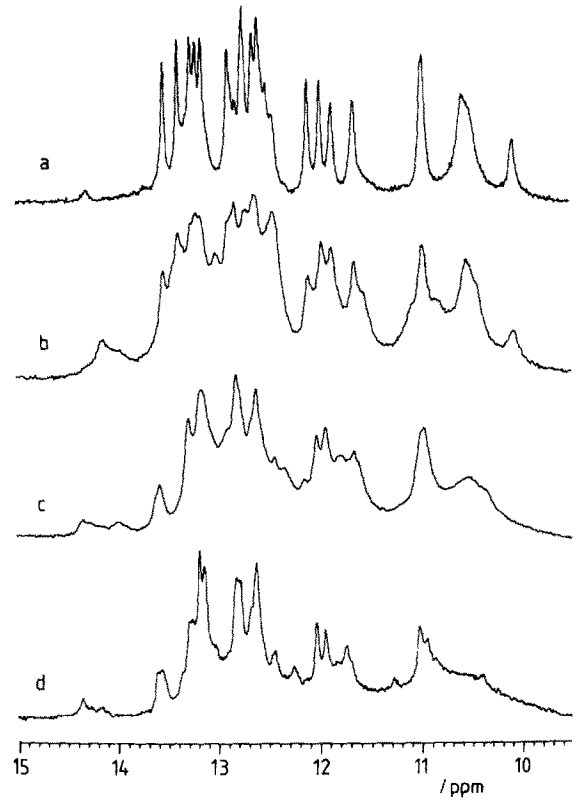

Fig.2. The downfield spectra of the RNase-resistant fragment and intact 5 S RNA in several conformations at 500 MHz. Samples were prepared as in section 2. Each spectrum required 1800 transients accumulated at 303 K with a 5 s pulse cycle time: (a) 1.03 mM RNase-resistant fragment; (b) 0.55 mM H form 5 S RNA; (c) 0.74 mM L form 5 S RNA; (d) 0.32 mM B form 5 S RNA.

estimated by integration. Integrated intensities were scaled from the absorbances at 260 nm of the different samples, and absolute values set by comparison with the fragment spectrum. The fragment spectrum contains a number of resonances of roughly equal intensity which were taken as representing 1 proton each.

The values obtained were: fragment, 19–21 protons; A form 5 S ($+Mg^{2+}$), 34–38 protons; A form ($-Mg^{2+}$), 28–32 protons; B form 5 S, 26–30 protons. Similar results have been obtained in [9] and [8] for A form 5 S in Mg^{2+} and for B form 5 S, respectively (see also [10,11]).

3.3. *H Form 5 S RNA and fragment compared*

It is clear from fig.2 that the fragment spectrum

in Mg^{2+} resembles that of the A form of the intact molecule, also in Mg^{2+}. Comparison of the two spectra can be facilitated by resolution enhancement (fig.3). Virtually every resonance in the fragment spectrum (a) has an obvious correspondent in the intact 5 S spectrum (b). The only region where the correlation cannot be clearly traced is between 11.3 and 12.0 ppm where the 5 S spectrum is inadequately resolved.

However, not all resonances in the intact 5 S RNA molecule have correspondents in the fragment spectrum (cf. fig.2b and 3b with fig.2a and 3a). This shows that there must be hydrogen-bonded structures within the loop consisting of bases 12–68, which is missing from the fragment, or between that loop and the fragment sequences. The fragment spectrum is a subset of the whole molecule spectrum.

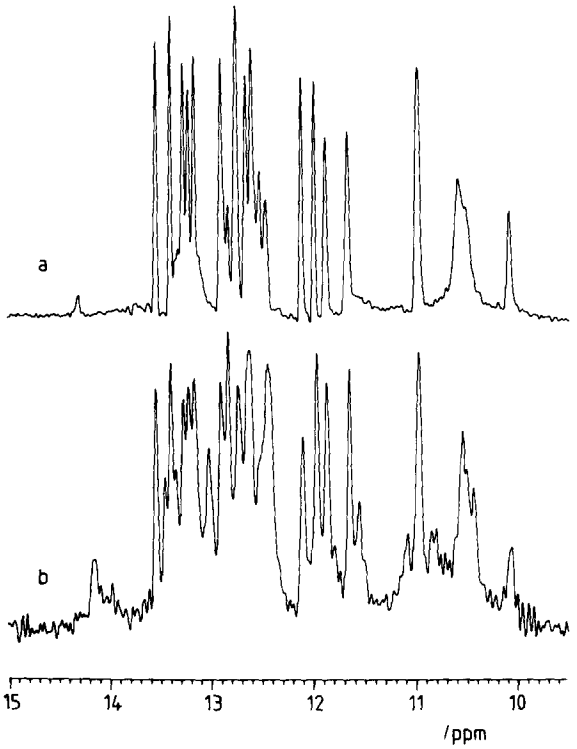

Fig.3. The resolution enhanced downfield regions of the RNase resistant fragment and H form 5 S RNA spectra at 500 MHz. The same data was used as for fig.2. Enhancement was achieved by Gaussian multiplication: (a) the RNase-resistant fragment; (b) H form 5 S RNA.

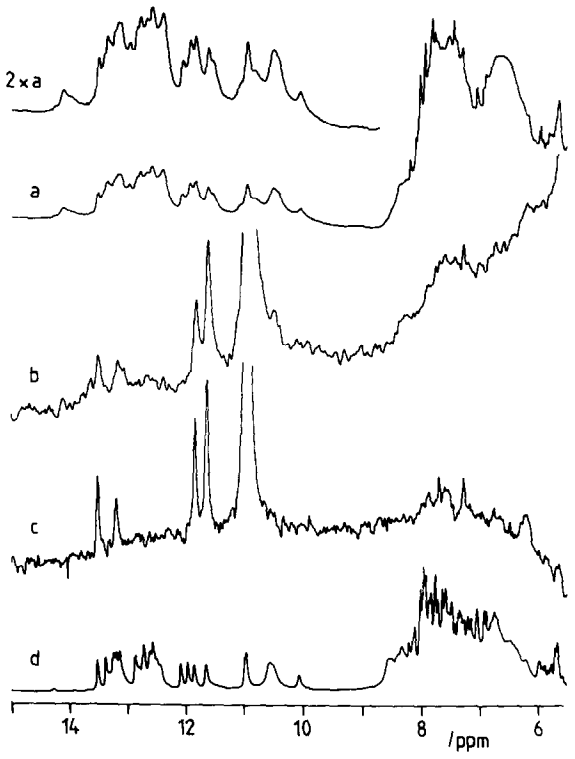

Fig.4. Comparison of a selective NOE experiment for the RNase-resistant fragment with one from H form 5 S RNA at 500 MHz. The samples used for this experiment were those used to accumulate the data in fig.2. The NOE pulse sequence involved a 0.2 s presaturation pulse at a specified frequency followed by a 45°–t–45° observe sequence. The presaturation power level was adjusted so that it reduced the presaturated resonance's intensity by 30–50%. NOE difference spectra were calculated by subtracting the spectrum obtained when the resonance of interest is presaturated (the 'on-resonance spectrum') from that accumulated when the presaturation pulse is delivered at a point where there are no resonances in the spectrum (the 'off-resonance spectrum'). Spectra were accumulated by a routine which accumulated FIDs cyclically, obtaining first 8 on-resonance FIDs followed by 8 off-resonance FIDs: (a) The off-resonance spectrum of H form 5 S RNA; 8000 scans (inset magnifies the downfield region 2 ×); (b) the NOE difference spectrum of H form 5 S RNA; 16000 scans. The on-resonance presaturating pulse was delivered so as to 'hit' the strong resonance at about 11 ppm. (The presaturated resonance is the largest feature in the spectrum.); (c) an NOE difference spectrum from the RNase + resistant fragment of 5 S RNA; 8000 scans; (d) the off-resonance spectrum of the RNase-resistant fragment of 5 S RNA; 4000 scans.

3.4. *Nuclear Overhauser experiments*

In order to establish that the chemical shift correspondences between the spectra of 5 S and its fragment are indicative of structural similarity a series of nuclear Overhauser experiments have been done. In double-helical structures presaturation of the hydrogen-bonded ring NH resonance of one base pair elicits NOEs from the hydrogen-bonded ring NH protons of base pairs on either side of the one whose resonance was saturated [12–14]. If the structures of 5 S and its fragment are the same, the NOEs seen when corresponding resonances are presaturated should be identical. Fig.4 presents the results of a typical experiment. Presaturation of the resonance at about 11 ppm produces 4 NOEs in both spectra which have identical chemical shifts. Many other experiments of this kind have been done with the same outcome in every case. The two structures are effectively the same in the sequences they share in common.

4. DISCUSSION

It is often observed that enzymes can be divided by proteolytic digestion into large fragments, which retain partial enzymatic activity. Correspondingly, the three-dimensional structural studies of enzymes commonly reveal division of the molecule into regions whose structures depend primarily on internal interactions, 'domains'. The observations on the RNase-resistant fragment of 5 S RNA [8] are typical of the kinds of chemical data which lead one to suspect domain structure in proteins. The above NMR data provide strong physical evidence that the fragment is a domain in the structural sense as well. The environments of the ring NH protons in hydrogen-bonded base pairs in the fragment sequence are not altered by deletion of the distal parts of 5 S RNA. Given the importance of hydrogen binding for nucleic acid structure it is hard to see how this could be true unless the three-dimensional structure of the fragment sequences within 5 S RNA is independent of the rest of the molecule.

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