NMR evidence for the existence of two native conformations of 5S RNA

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SUMMARY

NMR spectra of the non-exchangeable protons in 5S RNA from *E. coli* show the existence of two distinct conformers of the molecule which meet the operational definition of "A form" or native 5S RNA. Both are easily distinguished spectroscopically from denatured, "B form" 5S RNA. The conditions which interconvert the two A form conformers strongly suggest that the transition between them gives rise to the low temperature optical melting transition first reported in 5S RNA by Kao and Crothers (1).

INTRODUCTION

5S RNA is the smallest of the three RNAs found in procaryotic ribosomes. In *E. coli* it is an RNA of about 40,000 molecular weight consisting of 120 bases. While many activities of the 50S subunit persist at measurable levels in particles lacking 5S RNA, all are markedly stimulated by its presence (2). However, beyond its contribution to the structural integrity of the 50S subunit, its role in protein synthesis remains obscure (for review see reference 3).

It has been known for many years that 5S RNA can be obtained in two states, the A and B forms (4). The two forms are separable chromatographically, and differ both in their electrophoretic mobilities (5) and in their affinity for proteins L5, L18 and L25, the three found associated with 5S RNA in the ribosome (see (6) and references therein). Because A form 5S RNA participates in ribosome reconstitution while B form does not (4), and A form is also the one favored thermodynamically under physiological conditions (7), it is believed to correspond to the state of 5S RNA in the intact ribosome.

In the course of a series of experiments on protein interactions with 5S RNA we have obtained NMR data which indicate that there exist two readily distinguishable conformations of the molecule which meet the operational definition of "A form". The conditions required for eliciting

these two forms and interconverting them strongly suggest that they correspond to the high and low temperature states of 5S RNA identified optically by Kao and Crothers (1). The transition between these two states produces a detectable hyperchromic effect in 5S RNA preparations at near physiological temperatures and ionic conditions (1). The NMR data indicate that a substantial difference in the arrangement of bases must exist between these two forms, consistent with the optical observations. Both forms bind protein L25.

MATERIALS AND METHODS

5S RNA. Ribosomes were prepared both from *E. coli* MRE 600 and from *E. coli* PR13 by methods described elsewhere (8). RNA was obtained by phenol extraction of intact 70S particles or of LiCl-urea precipitates derived from 50S subunits. 5S RNA was purified from the resulting rRNA mixtures by the method of Kao & Crothers (1), except that the buffer used for Sephacryl chromatography was 0.15M NaCl, 1% methanol, 0.1M sodium acetate, pH 5.0. Purity was assessed by acrylamide gel electrophoresis.

5S preparations used in this work contained no detectable traces of other RNA species, and were entirely in the A configuration. B form 5S RNA was prepared by urea denaturation followed by gel filtration as described by Aubert, Scott, Reynier and Monier (4). Samples were stored at -80° C in Sephacryl buffer, and appeared to be stable indefinitely. Concentrations of 5S RNA were determined by absorbance at 260 nm assuming that an OD_{260nm} of 1.0 corresponds to a 1.18×10^{-6} M solution of 5S RNA (9), regardless of conformation.

Gel Electrophoresis. RNA and RNA/protein complexes were examined on acrylamide gels. The gel composition was 10% acrylamide, 0.5% bisacrylamide, 0.08% TEMED, 0.05% ammonium persulfate. The buffer used, unless otherwise noted, was 0.1 M KCl, 5 mM MgCl₂, 50 mM Tris-borate, pH 7.8. Gels were run at 3V/cm for 16 hours either at 4° C or at room temperature. Buffer was circulated between reservoirs to stabilize the pH. RNA was stained with methylene blue (10) and protein with Coomassie blue (.025% in a 50:50 mixture of 7% acetic acid and ethanol). The two stains are specific for RNA and protein respectively.

Protein L25. The preparation of L25 from 50S subunits has been described previously (11). The same techniques suffice to purify L25 from total 70S protein as well, since it elutes from carboxymethyl cellulose, at pH 5.6 in urea, separate from all other species. Concentrations

of L25 were estimated from absorbance at 276 nm. The absorbance of a 1% solution of L25 at 276 nm is 3.7 (Moore, unpublished observations).

NMR Spectroscopy. All spectra were accumulated on a Bruker WM 500 NMR spectrometer, operating in the Fourier transform mode. Typical spectra required the accumulation of 1000 transients with a cycle time of about 1.5 sec. Increasing the cycle time to 2.5 sec had no influence on the spectra. The residual HOD resonance was not suppressed by preirradiation. Spectra were obtained on samples held at 25° or 27° C at concentrations ranging from 0.5 to 1.0 mM. Dioxane was used as a chemical shift standard. Its (single) proton resonance has a chemical shift of 3.741 ppm relative to the methyl resonance of 3-(trimethylsilyl)propane sulfonic acid.

X-ray Scattering. X-ray solution scattering measurements were done using an instrument based on focussing mirror optics and a linear position-sensitive detector (12). Copper K α X-rays were used ($\lambda = 1.54 \text{ \AA}$). Data were corrected for smearing effects by indirect transformation (13).

RESULTS

Mg⁺⁺ Dependence of the NMR Spectrum of A form 5S RNA. An aliquot of 5S RNA was dialyzed into 0.1 M KCl, 10 mM Tris-HCl, pH 7.6 in H₂O at 4° C. The sample was concentrated by ultrafiltration to bring the RNA concentration to 0.65 mM. It was then divided into two parts and one of the portions dialyzed into 0.1 M KCl, 10 mM deuterotris-HCl, pH 7.6, D₂O. The remaining portion was dialyzed into the same buffer with 2 mM MgCl₂. The spectra given by the two samples at 27° C are shown in Figure 1, traces a and b.

Figure 1 shows the non exchangeable proton spectra of the two samples downfield of HDO (~4.7 ppm). The cluster of resonances around 5.5 ppm are those of the ribose 1' protons and the pyrimidine 5 protons. The resonances of the remaining nonexchangeable base protons appear in the aromatic region around 7.5 ppm. The two spectra shown in Figure 1 differ significantly in both these regions. The third spectrum in Figure 1 is the low Mg⁺⁺ spectrum, (a), less the high Mg⁺⁺ spectrum, (b). The ratio of the integrated intensity of the positive (or negative) regions of the difference spectrum to that of the whole spectrum indicates that the environments of at least 15% of the bases in the molecule are affected by the presence of Mg⁺⁺. Since the chemical shifts of these protons are largely determined by ring current effects, these spectral alterations

Figure 1: The Effect of Mg^{++} on A form 5S RNA. 5S RNA samples were prepared in 0.1M KCl, 10 mM deuterotris-DCI, pH 7.6, D_2O , in the presence and absence of 2 mM $MgCl_2$. Both samples were equilibrated with their buffers by dialysis. 1000 transients were accumulated on each at 300 K. The chemical shift scales are set by reference to dioxane.

Spectrum (a): 0.66 mM 5S RNA in the absence of Mg^{++}

Spectrum (b): 0.55 mM 5S RNA in the presence of Mg^{++}

Spectrum (c): The difference between spectra a and b. [Spectra were scaled so that the difference spectrum in the region upfield of H₂O (i.e. the sugar region) is zero.]

suggest a change in the base stacking arrangements in the molecule. Spectra obtained at 27° C, at neutral pH, and Mg^{++} concentrations above 2 mM are indistinguishable from the one shown in Figure 1b.

The State of the 5S RNA Preparations. RNA preparations at high concentration can display ionic strength-dependent aggregation effects. The possibility that aggregation might be responsible for the differences in NMR spectra shown in Figure 1 was examined by X-ray solution scattering. A sample of 5S RNA was prepared in 0.1 M KCl, 1 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.5 and X-ray data collected on it at a series of concentrations from 0.84 mM to 0.25 mM at both 25° C and 35° C. No temperature dependence

was noted. The radius of gyration at zero concentration was $34.7 \pm 1 \text{ \AA}$ which compares favorably with published values of $36.1 \pm 1 \text{ \AA}$ (14) and $34.5 \pm 1.5 \text{ \AA}$ (15). The observed radius fell with increasing RNA concentration.

At the same time as these measurements were done data were collected on a sample of 50S subunits known to be monodisperse by ultracentrifugation. Knowing the chemical compositions of both 50S subunits and 5S RNA, the ratios of their molecular weights can be estimated from the ratios of their forward scattering intensities per unit concentration. The forward scattering of 5S RNA, per unit concentration, extrapolated to zero concentration, indicated a molecular weight of 44,000 which is the value expected, 40,000, within the error of this method. Again the concentration dependence was such that the apparent molecular weight fell with increasing concentration.

A preparation of 5S RNA in 0.1 M NaCl, 0.01M EDTA, pH 5.6 in D_2O was examined at a single concentration (0.6 mM) at 25°, 35° and 45° C. At all temperatures both its radius of gyration and forward scattering were the same as those predicted from the data obtained with the Mg^{++} sample, within error.

These data indicate that 5S RNA should be monodisperse under the conditions explored in this study and that changes in aggregation state should not be contributing to the spectroscopic effects seen.

Interconversion of the Two Forms of 5S RNA. $MgCl_2$ was added to samples which gave spectra like that of Figure 1a and spectra recorded at several ratios of Mg^{++} ions per RNA molecule. The results are shown in Figure 2. The top trace (a) shows a typical low Mg^{++} spectrum which persists until the ratio of Mg^{++} to RNA reaches about 5 ions/molecule. At 5 ions/molecule (trace b), small alterations in the spectrum become apparent which are quite pronounced at 9 ions/molecule (trace c), and by 14 ions/molecule (trace d), the conversion is complete. [Note that since Mg^{++} is being added from the outside, these Mg^{++} levels are not equivalent to Mg^{++} ion activities.] Thus the conversion of the low Mg^{++} form of the molecule to the high Mg^{++} form occurs within a relatively narrow range of Mg^{++} ion concentration, suggestive of a cooperative transition. Furthermore, it is clear that the molecule is capable of making the transition at room temperature within a time which is short compared to the time required to collect NMR data (~1/2 hour).

L25 Binds to Both Forms of 5S RNA. With respect to the interaction

Figure 2: Titration of Mg^{++} -free A form 5S RNA with Mg^{++} . Mg^{++} was added as $MgCl_2$ to the 5S RNA sample which gave the spectrum shown in Figure 1a. The data collection conditions were the same as in Figure 1.

Spectrum (a): No Mg^{++} added. This spectrum is identical to Figure 1a.

Spectrum (b): The same sample as spectrum (a) after the addition of 5.1 Mg^{++} ions/5S RNA.

Spectrum (c): As in spectrum (b), with the Mg^{++} level raised to 9.1 Mg^{++} ions/5S RNA.

Spectrum (d): As above except that the Mg^{++} level is now 14.5 ions/5S RNA.

of L25 and 5S RNA there are two questions to ask: (1) is Mg^{++} required for the formation of a complex between the two, and (2) is Mg^{++} required for the continued stability of whatever complex is formed. To find the answers to these questions mixtures of L25 and 5S RNA were prepared with and without Mg^{++} and both mixtures were analyzed on two kinds of gels, one containing Mg^{++} and the other Mg^{++} free.

The gels shown in Figure 3 were run at room temperature. Similar results were obtained at 4° C. Tracks (a)-(g) are from a gel containing Mg^{++} . Tracks (a)-(d) are stained for RNA and (e)-(g) are stained for protein. Lanes (a) and (d) compare the mobility of a 5S RNA sample

Figure 3: The Protein Binding Properties of 5S RNA in the Presence and Absence of Mg^{++} . Acrylamide gels were run to analyze 5S RNA samples and mixtures of 5S RNA and L25. Two gels are shown here: tracks a-g are from a gel run in the presence of 5 mM Mg^{++} ; tracks h-n are from a gel run in the absence of Mg^{++} . The conditions for these gels were otherwise as given in Materials & Methods. The temperature during these runs was about 23° C. The samples in each case contained about 0.3 $A_{260\text{ nm}}$ of RNA and 7 μg of L25 per sample of 40 μl . The ionic conditions in the samples were those of the gel running buffer unless otherwise noted. Samples were kept at 0° C prior to loading on the gels.

First gel (5 mM Mg^{++}): Tracks a-d are stained with methylene blue to show RNA. Tracks e-g are stained with Coomassie blue to show protein. (a) 5S RNA alone, (b) 5S RNA with L25, (c) 5S RNA, previously dialyzed to remove Mg^{++} , plus L25, (d) 5S RNA, initially without Mg^{++} , no L25, (e) same as (b), (f), same as (c), (g) same as (a).

Second gel (0 Mg^{++}): Tracks h-k are stained with methylene blue. Tracks l-n are stained with Coomassie blue. (h) 5S plus L25 and Mg^{++} , (i) Mg^{++} -free 5S plus L25, (j) Mg^{++} -free 5S plus 2 μg L25, (k) 5S RNA with Mg^{++} , no protein, (l) same as (h), (m) same as (i), (n) same as (j).

containing Mg^{++} with that of a sample which was Mg^{++} free at the time it was loaded on the gel. Lanes (b) and (c) show corresponding samples containing L25. The reduced mobility of the RNA in (b) and (c) is due to of complex formation as proven by tracks (e) and (f) which show the presence of protein in those bands. Lane (g) is a sample of protein-free 5S RNA stained for protein.

Lanes (h)-(n) in Figure 3 repeat the observations of tracks (a)-(g)

in gels which lack Mg^{++} ion. Tracks (h) and (l) show a mixture of 5S RNA, Mg^{++} and a saturating amount of L25 stained for RNA, (h), and protein, (l). Tracks (i) and (m) are the corresponding experiments in the absence of Mg^{++} . Track (j) shows Mg^{++} free 5S RNA mixed with a less than saturating quantity of L25 and track (k) is 5S RNA with no L25 added, initially in the presence of Mg^{++} .

Clearly Mg^{++} -free and Mg^{++} containing samples of A form RNA cannot be distinguished by gel electrophoresis under these conditions consistent with the view that interconversion between the two forms is fast compared to the time of electrophoresis. Moreover, both forms bind to L25 regardless of the presence or absence of Mg^{++} in the binding mixture or in the analyzing gel subsequently. Mg^{++} is not required for binding or stabilization of the complex. This latter observation contradicts the finding of Spierer and Zimmermann (16) that there is a Mg^{++} requirement for this interaction, but is in accord with other more recent observations (3). It is possible that differences in the binding assay methods used may be the cause of the disagreement with Spierer and Zimmermann, in addition there are some small differences in the ionic conditions examined.

B form 5S RNA. To assure ourselves that the two states of 5S RNA seen here do not involve B form 5S RNA (4), a sample of B form 5S RNA was prepared and its spectrum taken, with the result seen in Figure 4. Clearly B form 5S RNA is unlike either Mg^{++} containing or Mg^{++} -free A form 5S RNA.

The samples in question were examined on acrylamide gels (Figure 5). Figure 5, track (a) is a sample of A form 5S RNA containing roughly 10% B form RNA. It was the A form residue from a urea denaturation mixture of 5S RNA which was resolved on G100 Sephadex (4). Track (b) is the B form product from the same column. Its mobility on gels is slightly greater than A form 5S RNA, as expected. Track (c) is an aliquot from the sample

Figure 4: 1H NMR spectrum of B form 5S RNA. A sample of B form 5S RNA was prepared as given in Materials and Methods. It was suspended in 50 mM KCl, 10 mM deuterio-tris-DCI, pH 7.5 at 0.55 mM in D_2O . 2000 transients were accumulated.

Figure 5: Gel analysis of A and B form samples of 5S RNA. The results of two different gels are shown: tracks (a-d) and tracks (e-g). Samples of A and B form 5S RNA were prepared by Sephadex chromatography as given in Materials and Methods for use as markers. All gels were stained with methylene blue. Gels contained 5 mM Mg^{++} and were run at room temperature. Tracks: (a) partially purified A type RNA, (b) pure B type RNA, (c) an aliquot from the B type sample used for NMR (see Figure 4), (d) a mixture of A and B type RNAs produced by heating an A form RNA sample in the absence of Mg^{++} . Tracks: (e) an aliquot from an A form sample used for NMR, (f) the sample in (c) after 30 minutes at 55° C with Mg^{++} added to give a nominal 10 mM concentration, (g) as in (a).

which gave the spectrum of B form RNA shown in Figure 4. Track (d) is an A form/B form mixture produced by heating a sample of A form 5S RNA in the absence of Mg^{++} . Track (e) is an aliquot from an A form 5S sample used for NMR. Track (f) is the B form sample after annealing in the presence of Mg^{++} . [Note it has regained A form mobility as a result, as anticipated.] Track (g) is the same sample as track (a).

The B form material used to produce the spectrum in Figure 4 has the electrophoretic properties it should and responds to heating in Mg^{++} as B form is supposed to. It is not the same as A form 5S RNA electrophoretically just as it is not the same as either of the two A forms spectroscopically.

DISCUSSION

In 1980 Kao and Crothers reported the existence of a low temperature melting transition in 5S RNA (1). Its salient features are that it occurs in buffers of moderate ionic strength and neutral pH, at temperatures close to room temperature, that it is fast, having a half time of milliseconds, and that the high temperature form of the molecule is stabilized by Mg^{++} ion. Recent tritium exchange results also point to the existence of a Mg^{++} dependent conformational change in 5S RNA (17).

The two states of 5S RNA presented in this study behave as would be expected if they corresponded to those identified by Kao and Crothers (1). The transition between them is induced by Mg^{++} ion at room temperature, and the interconversion between them is fast. An attempt was made to induce the same conformational change by raising the temperature. However under the conditions tested, the material converted into the B form upon heating rather than into the high Mg^{++} form hoped for. Given the sensitivity of the Kao-Crothers transition to ionic conditions it is possible that under different conditions of pH and ionic strength, the appropriate transition would be observed.

Several kinds of evidence indicate that the two A states examined here have nothing to do with the classical B form of 5S RNA. First, both forms have NMR spectra distinctly different from that of B form 5S RNA. Second, neither coelectrophoreses with B form RNA. Third, the rate of interconversion between these two forms at room temperature is orders of magnitude faster than the A to B conversion which under the conditions investigated here should take days (19). It is not possible kinetically that one form could be the A form and the other the B form.

We suggest that the two conformers of A type 5S RNA first recognized by Kao & Crothers (1), and identified here by NMR spectroscopy, be called "H" and "L". The H form would be that which is favored by high temperature and high Mg^{++} concentration, and the L form would be the one which is stable at lower temperatures in the absence of Mg^{++} .

Preliminary data on the NMR spectra of complexes of 5S RNA and L25 show that the mode of binding of L25 to the H and L forms is the same and that L25 has no effect on their interconversion (Moore, unpublished data). Furthermore, partial digestion experiments done with RNase A show that the large RNase resistant fragment isolatable from H form 5S RNA (13) can also be isolated from the L form (Chabot & Moore, unpublished observation). This fragment comprises nucleotides 1-11, 69-87 and 89-120 in the intact 5S molecule and includes the L25 binding site (13). Thus

it would appear that the spectral changes observed here must relate either to 5S tertiary interactions which do not affect L25 binding or RNase resistance or be localized in the 12-68 loop. Much more work will have to be done to further identify the part of the molecule where these changes are taking place.

Since, as Kao and Crothers (1) pointed out, the two states, H and L, interconvert under conditions very close to physiological, the possibility exists that the transition between them is related to 5S RNA function. Thus the characterization of the transition could turn out to be of more than passing interest.

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