Probing the α -sarcin region of *Escherichia coli* 23S rRNA with a cDNA oligomer

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Received December 24, 1987; Revised July 15, 1988; Accepted October 24, 1988

ABSTRACT

The cause of 50S ribosomal subunit collapse reportedly triggered by hybridization of a 14-base cDNA probe to the α -sarcin region of 23S rRNA (6) was investigated by physical measurement of probe-subunit complexes in varying buffer conditions. The results reported here show that this probe was unable to hybridize to its target site in the intact 50S subunit and the physical characteristics of 50S subunits remained unchanged in its presence. Subunit collapse was induced in buffer containing 20mM Tris-HCl (pH 7.5), 600 mM NH_4Cl , 1 mM MgCl_2 , 1 mM DTT, and 0.1 mM EDTA in the absence of probe. The probe bound specifically to its target site in the collapsed particle, but did not promote further unfolding. The results demonstrate that a DNA probe bound to the α -sarcin region cannot cause the 50S subunit to unfold or cause 23S rRNA to degrade. We suggest that the previously reported collapse was most probably the result of the ionic conditions used.

INTRODUCTION

One ribosomal RNA (rRNA) sequence of interest is the α -sarcin region of *Escherichia coli* 23S rRNA. This region spans nucleotides 2653-2667 and contains the cleavage site for the cytotoxin α -sarcin (1,2,3). Alpha-sarcin possesses both single- and double-stranded ribonuclease activities specific for purines. Although it causes extensive degradation of naked RNA, α -sarcin hydrolyzes only a single phosphodiester bond in 23S rRNA when incubated with 50S subunits or 70S ribosomes (2). The hydrolysis occurs 242 nucleotides from the 3' terminus of 23S rRNA between bases G₂₆₆₁ and A₂₆₆₂ (1,2,3). Cleavage of the 23S rRNA at the α -sarcin site inactivates the ribosome specifically by inhibiting EF-Tu-catalyzed aminoacyl-tRNA binding at the ribosomal A site (4,5). The region surrounding the cleavage site shares two characteristics with other functional rRNA sequences (2): i) its

sequence is conserved phylogenetically and ii) it is single-stranded.

We have probed the α -sarcin-sensitive region with cDNA oligomers to determine the cause of "ribosomal collapse" reportedly triggered by hybridization of a 14-base DNA probe complementary to nucleotides 2654-2667 (6). During collapse, the 50S subunit reportedly releases 5S rRNA and a distinct set of five ribosomal proteins and yields a 43S subparticle called the "alpha particle". In this paper we report that 50S subunit structure is not affected by the presence of an oligonucleotide complementary to the α -sarcin region, but is altered considerably by the buffer conditions used.

MATERIALS AND METHODS

Preparation of Ribosomal Subunits

Native 70S ribosomes were isolated from E. coli strain MRE 600 by the method of Hill, et al. (7). The 50S subunits were isolated by zonal centrifugation as outlined by Tam and Hill (8), with minor modification. Subunits were pelleted from the zonal fractions by centrifugation for at least 9 hrs, 60,000 rpm, 4°C in a Beckman Ti70 rotor. Pellets were resuspended in 10 ml buffer B (10 mM Tris-HCl, pH 7.4; 100 mM KCl; 1.5 mM MgCl₂) and dialyzed 24 hr against 1 L buffer B to remove residual sucrose. Subunit samples were stored at -70°C in small aliquots.

Alteration of Buffer Conditions

The 50S ribosomal subunits were transferred into alpha buffer (20 mM Tris-HCl, pH 7.5; 600 mM NH₄Cl; 1 mM MgCl₂; 1 mM DTT; 0.1 mM EDTA) via three different methods:

(1) The subunits were dialyzed against alpha buffer (1/500 v/v) for 24 hr at 4°C with three buffer changes.

(2) The subunits were pelleted (30 min, 100,000 rpm, 4°C in a Beckman TLA 100.2 rotor), the pellets washed and resuspended in alpha buffer.

(3) The subunits were diluted directly into alpha buffer conditions. (Note that buffer B contains some KCl whereas alpha buffer does not. Thus, diluting directly to alpha buffer conditions necessarily resulted in a final concentration of 20 mM KCl in addition to the other constituents of alpha buffer.)

In all cases, the subunits were analyzed immediately using the physical methods described below.

Preparation of DNA Probe

The α -sarcin probe [23S(2654-67), sequence 5'-(GGTCCTCTCGTACT)] was synthesized on a Biosearch Model 8600 DNA synthesizer using β -cyanoethyl phosphoramidite chemistry. Probes were purified before and after detritylation by reversed-phase HPLC on a Gilson HPLC system using a Column Engineering 10 μ m ODS column. Oligomers were stored at -70°C. Purified DNA probe was 5'-end labelled using T4 polynucleotide kinase (Bethesda Research Laboratories) and [γ - 32 P]ATP (New England Nuclear) as described by Chaconas and Van de Sande (9) with the omission of the dephosphorylation step.

The purity of probe preparations was monitored by gel electrophoresis. DNA oligomer (25 μ g) was evaporated to dryness, resuspended in 15 μ l of gel loading dye (7M urea, 5% glycerol, 0.025% bromphenol blue, 0.025% xylene cyanol) and loaded onto a 20% polyacrylamide gel (12.5 cm X 13.5 cm X 1.5 mm) containing 89 mM Tris-borate (pH 8.3) and 1mM EDTA (TBE). The sample was electrophoresed at 40 mA for 50 min at 4°C. Probe was visualized by methylene blue staining. Radiolabeled probe was also monitored for purity in a 20% polyacrylamide gel and visualized by autoradiography.

Hybridization Assays

Probe hybridization assays were performed by incubating 25 pmol 50S subunits with 5'- 32 P end-labeled probe for 4 hr at 4°C in 50 μ l binding buffer (10mM Tris HCl, pH 7.4; 100 mM KCl, 5 mM MgCl₂) or alpha buffer. The reactions were filtered through Millipore HAWP 0.45 μ m nitrocellulose filters and washed twice with 1-ml aliquots of reaction buffer. The radioactivity retained by the filters was determined by liquid scintillation.

Probe hybridization was also assayed using sucrose gradient centrifugation. In this experiment binding reactions were layered onto an 11 ml 10-30% sucrose gradient in alpha buffer or in binding buffer. Gradients were centrifuged in a Beckman SW41 rotor at 37,000 rpm for 12 hr at 4°C. Gradient fractions were assayed for subunit migration by measuring absorbance at 260 nm and probe migration by liquid scintillation.

RNase H Assays

To determine the exact site(s) of cDNA probe hybridization, the probe-subunit complex was incubated with RNase H and the digestion products analyzed. The conditions used were similar to those outlined by Donis-Keller (10). Digestion reactions containing 25 μ g ribosomal subunits, 2.5 μ g cDNA probe and 3-5 units of RNase H (P-L Biochemicals) in 20 μ l of 40 mM Tris-HCl (pH 7.9), 10 mM $MgCl_2$, 60 mM KCl and 1 mM DTT were incubated at 4°C for 24 hr.

Alpha-sarcin was used to generate the RNA fragment (the alpha fragment) used as the marker in this assay. In this reaction, 25 μ g 50S subunits and 0.25 μ g α -sarcin were incubated in 20 μ l of buffer A (50 mM Tris-HCl, pH 7.6; 50 mM KCl) for 15 min at 37°C.

Ribosomal RNA digestion products were analyzed by gel electrophoresis. The RNA was purified by extracting subunit samples three consecutive times with buffer-equilibrated phenol, precipitating with 2.5 volumes 95% ethanol at -20°C for 1 hr and pelleting by centrifugation. RNA pellets were resuspended in 15 μ l of gel loading dye and electrophoresed in a 5% polyacrylamide gel (12.5 cm X 13.5 cm X 1.5 mm) containing 7M urea and TBE, at 12.5 mA for 4 hr at room temperature. RNA products were visualized by staining with methylene blue.

Physical Methods

Sedimentation velocity experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with schlieren optics. Dual-cell experiments were carried out in all cases to allow direct comparison of samples; typically, 500-800 μ l of control samples were injected into a standard sample cell and the same volume of test sample was injected into a cell having a 1° wedge-shaped window. Both cells were centrifuged simultaneously in a Beckman ANH rotor at 52,000 rpm and 4°C. Photographs were taken at 4-minute intervals beginning 2 min after the rotor had reached 52,000 rpm.

Diffusion coefficients of subunit samples were obtained using quasi-elastic light scattering (QLS) (11). Control samples for quasi-elastic light scattering experiments contained 100 μ g 50S subunits in 200 μ l binding buffer or alpha buffer. Test

samples contained an additional 5.2 μg of probe. Prior to scattering experiments, all samples were centrifuged overnight at 5000 rpm, 4°C in a Sorvall HB4 rotor to remove dust particles.

The correlation function was obtained using a Malvern 4300 spectrometer system and a Langley-Ford 1096 autocorrelator and a Lexel 4 watt argon laser. The correlation functions were analyzed by the method of Blair *et al.* (12) to obtain diffusion coefficients.

RESULTS

Alpha Particle Formation

The sedimentation behavior of 50S subunits in alpha buffer was compared to that of 50S subunits in buffer B. A 25 mg/ml stock solution of 50S subunits in buffer B was divided into two aliquots: one was dialyzed against alpha buffer for 24 hrs at 4°C, while the other was left unfrozen at 4°C for the same period. Each sample was diluted in the respective buffer to 5 mg/ml. An 800 μl portion of buffer B containing 50S subunits was injected into a standard sample cell and the same volume of alpha buffer containing 50S subunits was injected into a cell having a 1° wedge-shaped window. Both cells were centrifuged simultaneously to allow direct comparison. As Figure 1A shows, 50S subunits migrated as two distinct boundaries in alpha buffer, while subunits in buffer B migrated as a single boundary. Also, both particles in alpha buffer sedimented more slowly than the particle in buffer B. Both sample cells were then removed from the rotor and incubated at 51°C for 40 min, cooled to 4°C, and centrifuged as before. The boundary representing 50S subunits in buffer B was much smaller and broader after heating, while the boundaries in alpha buffer disappeared completely (Fig. 1B).

The effect of probe 23S(2654-2667) on subunit sedimentation is shown in Figure 2. In the first experiment (Fig. 2A), a 2.5 mg sample of 50S subunits in 500 μl of buffer B was compared to a similarly prepared sample preincubated with an eight-fold excess (mol/mol) of probe for 4 hrs at 4°C. The schlieren patterns of both samples were identical. The samples in the

Figure 1. Schlieren patterns of 50S ribosomal subunits in alpha buffer (upper) and buffer B (lower) immediately following 24-hr dialysis against alpha buffer (A) and incubation at 51°C for 40 min (B). Samples (800 μ l vol.) were centrifuged in a Beckman ANH rotor at 52,000 rpm. Sedimentation is from left to right. Photographs taken 14 min after reaching 52,000 rpm using 70° phase plate angle. Temperature was 6.0°C.

second experiment (Fig. 2B) were identical to the first, except that alpha buffer was substituted for buffer B. The alpha particle again formed in the absence of probe and the sedimentation behavior of the sample was essentially the same as that of the sample containing probe.

In an attempt to reproduce the starting conditions of the earlier study (6), i.e. intact 50S subunits in alpha buffer, two alternatives to dialysis for transferring the subunits into alpha buffer were used.

First, the subunits in buffer B were pelleted and resuspended in alpha buffer. Their sedimentation behavior was then analyzed using analytical ultracentrifugation as before. These subunits did migrate as a single boundary although they sedimented somewhat more slowly than did 50S subunits in buffer B. The addition of probe, however, had no effect. The subunits still migrated as a single boundary.

Secondly, the 50S subunits were diluted directly to alpha

Figure 2. Schlieren patterns of 50S ribosomal subunits in absence of probe (lower) and after preincubation with 8-fold excess (mol/mol) of probe (upper) in buffer H (A) and alpha buffer (B). Samples (500 μ l vol.) were centrifuged in a Beckman ANH rotor at 52,000 rpm. Sedimentation is from left to right. Photographs taken 10 min after reaching 52,000 rpm using 75° phase plate angle. Temperature was 6.8°C.

buffer and their sedimentation behavior analyzed as before. Again, these subunits sedimented as a single boundary but somewhat more slowly than 50S subunits in buffer B, and their sedimentation behavior was unaffected by the addition of probe.

Quasi-elastic Light Scattering

Quasi-elastic light scattering was utilized to measure the diffusion coefficients of subunits under different experimental conditions (see Table I). Each sample contained 100 μ g 50S subunits in 200 μ l of buffer and a 20-fold excess (mol/mol) of probe where indicated. Consistent with the sedimentation data, there was an insignificant difference in the diffusion coefficient of 50S subunits in binding buffer ($1.94 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$) and the diffusion coefficient of 50S subunits incubated with probe in binding buffer ($1.96 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$). The diffusion coefficients for 50S subunits in alpha buffer were considerably

TABLE I.
50S Subunit Diffusion Coefficients in Binding Buffer and Alpha Buffer*

Binding Buffer	$D_{20,w}$ ($\text{cm}^2 \text{sec}^{-1}$)
Control 50S subunits	1.94×10^{-7}
50S subunits + probe	1.96×10^{-7}
Alpha Buffer	
Control 50S subunits	1.67×10^{-7}
50S subunits + probe	1.81×10^{-7}

*see Materials and Methods for experimental procedures

smaller than for those in binding buffer. Unexpectedly, the diffusion coefficient of subunits coincubated with probe in alpha buffer ($1.81 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$) was slightly larger than the diffusion coefficient of subunits in alpha buffer alone ($1.67 \times 10^{-7} \text{ cm}^2 \text{ s}^{-1}$).

Probe Binding

Probe hybridization was quantified by filter binding assays as previously described (13,14). Hybridization of the 14-base DNA probe to 50S subunits in alpha buffer is shown graphically in Figure 3. The graph demonstrates that as the amount of probe in the binding reaction was increased, about 30% of subunits in the reaction mixture complexed with probe. Similar values were obtained when reactions were incubated for 30 min at 37°C or 51°C. We were unable to detect any measurable binding of probe to the 50S subunit in other buffers containing 10 mM Tris-HCl (pH 7.4), 60-200 mM KCl, and 5-15 mM MgCl₂. We were also unable to induce binding in any of these buffers by varying the incubation period from 2-24 hrs at 4°C or 10°C, or for 30 min at 37°C or 51°C.

Probe hybridization was also assayed by sucrose gradient centrifugation. Hybridization is qualitatively demonstrated if radiolabeled probe comigrates with subunits in sucrose gradients. Migration of probe and subunits in a 10-30% sucrose gradient containing alpha buffer is shown in Figure 4. The two peaks correspond to 50S subunits and alpha particle,

Figure 3. Filter binding assay of 14-base DNA probe and alpha particles in alpha buffer. Experimental conditions were as described in Materials and Methods.

respectively. The labeled probe always comigrated with the alpha particle, and not with the 50S subunit. In experiments using binding buffer in place of alpha buffer, only an insignificant amount of probe sedimented with 50S subunits (data not shown).

Figure 4. Gradient binding assay of 14-base DNA probe and alpha particles in alpha buffer. The 50 μ l reaction was layered on an 11 ml 10-30% (w/v) sucrose density gradient and centrifuged in an SW41 rotor 12 hrs, 37,000 rpm, 4°C. Sedimentation is from right to left.

Figure 5. RNase H digestion of probe-alpha particle complex. RNA was electrophoresed in a 12.5cm X 13.5cm X 1.5mm 5% polyacrylamide gel containing 89 mM Tris-borate (pH 8.3) and 1 mM EDTA for 4 hrs at 12.5 mA. Lane 1: control alpha particles; Lane 2: alpha particle + probe; Lane 3: alpha particle + probe + RNase H; Lane 4: α -sarcin digest of 50S subunit.

RNase H Assays

RNase H was used to demonstrate that the probe was binding specifically to its target site. If the probe hybridized specifically at the α -sarcin region, RNase H would cleave a 240-base 3' fragment from 23S RNA. The cleavage products from that reaction are shown in Figure 5. The additional fragment in Lane 3 shows that 23S RNA is cleaved by RNase H when the alpha particle is coincubated with probe, demonstrating that the probe binds to 23S rRNA. The new fragment was also the same size as the 240-base fragment generated by an α -sarcin digest of 50S subunits (Lane 4), indicating the probe bound specifically to the target site. This contrasts the results of other experiments, where RNase H was unable to cleave 23S when incubated with the probe and intact 50S subunits (data not shown).

Figure 6. Electrophoretic analysis of ribosomal RNA from 50S subunits incubated in binding buffer at various temperatures in the presence or absence of probe 23S(2654-2667). Subunits were coincubated with 350 pmol probe where indicated. Samples were electrophoresed in a 12.5 cm x 13.5 cm x 1.5 mm 5% polyacrylamide gel containing 89 mM Tris-borate (pH 8.3) and 1 mM EDTA for 4 hrs at 12.5 mA. Lane 1: incubated 30 min, 4°C; Lane 2: incubated 30 min, 37°C; Lane 3: incubated 30 min, 51°C; Lane 4: plus probe, 30 min, 4°C; Lane 5: plus probe, 30 min, 37°C; Lane 6: plus probe, 30 min, 51°C.

Ribosomal RNA Analysis

Ribosomal RNA from 50S subunits incubated in binding buffer at various temperatures in the presence or absence of probe 23S(2654-2667) was isolated and analyzed electrophoretically (Figure 6). Each reaction contained 25 pmol 50S subunits in 50 ul of buffer containing 10 mM Tris-HCl, 100 mM KCl, and 5 mM MgCl₂. Subunits were coincubated with 350 pmol probe where indicated. The RNA was purified by phenol extraction and electrophoresed in a 12.5 cm x 13.5 cm x 1.5 mm 5% polyacrylamide gel containing 89 mM Tris-borate (pH 8.3) and 1 mM EDTA for 4 hrs at 12.5 mA. Clearly, the probe was unable to cause fragmentation of 23S rRNA, even when incubated at 51°C.

In a parallel experiment, electrophoretic analysis of rRNA isolated from 50S subunits incubated in alpha buffer in the same conditions as those outlined above revealed that in all cases, 23S rRNA did not undergo further degradation (data not shown).

DISCUSSION

The goal of this study was to investigate the ribosomal collapse described by Henderson and Lake (6). Reportedly, hybridization of a 14-base DNA probe complementary to the α -sarcin region of 23S rRNA triggers collapse of the 50S subunit. The ionic conditions used in that study were identical to those of alpha buffer and the hybridization reactions were incubated at either 37°C or 51°C for 30 min. We have probed the α -sarcin region of the 50S subunit in differing buffers and temperatures with a cDNA oligomer and used physical studies to characterize the resulting probe-subunit complexes. The results show that the ribosomal collapse was not caused by hybridization of a 14-base DNA probe complementary to the α -sarcin region, but probably by the ionic and thermal conditions utilized in the earlier study. The probe does not bind readily to the α -sarcin region or any other region of the 50S subunit in binding buffer, even though the ionic conditions of binding buffer are similar to those of buffers used in successful probing studies of sites in 30S and 50S subunits (13,14,15). The inability to detect more than trace probe binding to the α -sarcin region in binding buffer suggests this site is not accessible to the 14-base probe.

Although the 14-base probe is unable to bind the intact 50S ribosomal subunit, the α -sarcin region was available to the probe in the unfolded particle. Gradient binding assays (Fig. 4) showed that the probe can bind only to the alpha particle and RNase H assays showed that the nuclease, which degrades RNA in DNA-RNA heteroduplexes (10), cleaved 23S rRNA when incubated with a probe-alpha particle reaction mixture (Fig. 5). The RNA fragment generated by RNase H confirmed that the probe binds to 23S rRNA, and its size matches the size of the alpha fragment, indicating binding specificity at the α -sarcin site. The inability of RNase H to generate a similar RNA fragment when incubated with cDNA probe and intact 50S subunits demonstrates the probe does not hybridize even transiently to the α -sarcin region in the intact 50S subunit. The data indicate the probe does not bind to the α -sarcin site and cause the 50S subunit to collapse, but rather, the probe binds specifically to its target site in the alpha particle after the subunit unfolds.

The sedimentation velocity data shown in Figure 1 demonstrated that alpha buffer was directly responsible for disruption of subunit structure. This result is consistent with those from early studies of the physical characteristics of ribosomes at various magnesium ion concentrations (16,17,18,19,20). In those studies, the stability of 50S ribosomal subunits decreased in buffers containing less than 2.5 mM Mg^{2+} , especially when the monovalent/divalent cation ratio is greater than 60. In low-magnesium buffers, the subunit unfolded into several intermediates ranging from 16S to 45S without suffering any rRNA degradation or loss of ribosomal material. The unfolding process generally occurred over extended periods of time, but was accelerated at elevated temperatures (19). High concentrations of monovalent cations, particularly ammonium ion, also affect the physical characteristics of the ribosome (21). The sedimentation coefficient of the 50S subunit has been observed to decrease to 45S in 0.5 M NH_4Cl having nominal Mg^{2+} concentrations (21). There was no apparent shape change of the subunit or loss of subunit material, but there was a decrease in the density of the particle. In light of the results presented here, the ionic conditions of alpha buffer and elevated temperature were probably the cause of the reported subunit collapse. These results indicate that in alpha buffer, the 50S subunit unfolds, and upon heating the subunits undergo degradation as well.

The presence of the probe in these ionic conditions had no further effect on the physical characteristics of the alpha particle. The results reported above do not necessarily preclude the possibility that probe hybridization at the α -sarcin region could aid subunit unfolding. This possibility was tested by QLS and sedimentation velocity experiments. The schlieren patterns in Figure 2 demonstrate that the sedimentation behavior of 50S subunits in binding buffer did not change in the presence of probe and no further subunit degradation was observed when the probe was incubated with alpha particles in alpha buffer. Similarly, the diffusion coefficient of subunits in binding buffer was unaffected by the presence of probe. Unexpectedly, the diffusion coefficients of subunits in alpha buffer increased

slightly when probe was added. We cannot provide an explanation for this observation, but we note that the increased diffusion coefficient is the opposite effect one would expect if the probe actually triggers ribosomal collapse.

Because the probe reportedly triggered 23S rRNA degradation (6), we isolated and analyzed electrophoretically the ribosomal RNA from 50S subunits incubated in binding buffer at various temperatures in the presence or absence of probe 23S(2654-2667) (Figure 6). The probe was unable to cause fragmentation of 23S rRNA, even when incubated at 51°C. Ribosomal RNA isolated from 50S subunits incubated in alpha buffer in the same conditions as those outlined in the legend of Figure 6 is also undegraded, indicating that neither increased temperatures nor the cDNA probe could induce rRNA cleavage in alpha buffer.

Acknowledgements. We thank Dr. Ira G. Wool for his generous gift of α -sarcin and Mssrs. Thomas Gluick, David Camp, and Richard Marconi for helpful discussions and proofreading. This work was supported in part by NSF Grant DMB-8417297 and NIGMS Grant GM 35717.

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