The RNA-binding domain of ribosomal protein L11 recognizes an rRNA tertiary structure stabilized by both thiostrepton and magnesium ion

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ABSTRACT

Antibiotics that inhibit ribosomal function may do so by one of several mechanisms, including the induction of incorrect RNA folding or prevention of protein and/or rRNA conformational transitions. Thiostrepton, which binds to the ‘GTPase center’ of the large subunit, has been postulated to prevent conformational changes in either the L11 protein or rRNA to which it binds. Scintillation proximity assays designed to look at the binding of the L11 C-terminal RNA-binding domain to a 23S ribosomal RNA (rRNA) fragment, as well as the ability of thiostrepton to induce that binding, were used to demonstrate the role of Mg2+, L11 and thiostrepton in the formation and maintenance of the rRNA fragment tertiary structure. Experiments using these assays with both an Escherichia coli rRNA fragment and a thermostable variant of that RNA show that Mg2+, L11 and thiostrepton all induce the rRNA to fold to an essentially identical tertiary structure.

INTRODUCTION

Many antibiotics that interfere with ribosome function protect specific regions of rRNA from chemical modification (1) and probably derive much of their binding affinity from direct contacts with the rRNA. It is attractive to think that antibiotic-induced stabilization of rRNA structure could be a common mechanism of antibiotic action, for instance by folding the RNA into a non-functional structure or by preventing a conformation change that normally takes place during the ribosome cycle. One candidate for such a mechanism is thiostrepton, which binds to the ‘GTPase center’ of the large subunit, comprised of a highly conserved region of large strepton, which binds to the ‘GTPase center’ of the large ribosome cycle. One candidate for such a mechanism is thioconformation change that normally takes place during the RNA into a non-functional structure or by preventing a mechanism of antibiotic action, for instance by folding the induced stabilization of rRNA structure could be a common contacts with the rRNA. It is attractive to think that antibiotic-probably derive much of their binding affinity from direct specific regions of rRNA from chemical modification (1) and

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enhanced 100–1000-fold when L11 protein is present (6,19,20), but this stimulation depends on the presence of an N-terminal domain in L11 (19). In the present studies we have used only L11-C76, the C-terminal domain of L11 lacking any intrinsic interactions with thiostrepton, in order to focus on the effects of L11 and thiostrepton on the rRNA tertiary structure. Although thiostrepton and L11-C76 both stabilize tertiary structure within the rRNA domain, it is not known whether the stabilized conformation is the same in both cases (12,18,19).

A nitrocellulose filter binding assay has been used in previous studies of L11 and thiostrepton interactions. As L11 and thiostrepton both retain rRNA on filters, the assay cannot be used to measure binding of one ligand in the presence of the other. Thus we have turned to scintillation proximity assays (SPAs) (21,22), which have several advantages over filter binding assays for making equilibrium measurements and have been shown to give reliable and reproducible results with well-characterized interactions (see 23,24 as just two such examples). Here we use SPAs to examine the way rRNA tertiary folding is linked to binding of Mg ions, L11-C76 and thiostrepton, and show that all three ligands stabilize essentially the same rRNA tertiary structure.

MATERIALS AND METHODS

L11-C76 protein

L11-C76, which contains the C-terminal 75 residues of the Bacillus stearothermophilus L11 protein, was prepared from an overproducing strain as described (19). L11-C76 (2–5 mg) was first concentrated in a Centricon-3 (Amicon) for 3 h at 7000 r.p.m. and resuspended in 500 µl of storage buffer [20 mM phosphate (pH 7.5), 100 mM KCl]. The protein was then biotinylated using the EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce). A 5-fold molar excess of Sulfo-NHS-LC-biotin was added and incubated on ice for 2 h. Biotinylated protein was purified away from free biotin using a desalting column (Pierce). Protein concentration was determined by BCA assay (Pierce). Percent biotin incorporation was determined using the HABA [2-(4’-hydroxyazobenzene) benzoic acid] assay (Pierce). Incorporation of biotin in the L11-C76 protein was typically 1–2 mol biotin/mol L11-C76. Higher levels of biotinylation were generally inhibitory to binding activity (data not shown). The biotinylated protein concentration was adjusted to 1.5 mg/ml (175 µM) with storage buffer and stored at –80°C.

3P-labeled RNA

3P-labeled RNA was prepared by T7 RNA polymerase transcription of BamHI linearized pLL1 or its BstUI linearized U1061A variant (16) in the presence of [α-33P]UTP (Amersham, 2500 Ci/mmol, 10 mCi/ml) using a MAXIScript™ T7 In Vitro Transcription Kit (Ambion). Transcripts were purified away from unincorporated label by passage over a G-10 spin column. Specific activity of transcripts was ~5.0 × 105 c.p.m./µg.

Scintillation proximity assays

Assays are performed in a 96 well Packard Optiplate. The following protocol details assay components per well. The final concentrations of assay components per well are noted below.

U1061A RNA assays. U1061A RNA (concentration varied, noted in figure legends), L11-C76 (concentration varied, noted in figure legends), 0.02 a.u. tRNA at 260 nm and 120 000 c.p.m. 3P-RNA (~1.4 nM final assay concentration) are added to annealing buffer (30 mM Tris, pH 7.6, 350 mM KCl, 20 mM MgSO4) to a final volume of 15 µl. The mixture is heated for 10 min at 65°C then placed on ice. Biotinylated L11-C76 (concentrations varied, noted in figure legends) is combined with assay buffer (30 mM Tris, pH 7.6/175 mM KCl, 2 mM MgSO4) to a final volume of 35 µl and 15 µl reannealed RNA mixture is added. The reaction is mixed and incubated for 15 min at room temperature (22–26°C). Ten microliters of 50 µg/ml streptavidin coated SPA beads (Amersham) are added and incubation is continued for 10 min at room temperature. Beads are settled by spinning for 5 min at 1000 r.p.m. in a Beckman tabletop centrifuge. Signals are read on a Packard TopCount with the following settings: data mode, DPM; radio-nuclide, 3P-PVT-SPA; count time, 1 min; quench indicator, tSIS; scintillator, liquid/plastic; efficiency mode, normal efficiency; energy range, low energy; region A, 2.90–256.00.

Wild-type E.coli RNA assays. Performed as above except for the addition of 10% DMSO to all buffers other than annealing buffer. Ten percent DMSO was the minimum amount determined to maintain the solubility of the thiostrepton at all concentrations used. Annealing buffer is 30 mM Tris, pH 7.6, 350 mM KCl, 20 mM MgSO4, 0.1% Triton. Assay buffer is 30 mM Tris, pH 7.6/400 mM KCl, 0.1% Triton/10% DMSO.

Data analysis

Kaleidagraph software was used to fit titration curves to single site binding isotherms, allowing both Kd and the extrapolated counts at infinite protein concentration to be variables (25).
Experimental rationale

We begin with the hypothesis that Mg$^{2+}$, thiostrepton and L11 all individually promote folding of the same, unique rRNA tertiary structure. Figure 2A shows the binding scheme that is expected if this assertion is true. The first step, $K_I$, is folding of a specific tertiary structure, which is known to be strongly dependent on Mg$^{2+}$ concentration in this RNA (20,28). In the next two steps, this folded structure may bind L11 (via its C-terminal domain) and thiostrepton, in either order. Finally, interaction of the L11 N-terminal domain further stabilizes the complex. Complex 4 would not normally be observable, as the strong interaction of the L11 N-terminus with thiostrepton would heavily favor formation of complex 5. However, a complex of L11–thiostrepton–RNA, which lacks the L11–thiostrepton interaction, would be equivalent to complex 4. This scheme makes two important predictions: (i) if sufficient Mg$^{2+}$ is added to drive the RNA entirely into the tertiary structure 2, then L11–C76 should be independent of L11-C76; (ii) if the RNA is largely in the unfolded state 1, then addition of thiostrepton should promote folding of the L11 secondary structure and enhance binding of L11–C76, and vice versa. This is the simplest possible form of allostery, in which a ligand drives a macromolecular conformational transition and thereby affects the binding of another ligand at a second site (29).

There are several more complicated binding schemes for which the above two predictions would not hold. Two are important to consider here. (i) Suppose that thiostrepton and L11 bind different RNA conformations, both of which are stabilized by Mg$^{2+}$ (Fig. 2B). In that case, thiostrepton and L11 should compete for binding and complex 4 would be less stable than the individual complexes. However, the ternary L11–thiostrepton–RNA complex 5 could still form because of the additional interaction contributed by the N-terminus. (For simplicity, Figure 2B shows complex 5 in the thiostrepton-bound conformation, since the main interaction with L11 is via its N-terminus and not the C-terminal RNA binding domain. Other conformations could be imagined.) This model predicts that L11-C76 and thiostrepton should show competition (or anticooperativity) in RNA binding (complex 4). (ii) Instead of promoting formation of a tertiary structure encompassing the entire rRNA domain, thiostrepton and L11–C76 could induce local conformational changes (Fig. 2C). Binding of one ligand would then be independent of the other, but, in contrast to the situation in Figure 2A, independent binding would persist even at low Mg$^{2+}$ concentrations that destabilize tertiary structure.

The experiments reported below are consistent with the rRNA fragment adopting a unique tertiary structure, as in Figure 2A, and rule out the schemes in Figure 2B and C. To discuss these binding experiments, we define the necessary equilibrium constants and derive equations that predict L11-C76 binding isotherms at various Mg$^{2+}$ and thiostrepton concentrations.

We start with the equilibrium between a folded RNA with secondary structure (RNA$_S$) and one with tertiary structure (RNA$_T$):

$$RNA_T = RNA_S \quad K_I = [RNA_T][RNA_S]$$

In many RNAs, folding of tertiary structure is very dependent on the concentration of Mg$^{2+}$, and this sensitivity has been demonstrated for the RNA considered here (20,28). We now suppose that L11-C76 (P) and thiostrepton (TS) both bind RNA$_T$:

$$P \cdot RNA_T = P + RNA_T$$

$$TS \cdot RNA_T = TS + RNA_T$$

The last equation assumes that there is no intrinsic interaction between protein and antibiotic when bound to RNA.

To calculate the extent of protein binding under different conditions, we first write a binding polynomial by adding up the concentrations of all the bound and free forms of RNA, and then dividing by [RNA$_T$]:

$$\Sigma = 1 + [P]K_T + [TS]K_{TS} + [P][TS]/K_{TS}$$

Each term on the right gives the concentration of an RNA species relative to the concentration of free RNA$_T$ (the reference state). Appropriate derivatives of the polynomial predict the extent of protein or antibiotic binding in the presence of the other ligand (29,30). Since the assay described below detects protein bound to RNA, the needed derivative is the fractional saturation of RNA with protein:

$$v_p = \partial \ln(\Sigma)/\partial \ln[P] = (([P]/K_T)(1 + [T]/K_{TS}))/\Sigma$$

RESULTS

L11-C76 binding to RNA with stable tertiary structure

SPAs involve the use of a scintillant-filled bead coated with a capture molecule such as streptavidin (26). When a probe labeled with $^3$H, $^{125}$I or $^{33}$P is captured by the bead it brings the
isotope close to the scintillant resulting in light emission. Free probe does not excite the scintillant and does not contribute to the signal. This obviates the need for separation of captured and free probe from a reaction mixture. For the work reported here, L11-C76 is biotinylated to interact with a streptavidin coated SPA bead, and the RNA used is homogeneously labeled with \(^{33}\)P[UTP. The concentration of labeled RNA is insignificant in comparison to that of the added protein, so that the concentration of free protein is approximately equal to the total protein concentration.

To test the reliability of the SPA, we first used a 103 nt fragment of the *E.coli* rRNA sequence, U1061A, in which a single U→A mutation has greatly stabilized the RNA tertiary structure relative to the wild-type *E.coli* sequence (Fig. 1) (27). The SPA assay measures the ability of the biotinylated L11-C76 protein to bind, or capture, the labeled RNA fragment. Recognition and binding of the RNA by the protein requires that the RNA be folded in a favorable conformation. Titration of the RNA with biotinylated L11-C76 gave a strong signal over background (Fig. 3A). Inclusion of tRNA in the reaction buffer did not alter the signal, indicating the binding reaction was specific (data not shown). Based on the recognition of the RNA fragment by the L11-C76 protein, the tertiary structure appears to be fully folded at Mg\(^{2+}\) concentrations ≥ 2 mM. Dissociation constants calculated from these data, 28 ± 3.9 nM at 5 mM Mg\(^{2+}\) and 44 ± 3.4 nM at 2 mM Mg\(^{2+}\), are quite comparable to the 36 ± 5 nM determined by filter binding assay for the same RNA under nearly identical conditions (10 mM MOPS pH 7.0, 3 mM MgCl\(_2\), 175 mM KCl) (28), but using unmodified L11-C76. Biotinylation of the L11-C76 protein (at 1–1.5 biotins per molecule) does not significantly affect the dissociation constant as determined by filter assay (data not shown). A SPA titration done at 0.5 mM Mg\(^{2+}\) does not as cleanly fit a binding isotherm, but clearly indicates that binding is taking place in approximately the same concentration range as the titrations at higher [Mg\(^{2+}\)]. (A fraction of misfolded RNAs may account for the lower extent of binding at 0.5 mM Mg\(^{2+}\), as the RNA renatures to form the tertiary structure very slowly under these conditions.) We conclude from these assays that SPA using biotinylated L11-C76 gives reliable binding constants, and that protein binding to U1061A RNA is not strongly dependent on magnesium ion concentration over the range 0.5–5 mM.

Mg\(^{2+}\) stimulates L11-C76 binding to an RNA with unstable tertiary structure

The *E.coli* rRNA fragment binding L11 has a marginally stable tertiary structure: in melting studies, a distinct tertiary unfolding transition is only observed in the presence of >2 mM Mg\(^{2+}\), and >10 mM Mg\(^{2+}\) is required before the structure is completely formed at room temperature (10). Binding of full-length L11 to this RNA was previously shown to depend strongly on Mg\(^{2+}\) concentration (12). Half-maximal stimulation of protein binding occurred at 0.6 mM Mg\(^{2+}\) when assayed at 0°C; this concentration is lower than that required to observe the presence of tertiary structure in melting experiments presumably because the protein helps drive the RNA into a folded state.

SPA titrations with biotinylated L11-C76 and the *E.coli* rRNA fragment also show a large effect of Mg\(^{2+}\): binding is nearly undetectable at 0.5 mM Mg\(^{2+}\), but is detectable at 2 mM Mg\(^{2+}\) with a dissociation constant of 140 ± 23 nM (Fig. 3B). The titration could not be carried out to high enough protein concentrations to reliably measure a \(K_d\) in 1 mM Mg\(^{2+}\). The \(K_d\) at 2 mM Mg\(^{2+}\) is only 3.2-fold weaker than seen with U1061A RNA, suggesting that the two RNAs are not intrinsically different in their ability to bind L11-C76 as long as sufficient Mg\(^{2+}\) is present to promote folding of the required tertiary structure.

**TS stimulates L11-C76 binding to RNA with unstable tertiary structure**

It has not been possible to quantitate L11 binding in the presence of thiostrepton by a filter binding assay since thiostrepton and L11 both retain RNA fragments on filters (16). Here we have circumvented this problem by developing a more versatile protocol based on SPA technology, and use this assay to ask whether thiostrepton affects L11-C76 binding to the two RNAs. With U1061A RNA, addition of 20 μM thiostrepton had only a marginal effect on the apparent protein affinity (16 ± 1 nM without thiostrepton, 12.2 ± 1 nM with thiostrepton;...
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Fig. 4A). Ten percent DMSO, needed to solubilize thiostrepton, was included in both titrations; this accounts for the tighter affinity measured here than in Figure 3A. Though we do not have an independent measure of thiostrepton interaction with the RNA in the SPA, the antibiotic is known to bind U1061A RNA with an affinity of 1.1 µM under similar buffer conditions as used here (2% DMSO, 30 mM Tris pH 7.6, 175 mM KCl, 11 mM MgSO₄) (16). The weak effect of thiostrepton on L11-C76 binding at this high concentration argues that binding of the two ligands is nearly independent.

When titrating the E.coli RNA fragment with L11-C76 at 0.5 mM Mg²⁺, 20 µM thiostrepton dramatically stimulates L11-C76 binding (Fig. 4B). In the presence of thiostrepton, the apparent Kₐ is 312 ± 48 nM. The titration in the absence of thiostrepton does not reach a high enough level of RNA binding to fit a binding curve, so we cannot quantitate the degree of stimulation.

At 5 mM Mg²⁺, the effect of thiostrepton on L11-C76 binding to E.coli RNA is nearly eliminated (Fig. 4C), with 20 µM thiostrepton decreasing the Kₐ by a factor of only 1.2. This is the expected result if Mg²⁺ stabilizes a conformation recognized by both protein and antibiotic, and if there is no interaction between the two ligands.

Apparent affinity of TS for unfolded RNA

An alternative way to examine the interplay between RNA folding, thiostrepton and L11-C76 is to titrate protein with thiostrepton in the presence of a constant amount of RNA. Under conditions such that the RNA is largely unfolded, the extent of protein–RNA complex formation is then a readout of thiostrepton interaction with RNA. Such a titration of E.coli RNA at low Mg²⁺ concentration (0.5 mM) is shown in Figure 5. As expected, the extent of RNA binding starts at a low value in the absence of thiostrepton and saturates at high antibiotic concentrations (>20 µM). The data have been fit to equation 2 using an estimate of Kₛₐ as 24 nM, the affinity measured in 5 mM Mg²⁺ (Fig. 4C). The fitted value of Kₛₐ is then 29.0 ± 2.3 nM (+ thiostrepton) and 40.2 ± 2.5 nM (– thiostrepton).

Figure 5. Apparent affinity of TS for unfolded E.coli RNA fragment. ³²P-labeled E.coli RNA (no cold RNA added) and increasing amounts of thiostrepton at 0.5 mM Mg²⁺ in the presence of 0.4 µM L11-C76 (constant), 0.02 a.u. tRNA at 260 nm, and 400 mM KCl. Background level has been subtracted from the data points. The curve is best fit using equation 2.
DISCUSSION

We have used a SPA assay to examine the ability of L11-C76 to recognize the tertiary structures of a fragment of the 23S rRNA formed in the presence of various amounts of Mg²⁺ and thiostrepton. When we used the E.coli rRNA variant U1061A in the assay, the binding of L11-C76 was virtually independent of Mg²⁺ concentration over a wide range (Fig. 3A). The K_d of thiostrepton (at 2 mM Mg²⁺) is 44 nM. U1061A has been shown previously to fold into a very stable tertiary structure recognized by L11-C76 (27). When the wild-type E.coli RNA fragment corresponding to U1061A is used, binding of L11-C76 is highly dependent on Mg²⁺ concentration (Fig. 3B). Under relatively high Mg concentrations (2–5 mM) the RNA assumes a conformation recognized by L11-C76, but under lower Mg²⁺ concentrations (<1 mM) binding is essentially eliminated. At 2 mM Mg²⁺ the K_d between the E.coli rRNA sequence and L11-C76 is ~140 nM. The similar K_d values obtained with the intrinsically stable RNA fragment and the E.coli fragment under higher Mg²⁺ conditions demonstrate that Mg²⁺ can induce the E.coli RNA fragment to adopt a tertiary conformation that is recognized by L11-C76 and essentially the same as that of the U1061A variant.

The binding of L11-C76 to U1061A RNA is not affected by thiostrepton (Fig. 4A), although recent work in our laboratory has demonstrated that thiostrepton does interact directly with this RNA (S.Hofstadler, L.B.Blyn, K.Lowery and J.Drader, unpublished results). The lack of competition between L11-C76 and thiostrepton in binding U1061A RNA argues against the unpublished results). The lack of competition between L11-C76 and thiostrepton (Fig. 4A), although recent work in our laboratory of the U1061A variant.

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Our conclusion from these results is that thiostrepton and L11-C76 both recognize essentially the same Mg²⁺-stabilized RNA tertiary structure, in agreement with the model presented in Figure 2A. Under high Mg²⁺ conditions, the two ligands bind nearly independently to the RNA, demonstrating that there is neither an intrinsic cooperative interaction between them nor competition for different RNA conformations. Under low Mg²⁺ conditions, binding of the two ligands is mutually stimulatory because of their binding to the same RNA structure. The RNA structures stabilized by L11, thiostrepton and Mg²⁺ can differ only in very minor ways: the apparent cooperativity factors between L11-C76 and thiostrepton in binding U1061A RNA is only 1.3-fold; L11-C76 binds Mg²⁺ and thiostrepton-stabilized E.coli RNA with K_ds differing by only 2-fold. The 3.2-fold difference between L11-C76 binding Mg²⁺-stabilized E.coli RNA and U1061A RNA, though still very small, could be due to subtle conformational differences between the two RNAs.) A difference of a single stacked or unstacked base in the recognized RNA structures could have caused these ratios to be as large as 10 (29).

We note that our conclusion in favor of the Figure 2A model for thiostrepton and L11-C76 binding argues against the simplest formulations of an ‘RNA-centric’ model of L11 and thiostrepton activity, i.e. there is no evidence that the RNA tertiary structure recognized by L11 switches between alternative conformations. Schemes in which the RNA binding domain of L11 transiently dissociates from the RNA and allows unfolding of the tertiary structure, or in which the N-terminal domain of L11 affects rRNA conformation elsewhere in the ribosome, have not been ruled out.

The information and assays described in this work will aid in the discovery of new types of antibiotics that function at the same site of action as thiostrepton. Solubility and other issues make thiostrepton a relatively poor commercial antibiotic that is mainly used in topical applications. The discovery of new antibiotics that function at the same site as thiostrepton, but are substantially different in nature would be highly valuable. The results described here demonstrate that the RNA structure recognized by thiostrepton and by L11 are fundamentally the same. The recent reports of the L11/23S rRNA fragment crystal structure provide an opportunity to model thiostrepton towards the effort to design and discover new drugs that bind and function in the same way. The assays described here also provide a mechanism for the high throughput screening of drug candidates for the ability to affect L11/rRNA binding and for their ability to stabilize RNA structure as in the case of thiostrepton.

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