Interactions of the N-terminal Domain of Ribosomal Protein L11 with Thiostrepton and rRNA

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Running title: rRNA complexes with L11 and thiostrepton

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Ribosomal protein L11 has two domains: the C-terminal domain (L11-C76) binds rRNA while the N-terminal domain (L11-NTD) may variously interact with elongation factor G, the antibiotic thiostrepton, and rRNA. To begin to quantitate these interactions, L11 from Bacillus stearothermophilus has been overexpressed and its properties compared with those of L11-C76 alone in a fluorescence assay for protein – rRNA binding. The assay relies on 2’amino-butyryl-pyrene-uridine incorporated in a 58 nt rRNA fragment, which gives ~15 fold enhancement when L11 or L11-C76 is bound. Although the pyrene tag weakens protein binding, unbiased protein – RNA association constants were obtained in competition experiments with untagged RNA. It was found that (i) intact Bst L11 binds rRNA with $K \approx 1.2 \times 10^9 \text{ M}^{-1}$ in buffers with 0.2 M KCl, about 100 fold tighter than E. coli L11; (ii) the N-terminal domain makes a small, salt-dependent contribution to the overall L11 – RNA binding affinity (~8 fold enhancement at 0.2 M KCl), (iii) L11 stimulates thiostrepton binding by 2.3 $\pm$ 0.6 x $10^3$ fold, predicting an overall thiostrepton affinity for the ribosome of $\sim 10^9 \text{ M}^{-1}$, and (iv) the yeast homolog of L11 shows no stimulation of thiostrepton binding. The latter observation resolves the question of why eukaryotes are insensitive to the antibiotic. These measurements also show that it is plausible for thiostrepton to compete directly with EF-G•GDP for binding to the L11-rRNA complex, and provide a quantitative basis for further studies of L11 function and thiostrepton mechanism.

Most ribosomal proteins cannot be assigned a specific functional role, beyond their potential for contributing to the stability and proper assembly of rRNA structures (1-3). L11 is unusual in this regard, in that functional roles have been hypothesized for each of its two domains. L11 is associated with a highly conserved 58 nucleotide domain of large subunit ribosomal RNA (referred to here as the L11 binding domain RNA, L11 BD RNA) which constitutes a “knob” on the surface of the large subunit (4-7). A C-terminal domain of the protein, L11-C76, sits in the distorted minor groove of the L11 BD RNA (Figure 1), binds the folded RNA with high affinity (8,9), and in E. coli ribosomes, is essential for the RNA to form its native tertiary structure (10). Biochemical evidence and direct observation by cryo-electron microscopy have suggested that the N-terminal domain of L11 (L11-NTD) occupies different positions during the ribosome cycle and variously contacts elongation factor G (EF-G) (11), release factors 1 and 2 (12-14), and stringent factor (15). E. coli cells lacking L11-NTD (i.e., having only L11-C76) grow two fold more slowly than wild type (13). It thus appears that both domains of L11 are important for optimum ribosome function, one domain stabilizing a necessary tertiary structure and the other promoting the function of protein factors.

The functional importance of the L11-RNA region of the ribosome is also suggested by the fact that it is a target for the thiazole class of antibiotics. The best-studied of these, thiostrepton, binds to ribosomal RNA fragments with affinities on the order of $10^6 \text{ M}^{-1}$ (6,16), although its affinity for rRNA is greatly stimulated when the N-terminal domain of L11 is present (17). Structure probing and NMR studies place thiostrepton near the hairpin loop containing A1067 (Figure 1), but structural details of the thiostrepton-L11-rRNA complex are unresolved (10,18,19). Whether thiostrepton inhibits translation by competing with EF-G for ribosome binding or by stabilizing an EF-G - ribosome complex is also unresolved; there is experimental support for each model (20-22). An additional intriguing question is why eukaryotic ribosomes...
are insensitive to thiostrepton, an observation which has variously been attributed to rRNA sequence differences (23) or occlusion of the ribosome thiostrepton binding site by the eukaryotic L11 homolog (24).

To enable quantitative studies of the roles of L11 in ribosome function, we have begun to characterize N-terminal domain interactions with ribosome components. Here we describe the development of a fluorescence assay that allows accurate measurement of RNA affinities for L11 or L11-C76 under a wide variety of solution conditions and in the presence or absence of thiostrepton. Using this assay, we show that the bacterial L11-NTD contributes a small, salt-dependent free energy to L11 - rRNA binding affinities, and enhances thiostrepton – rRNA interactions by factors of more than 10^3. This cooperative interaction does not take place with a eukaryotic L11 homolog, which accounts for the resistance of eukaryotic ribosomes to the drug.

**Experimental Procedures**

**Buffers and Materials.** The standard buffer for the various titration experiments was 10 mM MOPS pH 7.0, 5 mM MgCl₂, 5% DMSO and the indicated concentration of KCl in mM; e.g., M5K200D contains 200 mM KCl. 1 mM DTT was also present in all titrations that included L11. Thiostrepton was purchased from CalBiochem. Stock solutions were made in DMSO using an extinction coefficient of 0.027 cm⁻¹µM⁻¹ at 280 nm (25). To obtain reproducible titration data, solutions were made fresh on the day of use. The identity of the sample was confirmed by electrospray ionization mass spectrometry in positive mode.

**Cloning and Purification of Bacillus stearothermophilus L11.** Bst L11 was cloned from genomic DNA in two steps. First, the portion of the Bst genome containing the conserved gene cluster *rpmG* - *secE* – *nusG* – *rplK* - *rplA* was PCR-amplified and cloned. The following DNA oligomers were designed and synthesized: GT(G,C) AA(C,T) AT(C,T) AC(A,C,G) (C,T)TG GC(G,C) TG(C,T) AC(A,C,G) GA(A,G) TG(C,T) GG, on the basis of a conserved amino acid sequence of L33 (*rpmG*), and AAA CGT GAC CGT GCG GGT TTT CGG, based on a known segment of *rplA* (W. Liu & D.E.D., unpublished observations). 30 rounds of PCR were performed using Taq polymerase. The PCR product was purified by extraction from a 1% agarose gel and ligated into TOPO TA plasmid (Invitrogen). The L11 gene was sequenced from the TOPO plasmid and a PCR fragment containing the L11 coding sequence was cloned into a T7 promoter-based expression plasmid, pMFT, using Nde I and Bam HI restriction sites (26).

To purify Bst L11, a single colony of BL21(DE3) transformed with the pMFT L11 expression plasmid was grown to mid-log phase in 1 liter of broth, induced with 1 mM IPTG, and harvested four hours later. The cells were resuspended in 20 mls lysis buffer (50 mM Tris pH 7.6, 1 mM DTT, 1 mM EDTA, with 100 µM phenylmethylsulfonyl fluoride and 100 µM benzamidine added as protease inhibitors) and lysed by three passes through a French Press at 750 psi. L11 was found in the supernatant after pelleting cell debris. The supernatant was dialyzed against 10 mM potassium phosphate pH 6.0, 1 mM DTT, 0.1 mM benzamidine and 0.1 mM PMSF at 4 °C. The dialyzed protein was loaded onto a sulfopropyl cation exchange HPLC column (21.5 x 150 mm TSK SP-5PW; Toyohaas) and eluted with a 500 mL gradient of 0-200 mM KCl in dialysis buffer without the protease inhibitors. Protein was dialyzed into 10 mM Tris pH 7.6, 3 mM MgCl₂, 50 mM KCl, 1 mM DTT and stored at ~80 °C. A single cysteine in the L11 N-terminal domain causes dimerization of the protein if adequate concentrations of a reducing agent are not present. The overall yield was ~30 mg protein from 1 liter of cell culture; no impurities could be detected on a heavily overloaded SDS gel.

Protein concentration was determined by absorbance at 230 nm using an extinction coefficient of 45 x 10⁻³ M⁻¹ cm⁻¹, based on an average extinction per peptide bond (27) and on quantitative nitrogen analysis (AAA Service Laboratory, Inc., Boring, OR).

The C-terminal RNA binding domain of Bst L11, L11-C76, was purified as described (17). **Cloning and Purification of Saccharomyces cerevisiae L11 homolog.** The *Saccharomyces cerevisiae* gene *rpl12A* was amplified from yeast DNA by PCR and cloned into the inducible expression vector pET11a (Novagen). Upon transformation into BL21(DE3) cells and
induction of T7 RNA polymerase with IPTG, the protein was expressed at moderate levels but remained insoluble. The crude protein was redissolved in buffers containing 6 M urea, 20 mM potassium phosphate (pH 6.5) and 5 mM DTT, and purified by cation exchange chromatography as described for Bst L11, with the exceptions that the salt gradient was run from 0 to 400 mM KCl and the ion exchange buffers contained 6 M urea.

Purified protein was stored at –80 °C in the same buffer used for redissolving the protein from cell lysates. Before use in RNA binding experiments, it was diluted 10 fold with the buffer of interest and warmed at 37 °C for 30 min. The protein is fully active in binding its rRNA target; a more detailed account of its rRNA binding properties will be reported elsewhere (E.P. and D.E.D., ms. in preparation). The naming of ribosomal proteins is not consistent between bacteria and eukaryotes. To avoid confusion with the acidic ribosomal protein also called L12, here we call the S. cerevisiae L11 homolog yL11.

**Fluorescence titrations.** An RNA fragment based on nucleotides 1051-1108 of the E. coli 23S rRNA sequence was synthesized and purified by Dharmacon Research, Inc. (Lafayette, CO). The RNA sequence contained the stabilizing mutation U1061A (28) and a modified nucleoside, 2'-amino-butyryl-pyrene-uridine, at U1082 (py-U1082 RNA). Unlabelled RNA of the same sequence (U1061A RNA) was transcribed from a plasmid DNA template by phage T7 RNA polymerase and purified by gel electrophoresis as described (29). Before use, RNAs were heated in renaturation buffer (10 mM MOPS pH 7.0, 20 mM MgCl2, 175 mM KCl) at 65 °C for 5 minutes and then cooled to 25 °C for 15 minutes.

Buffers used for fluorescence experiments contained 10 mM MOPS pH 7.0, various concentrations of MgCl2 and KCl, 1 mM DTT, 5% DMSO and 0.4 µM ovalbumin (Sigma). Ovalbumin was necessary to prevent loss of L11 or L11-C76 on the cuvette walls. Measurements were performed on an Aviv Instruments ATF105 fluorimeter. Pyrene fluorescence was monitored with an excitation wavelength of 345 nm and an emission of 395 nm. All titrations reported here were conducted at 25 °C in 1 cm x 1 cm cuvettes. For binding studies with only L11 or L11-C76, the sample was incubated with stirring for 10 minutes between protein additions. Fluorescence binding data were fit to hyperbolic binding isotherms with the equilibrium constant and fluorescence intensity of the protein-RNA complex as variables (30).

For binding studies with L11 and thiostrepton (THS), a fixed concentration of THS, usually 7 nM, was present during the titration. For the first data points, the increase in fluorescence intensity was monitored for 30 minutes after addition of protein. After the RNA became ~50% saturated with protein, this time was reduced to 15 min. The time course of fluorescence increase was fit to a first order rate equation; each fluorescence intensity point on the equilibrium binding curve was calculated from an extrapolated of the rate equation to infinite time. Thiostrepton alone had no effect on py-U1082 RNA fluorescence in control titrations.

In the course of these titrations, three complexes between RNA, thiostrepton, and L11 are potentially formed, as shown by the Scheme I and II equilibria in Results. The polynomial expression that relates the concentrations of these different species to equilibrium constants and initial conditions cannot be solved for the fraction of RNA bound to L11 as a function of added protein. To generate equilibrium binding curves for comparison with titration data, a quadratic equation derived from the mass action and mass conservation equations was solved to give the free concentration of thiostrepton as a function of the equilibrium constants, total concentrations of thiostrepton and RNA, and various free concentrations of L11. The corresponding free concentrations of thiostrepton and L11 were then substituted in a binding polynomial describing all possible complexes of free and bound RNA, from which the fraction of RNA bound to L11 (with or without thiostrepton) was calculated. This latter value was then used to find the total L11 concentration supporting the calculated extent of RNA binding. These calculations were conveniently carried out in a spreadsheet on which a range of free L11 concentrations could be chosen to generate a binding curve over the appropriate range of total L11 concentrations for a particular titration.

The low solubility of thiostrepton prompted us to measure the maximum concentration that could be achieved under our experimental conditions. To do this, a concentrated stock of thiostrepton in...
DMSO was diluted with stirring into M5K200D buffer (5% final DMSO concentration) to a nominal concentration of 20 µM. Although the solution appeared clear to the eye, light scattering was detectable in the spectrometer or fluorimeter. After stirring for 2 hours at room temperature, the solution was centrifuged. The absorbance of the supernatant indicated that 5 µM of thiostrepton remained soluble.

Salt-back titrations and data analysis. The salt dependence of either L11 or L11-C76 affinities for py-U1082 RNA was determined by salt-back titrations, in which the pre-formed protein – RNA complex was titrated with KCl while keeping other buffer components constant. The fluorescence at each point defines the binding constant at that salt concentration, provided that the fluorescence intensities of the protein-RNA complex and the unbound RNA, which may vary with salt concentration, are known. Control titrations of py-U1082 RNA alone showed that there is a negligible change in the pyrene fluorescence as salt is added. To correct for potential salt dependence of the protein-RNA fluorescence signal, salt-back titrations done at two or three protein : RNA ratios were analyzed simultaneously (31). In all cases, all the curves were fit with the same salt dependence when the protein-RNA fluorescence was assumed to be independent of salt concentration.

CD and UV Experiments. Circular dichroism (CD) spectra were obtained on a Jasco J-810, using 22 µM L11-C76 or 36 µM L11 protein in 10 mM potassium phosphate buffer, pH 7.0, 175 mM KCl, and 1 mM DTT.

Melting experiments were performed on a Cary 400 spectrophotometer at a heating rate of 0.8 °C/minute and RNA concentration of ~1 µM as previously described (29,32). Py-U1082 RNA or U1061A RNA were renatured before use as described above for fluorescence titrations. 1µM L11-C76 was included in some experiments. The buffer for all melting experiments was 10 mM MOPS pH 7.0, 3 mM MgCl₂, 100 mM KCl.

Results

Cloning and Purification of L11. The L11 gene from Bacillus stearothermophilus (Bst) was previously cloned and expressed (17) based on a sequence of ribosome-purified protein reported by the Wittman-Liebold laboratory (33). In alignments of eubacterial L11 homologs, this protein was unique in its lack of approximately eight amino acids at the N-terminus. To see whether this was an unusual feature of the Bst L11 protein or an error in protein sequencing, a Bst chromosomal DNA fragment containing the L11 gene (rplK) was cloned and sequenced; the gene in fact encodes eight N-terminal amino acids not present in the originally reported amino acid sequence (see Experimental Procedures). It is possible that truncation of the previously reported sequence was an artifact of protease contamination, a problem if protease inhibitors are not present during L11 purification.

The full length Bst L11 was easily overexpressed and purified without the use of denaturing conditions previously required to obtain the truncated protein. The L11 CD spectrum showed a significant increase in β-sheet content, as compared to the protein missing the eight N-terminal amino acids (data not shown). Consistent with this observation, NMR studies of Thermus thermophilus L11 have suggested that there are three segments of extended strand conformation in the N-terminal domain, including residues 4-12 (34). The crystal structure of Thermotoga maritima L11 also shows a three-strand β-sheet at about the same positions, though residues 1-7 are unresolved (35). It is plausible that the eight residue truncation present in the previously described Bst L11 substantially destabilized the β-sheet structure of this domain.

Characterization of Pyrene-labeled L11 BD RNA. The filter binding assay previously used to measure L11-RNA binding affinities cannot be used to detect L11 binding in the presence of thiostrepton, since thiostrepton itself retains the RNA fragment on filters (6). To develop an alternative assay, 2′amino-butyryl-pyrene-uridine was introduced at position U1082 of the L11 BD RNA fragment (Figure 1); this tagged 58 nt RNA fragment is called py-U1082 RNA. U1082 was chosen because it is close to an α-helix in the C terminal domain of RNA-bound L11 (Figure 1B) (8).

Py-U1082 RNA is a derivative of the U1061A variant of the E. coli 23S rRNA, which has an unusually stable tertiary structure (8,28). Py-U1082 RNA and U1061A RNA were compared in
UV melting experiments to determine whether the pyrene tag disrupts the RNA structure. The unfolding of tertiary structure in this RNA is characterized by a transition showing hyperchromicity at 260 nm but not at 280 nm (28). Tertiary structure in the U1061A variant is very stable and unfolds at high temperature simultaneously with some of the secondary structure (Figure 2A). The pyrene tag has clearly destabilized the RNA: the melting profile of py-U1082 RNA is much broader than that of U1061A RNA, and a comparison of the 260 and 280 nm melting profiles gives no evidence for the presence of a tertiary unfolding transition (Figure 2A). However, melting of py-U1082 RNA in the presence of L11-C76 shows a transition at about 50 °C with the expected ratio of hyperchromicities at 260 and 280 nm (Figure 2B). In agreement with previous experiments (17), the U1061A RNA tertiary transition was also stabilized by L11-C76, though to a higher temperature than the L11-C76 - py-1082U RNA complex (Figure 2B). It thus appears that the pyrene tag destabilizes the RNA tertiary structure, but that L11-C76 can nevertheless bind the tagged RNA and induce correct folding of the tertiary structure. Specific L11-C76 binding was not observed when the pyrene tag was placed in the wild type E. coli sequence, which has a much less stable tertiary structure than the U1061A variant (data not shown). Presumably this RNA was too drastically misfolded or destabilized for the protein to bind well.

**L11 and L11-C76 Binding to py-U1082 RNA.** Representative titrations of py-U1082 RNA with L11 or L11-C76 are shown in Figure 3. An approximately 15 fold enhancement in the intensity of pyrene fluorescence is seen with either protein. Competition experiments were performed to determine the degree to which the pyrene tag perturbs L11 or L11-C76 binding to the 1051-1108 rRNA fragment. Titrations of equal concentrations of U1061A RNA and py-U1082 RNA with the proteins are shown in Figure 4 for buffer containing 700 mM KCl; similar experiments were also done at 200 mM KCl (Table 1). A lag at the beginning of the titration curves indicates preferential binding of the proteins to unlabeled U1061A RNA. The titrations in buffer containing 700 mM KCl were fit by theoretical curves which assume eight fold greater protein affinity for U1061A RNA over py-U1082 RNA (see Experimental Procedures). At lower salt the preference of the proteins for unlabeled RNA is ~20 fold. Because the pyrene tag destabilizes the RNA tertiary structure that is recognized by L11, weaker protein binding to py-U1082 RNA was expected. The diminished effect of the tag at higher salt concentration is consistent with salt stabilizing the correctly folded RNA structure. The fact that L11 and L11-C76 binding affinities are weakened to the same degree suggests that the tag does not affect any interactions of the L11 N-terminal domain with RNA.

Thiostrepton, which also stabilizes U1061A RNA tertiary structure (36), enhances py-1082 RNA fluorescence at least several fold (data not shown), even though the presumed thiostrepton binding site is distant from the U1082 tag. It is possible that the tag is more sensitive to RNA folding than to L11 proximity. We have been unable to make a definitive measurement of the fluorescence enhancement induced by thiostrepton, as its affinity for py-1082 RNA is so weak that its maximum solubility, ~5 µM in M5K200D buffer, is too low to permit reliable extrapolation of binding curves to complete saturation.

The binding constants for L11 or L11-C76 binding py-U1082 and U1061A RNAs at different salt concentrations are listed in Table 1. The N-terminal domain of L11 clearly enhances protein binding in a salt-dependent manner, a small effect not seen in studies with Bst L11 missing the first 8 amino acids (17).

**Salt dependences of L11 and L11-C76 binding RNA.** The salt dependence of L11-C76 and L11 binding RNA were measured by “salt-back” titrations (37). Py-U1082 RNA in buffer containing 0.2 M KCl was first titrated with either L11 or L11-C76 to 80 - 90% saturation. The complex was then titrated with KCl, keeping all other buffer components constant (Figure 5). The resulting data were compared to curves in which the only variable is the dependence of the binding constant on salt concentration, $\frac{\partial \log(K_a)}{\partial \log[KCl]}$ (31) (see Experimental Procedures). The binding constants calculated by this method at a given salt concentrations are within error of affinities derived from protein – RNA titrations carried out at constant salt concentration.
For L11-C76 binding py-U1082 RNA, ∂log(Ka)/∂log[KCl] is -1.8 ± 0.1. U1061A RNA competes for protein binding against py-U1082 RNA more effectively at lower salt concentrations, as shown in the preceding section. From these measurements, we estimate that the salt dependence for L11-C76 binding the unmodified U1061A RNA is 0.7 more negative than for py-U1082U RNA, i.e. the salt dependence for L11-C76 binding U1061A RNA is approximately -2.5. This is significantly more negative than the value of -1.3 determined by a filter binding assay in a buffer lacking DMSO (38). This difference from previous measurements is considered further in the Discussion.

The salt dependence of L11 binding py-U1082 RNA is -2.3 ± 0.1 (Figure 5B). At salt concentrations greater than ~1 M KCl, the apparent binding constants measured for L11 are larger than predicted by a simple linear dependence of log(Ka) on log[KCl]; the same is not true for L11-C76 (data not shown). There are a variety of reasons why a simple log-log dependence might not extrapolate to extremely high salt concentrations, for instance salt-induced conformational changes in the protein might promote different contacts with the RNA. To avoid possible complications from these kinds of effects, data taken between 1 and 1.4 M KCl have not been used in fitting the salt dependence curves of either L11 or L11-C76.

The salt-back titrations show that the L11 – py-U1082 RNA salt dependence is steeper than that of L11-C76 by 0.5. This increased sensitivity to salt is consistent with the different ratios of binding constants measured for the two proteins at 200 and 700 mM KCl (Table 1); L11 binds nearly 8 fold more tightly than L11-C76 at the lower salt concentration, and only 2.5 fold more tightly at the higher salt. It thus seems likely that there is a net favorable interaction between basic residues within the L11-NTD and the RNA.

**Cooperativity between L11 and Thiostrepton.**

Individual interactions of L11 and thiostrepton (THS) to rRNA (R) are defined by the following equilibria:

\[
K_{L11} = \frac{[R][L11]}{[R\cdotL11]} \\
K_{THS} = \frac{[R][THS]}{[R\cdotTHS]} \\
K_{app} = \frac{K_{L11}[(1+\omega K_{THS}[THS])/(1+K_{THS}[THS])]}{(1+K_{THS}[THS])} \\
\omega K_{L11} = \frac{\omega K_{L11} + \omega K_{THS}[THS]}{\omega K_{THS}[THS]} \\
\omega = \frac{\omega K_{L11} + \omega K_{THS}[THS]}{\omega K_{THS}[THS]} \\
\omega = \frac{\omega K_{L11} + \omega K_{THS}[THS]}{\omega K_{THS}[THS]} \\
\omega = \frac{\omega K_{L11} + \omega K_{THS}[THS]}{\omega K_{THS}[THS]}
\]

Either of these complexes can react further to form the ternary rRNA-thiostrepton-L11 complex:

\[
R\cdotL11 + THS \rightleftharpoons R\cdotL11\cdotTHS
\]

(Scheme II)

The cooperativity factor \(\omega\) is included to account for the possibility that thiostrepton affinity for the L11-RNA complex might be different than its affinity for the RNA alone. (Microscopic reversibility requires that L11 affinity for a thiostrepton-RNA complex be altered by the same factor \(\omega\).) Thus our strategy for detecting cooperative interactions between L11 and thiostrepton will be to compare the apparent affinity of L11 for RNA in the presence and absence of thiostrepton.

From the above definitions of equilibrium constant, it can be shown that titration of py-U1082 RNA with L11 in the presence of excess thiostrepton would give an apparent binding constant

\[
K_{app} = K_{L11}[(1+\omega K_{THS}[THS])/(1+K_{THS}[THS])]
\]

([THS] is the concentration of free thiostrepton, which will be approximately constant during a titration if the total amount of thiostrepton is in large excess over the RNA.) \(K_{THS}\) has been measured by two different methods to be 0.6 µM⁻¹, independent of KCl or MgCl₂ concentration (6,39), and \(\omega\) is on the order of 10³ (see below). \(K_{L11}\) is nearly 10⁹ M⁻¹ in buffers containing 200 mM KCl (Table 1); thus the above equation predicts an apparent binding constant on the order of 10¹¹ M⁻¹ in the presence of 0.1 µM thiostrepton. This is about two orders of magnitude larger than conveniently measureable with the py-U1082 RNA assay, which requires a minimum py-U1082 RNA concentration > 1 nM. We therefore carried out titrations at a series of salt concentrations greater than 0.5 M to reduce the L11-RNA affinity, and also used a low concentration of thiostrepton (7 nM) comparable to that of the py-U1082 RNA (5.6 nM).

An additional problem in analysis of L11:py-U1082U RNA titrations in the presence of thiostrepton was the finding that very long times were required to reach equilibrium, on the order of several hours for the first few titration points. The
approach to equilibrium was found to follow first order kinetics (Figure 6A). Thus, each titration point in an equilibrium binding curve was derived from a series of fluorescence readings extrapolated to infinite time (Figure 6B). Kinetic measurements made with various combinations of L11, L11-C76, and thiostrepton binding to py-1082 RNA suggest that RNA folding becomes rate limiting at saturating protein and thiostrepton concentrations; a fraction of the RNA binds rapidly (<1 min) but there are also very slow components (data not shown). Multiphasic kinetics are common for RNA folding (40); further analysis of this phenomenon is beyond the scope of this paper.

The equation for $K_{\text{app}}$ could not be used to extract $\omega$ from Figure 6B binding data, because thiostrepton was not in excess over the RNA. We therefore resorted to computer simulation of the equilibrium binding curves using ranges of $\omega$ values, as described in Experimental Procedures, for comparison with the binding data. Using this approach, $\omega$ was determined at three different salt concentrations (Figure 6B and Table 1). The average of seven measurements is $\omega = 2300 \pm 600$; no trend in $\omega$ with salt concentration is apparent within the error of the experiment.

**L11-C76 Interactions with Thiostrepton**. Previous studies have suggested that the L11 N-terminal domain mediates the cooperative binding of thiostrepton to rRNA (17). To verify this finding in the present system, the binding of L11-C76 to py-U1082 RNA was measured with 185 nM thiostrepton present in buffer containing 700 mM KCl (Figure 7A). A slight enhancement of L11-C76 binding is seen, though nearly within the error of the experiment; $\omega$ calculated from equation (1) is 2.5. Thiostrepton concentrations on the order of 10 $\mu$M have been observed to stimulate L11-C76 binding to rRNA fragments under conditions in which the rRNA tertiary structure is unstable (36); the high thiostrepton concentrations promote RNA folding into the structure recognized by L11-C76. The same phenomenon could account for the weak stimulation of L11-C76 binding by the much lower concentrations of thiostrepton used here, since py-U1082 RNA tertiary structure is similarly unstable. It appears that the protein N-terminal domain is responsible for essentially all of the L11-thiostrepton binding cooperativity observed in the experiments reported here.

*A eukaryotic L11 homolog does not stimulate thiostrepton – RNA binding.* It has been reported that yeast ribosomes are completely insensitive to thiostrepton, even though yeast ribosomal RNA binds thiostrepton (24). To see if cooperative L11 – thiostrepton interactions have been conserved in eukaryotes, the yeast homolog of rplK was cloned and overexpressed; we refer to this protein as yL11 (see Experimental Procedures). The protein itself is much less stable than the thermophilic Bst L11; consequently 15% glycerol has been included in all buffers to stabilize the protein and prevent aggregation. Titrations of py-U1082 RNA with yL11 under conditions otherwise similar to those used with Bst L11 showed binding constants of $\sim 40 \mu M^{-1}$ (Figure 7B), weaker than seen with Bst L11 at the same salt concentration but comparable to the affinity of *E. coli* L11 for *E. coli* rRNA (5). Inclusion of a relatively high concentration of thiostrepton in the titration had essentially no effect on the observed binding isotherm (Figure 7B). As a control for the possibility that yL11:thioistrepton cooperativity is only manifested when the RNA is unmodified or contains base changes conserved in eukaryotes, equal concentrations of py-1082U RNA and the 58mer RNA domain from a eukaryote were titrated in the presence and absence of thiostrepton. No effect of thiostrepton on yL11 affinity for the eukaryotic competitor RNA was observed (data not shown).

**Discussion**

*A fluorescence assay for L11-RNA Interaction.* The results presented here introduce a new fluorescence method for measuring L11-RNA binding affinities. The assay was developed primarily for the purpose of studying cooperative interactions between L11 and other ribosomal components and antibiotics. The disadvantage of a binding assay based on a fluorescence tag is that the tag may perturb the interaction being examined. The py-1082 location of the tag used here was chosen with the anticipation that its proximity to bound L11 would induce a change in fluorescence intensity. However, an equally likely explanation is that the pyrene fluorescence is quenched by stacking with bases in a partially unfolded form of the RNA. This would account for both tertiary structure destabilization and the
fluorescence enhancement induced by ligands. Similar behavior of pyrene tags has been seen in other nucleic acid systems (41,42). The weaker protein affinity for the py-U1082 RNA has actually been an advantage for measuring the large enhancement of protein binding in the presence of thiostrepton, and in any case affinities for unmodified RNAs can be obtained from competition experiments.

Bst L11 – RNA binding affinity. The Bst L11-rRNA affinity reported here, \( \sim 1.2 \times 10^9 \text{ M}^{-1} \) in M5K200D buffer, is about two orders of magnitude tighter than reported for \( E. coli \) L11 under roughly comparable conditions (5,6). We have found similar, \( \sim 100 \) fold differences in the affinities of \( E. coli \) and Bst ribosomal protein – rRNA complexes in two other cases, S4 (43) and L3 (U. Sengupta & D.E.D., unpublished data). A systematic study of ribosomal protein S8 homologs from meso-, thermo-, and hyperthermophilic sources also found a 100 fold range in rRNA binding affinities that correlated with growth temperature (44). Whether the higher RNA affinity of thermophilic proteins is entirely due to a more stable protein fold or also to a more favorable set of protein-RNA contacts has not been examined.

The salt dependence of the L11-C76 protein binding RNA as measured by the fluorescence assay, \(-2.5\), is much steeper than previously reported using filter binding assays, \(-1.3\) (38). This difference is not due to the presence of DMSO in the buffers used for the fluorescence assay (data not shown). We have found that L11 (and other basic ribosomal proteins) avidly bind to quartz and plastic, even after treatment of the surfaces with siliconizing agents; the problem is more severe in low salt buffers. Inclusion of micromolar concentrations of ovalbumin reduces the problem, as evidenced by more stable fluorescence signals with time and tighter apparent protein-RNA binding affinities. We believe the L11-C76 salt dependence measured by filter binding assays was biased by an underestimation of the protein binding affinity at lower salt concentrations, and that \(-2.5\) is a more accurate value. The conclusions of the previous study were based on relative salt dependences, and are unaffected by the underestimate of the binding constants.

**Participation of the L11-NTD in rRNA and thiostrepton binding.** A comparison of L11 and L11-C76 binding affinities for py-U1082 RNA and U1061A RNA shows that the N-terminal domain contributes to the overall binding affinity. The contribution is approximately \(-1.2 \text{ kcal/mol} \) at \( 0.2 \text{ M KCl} \); from the measured salt dependences, this number extrapolates to \(-0.7 \text{ kcal/mol} \) at \( 1 \text{ M} \) salt. These are small contributions compared to the overall \(-12.4 \text{ kcal/mol} \) binding free energy for L11-C76 binding rRNA in buffer with \( 0.2 \text{ M KCl} \). Such weak protein-RNA interactions may take place simply because the N-terminal domain is constrained near the rRNA surface.

In a crystal structure of *Thermatoga maritima* L11 bound to its cognate 58 nt rRNA recognition site, only one of the two complexes in the unit cell had resolvable electron density for the L11-NTD (35). Though the domain is close to the RNA surface, it is in position to make only a single hydrogen bond. Two lysine residues are within \( \sim 5 \text{ Å} \) of phosphate oxygens, which could potentially account for the increased salt dependence of L11 over L11-C76 in binding RNA. The paucity of RNA contacts with the N-terminal domain and the disordered state of the domain in the second unit cell complex are consistent with weak, non-specific RNA-protein interactions. The different positioning of the N-terminal domain in different ribosomes, as observed by cryo-electron microscopy and X-ray crystallography under different conditions, also suggests that the L11-NTD interactions with RNA are very weak, if not non-specific (11).

We observe a strong cooperative interaction of thiostrepton with the L11-NTD, such that thiostrepton binding to RNA saturated with L11 should show an affinity of \( \sim 10^9 \text{ M}^{-1} \). Tight binding was expected from the fact that thiostrepton binds stoichiometrically to micromolar concentrations of ribosomes or L11-23S rRNA complexes (45,46). The most likely origin of the cooperativity is direct interaction between the L11-NTD and thiostrepton, as suggested by a plausible structural model in which thiostrepton contacts both protein and RNA residues that are mutated in thiostrepton-resistant bacterial strains (19).

It has long been known that eukaryotic ribosomes are insensitive to thiostrepton (24,47). All bacteria have A at position 1067; 2'-O
methylation of this residue renders ribosomes resistant to thiazole antibiotics (48). 1067 is conserved as G in all eukaryotes, and an A1067G mutation in bacterial rRNA decreases its affinity for thiostrepton; conversely, a G1067A mutation in eukaryotic rRNA enhances thiostrepton binding (23). Thus the identity of position 1067 has been suggested to be a primary determinant of ribosome sensitivity to thiostrepton. However, bacterial ribosomes carrying G1067 are not thiostrepton resistant, which led to the suggestion that yeast ribosome resistance is due to occlusion of the thiostrepton rRNA site by L11 or other yeast ribosomal proteins (24). In the present study, we show that yeast L11 does not stimulate thiostrepton binding to rRNA, in the context of either a bacterial or eukaryotic rRNA sequence. Though weaker intrinsic affinity of thiostrepton for eukaryotic rRNA may certainly contribute to thiostrepton resistance, the lack of thiostrepton interaction with the eukaryotic L11-NTD is likely to render thiostrepton ineffective, even if it is bound to the ribosome (see the discussion of thiostrepton mechanism, below).

It is also noteworthy that the N-terminal domains of bacterial and eukaryotic L11 homologs have weak sequence homology, and that eukaryotic L11 is more effective than bacterial L11 in stimulating the activity of the eukaryotic translocation factor, EF-2 (49). Thus the specificity of thiostrepton for bacterial ribosomes may lie in its targeting a region of the L11-NTD that is divergent between bacteria and eukarya.

Implications for thiostrepton mechanism. As mentioned in the Introduction, the L11-NTD probably interacts with several factors that bind the ribosome. In particular, cryo-electron microscopy studies of EF-G - ribosome complexes show L11-NTD close to the A1067 hairpin loop before GTP hydrolysis, and displaced from this position by insertion of EF-G domain V between the L11-NTD and the RNA surface after GTP hydrolysis (11). The EF-G•GDP complex in these studies was stabilized on the ribosome by fusidic acid; similar complexes are known to protect the A1067 hairpin loop from reaction with chemical reagents (10,50,51). Insertion of thiostrepton between the L11-NTD and rRNA could thus sterically block EF-G interactions with the L11 BD region. The affinity of EF-G•GDP complex for ribosomes is on the order of 10^7 M^-1 (52), about two orders of magnitude weaker than the thiostrepton affinity for L11-rRNA complexes as measured in the present work. Therefore, under typical experimental conditions for in vitro translation (~ 1 µM ribosomes and a small excess of EF-G), amounts of thiostrepton stoichiometric with ribosomes have the potential to displace EF-G•GDP from the ribosome. In fact, apparent competition between the EF-G•GDP complex and thiostrepton for binding to ribosomes has been observed (21,53).

An alternative explanation for thiostrepton activity has been based on kinetic studies of GTP hydrolysis catalyzed by EF-G and ribosomes. It was found that thiostrepton did not inhibit EF-G – dependent GTP hydrolysis in single turnover experiments, but did prevent the release of both inorganic phosphate and EF-G from the ribosome after GTP hydrolysis (20). EF-G•GDP, kinetically trapped on post-translocation ribosomes in the presence of thiostrepton, has been located by cryo-electron microscopy at a site formed mostly by the 30S subunit and distinct from the fusidic acid-stabilized EF-G•GDP complex (54). This implies an allosteric effect of thiostrepton at some distance from L11, and it is not obvious how thiostrepton interactions with the L11 BD RNA might be coupled to EF-G - ribosome binding some distance away. (It should be noted that the nominal 100 µM concentration of thiostrepton used in these studies, well above the ~5 µM solubility limit, raises the possibility of the antibiotic acting at secondary sites.)

Thus, experimental data support two different models of thiostrepton action, one a thermodynamic competition between thiostrepton and EF-G•GDP binding to the same site and the other a kinetic entrainment of EF-G•GDP by thiostrepton binding at a different site. Equilibrium binding data presented here are consistent with the first mechanism, but are not directly relevant to the implied allosteric and kinetic changes of the second model. Further quantitative studies will be needed to resolve the thiostrepton mechanism on functioning ribosomes.
References

Footnotes

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1 Abbreviations: L11 BD RNA, nucleotides 1051-1108 of the 23S rRNA (E. coli numbering) that constitute the minimal binding domain for protein L11; L11-NTD, N-terminal domain of L11; L11-C76, C-terminal RNA binding domain of L11; Bst, Bacillus stearothermophilus; THS, thiostrepton.
Table 1

L11 and L11-C76 RNA Binding Affinities and Thiostrepton Cooperativity

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Protein</th>
<th>K (µM⁻¹) a</th>
<th>THS cooperativity b</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Py-U1082 RNA</td>
<td>U1061A RNA</td>
</tr>
<tr>
<td>M5K200D</td>
<td>L11</td>
<td>497 ± 15.4</td>
<td>1.0 x 10⁴</td>
</tr>
<tr>
<td></td>
<td>L11-C76</td>
<td>63.3 ± 1.8</td>
<td>1.2 x 10³</td>
</tr>
<tr>
<td>M5K500D</td>
<td>L11</td>
<td>68.3</td>
<td>2350</td>
</tr>
<tr>
<td>M5K700D</td>
<td>L11</td>
<td>30.0 ± 0.5</td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>L11-C76</td>
<td>11.8 ± 3.4</td>
<td>94</td>
</tr>
<tr>
<td>M5K1000D</td>
<td>L11</td>
<td>22.2</td>
<td>1750</td>
</tr>
</tbody>
</table>

aBinding constants are the averages of 2-4 separate titrations. U1061A RNA affinities were estimated from competition experiments between py-U1082 RNA and U1061A RNA.

bThe cooperativity factor ω (defined in the text, Scheme 1) was found from L11- py-1082 RNA titrations in the presence of thiostrepton. Reported values are the averages of two or three titrations.
Figure Legends

Figure 1. Structure of the L11 BD RNA fragment from 23S rRNA. A, secondary structure of the U1061A variant of the E. coli sequence. Horizontal red bars represent tertiary base-base hydrogen bonding and the vertical cyan bar represents a tertiary base stacking interaction. B, crystal structure of the same RNA fragment with L11-C76 bound (8). “N” indicates the N-terminus of the protein, the point from which the N-terminal domain would be attached. The 2′OH of U1082, which has been modified in py-U1082 RNA, is colored red. In both panels, nucleotides contacted by L11-C76 are in blue and A1067, which probably forms part of the thiostrepton recognition site, is colored tan.

Figure 2. UV Melting profiles of py-U1082 RNA (gray) and U1061A RNA (black) in M3K100 buffer. Melting profiles are shown at two wavelengths, 260 nm(—) and 280 nm (---). A, py-U1082 RNA or U1061A RNA alone. B, 1 µM py-U1082 RNA or 1µM U1061A, each with 1µM L11-C76.

Figure 3. Representative fluorescence binding curve of L11 (●) or L11C76 (○) to py-U1082 RNA in M5K700D. Lines are least squares fit of binding isotherms, as described in Experimental Procedures. Association constants calculated from these curves were 32.2 µM⁻¹ for L11 and 15.5 µM⁻¹ for L11-C76.

Figure 4. Competition between py-1082 and U1061A RNAs in binding L11 proteins. Equimolar amounts of the tagged and unmodified RNAs were titrated with L11 (●) or L11-C76 (○) in M5K700D buffer. The solid lines were calculated assuming that py-U1082 RNA binds 8 fold weaker to protein than U1061A RNA.

Figure 5. Salt-back titrations for L11 proteins binding py-1082 RNA. A, L11-C76 at 106 nM (●) or 209 nM (○) with 5.6 nM py-1082 RNA, was prepared in M5K200 buffer and titrated with a concentrated KCl solution containing the other buffer components at standard concentrations. B, same conditions as in panel A only using 13 (●), 29 (○), or 37 (■) nM L11. Lines are calculated assuming ∂log(Ka)/∂log[KCl] = -1.8 (panel A, L11-C76) or –2.3 (panel B, L11) (see Experimental Procedures).

Figure 6. Titration of py-U1082 RNA with L11 in the presence of thiostrepton. A, representative kinetics of fluorescence increase after adding L11 to py-U1082 RNA in M5K700D buffer with 7 nM thiostrepton. The solid line is the fit of a first order exponential to the time course data. B, Titrations of py-U1082 RNA with L11 in the presence of 7 nM thiostrepton. Buffers are M5K500D (●), M5K700D (○), or M5K1000D (■). Solid lines are calculated using ω = 2500, 3100, or 2000, respectively, as described in Experimental Procedures.

Figure 7. Thiostrepton does not significantly stimulate L11-C76 or yL11 binding to py-U1082 RNA. A, Py-U1082 RNA was titrated with L11-C76 in M5K700D buffer with no thiostrepton (●, K = 8.8 µM⁻¹) or 185 nM thiostrepton (○, K = 10.1 µM⁻¹). Curves are best fits of the data to single site binding isotherms. B, same conditions as panel A, but using yL11 protein and M5K700D buffer with 15% glycerol. Titration in the absence of thiostrepton (●, K = 39. µM⁻¹) or in the presence of 185 nM thiostrepton (○, K = 26. µM⁻¹).
Figure 2

(A) U1061A RNA

(B) U1061A RNA + L11-C76

py-1082 RNA + L11-C76

Temperature, °C
Figure 3

Fluorescence Intensity

[L11] or [L11-C76], μM
Figure 4

Relative Fluorescence Intensity

[L11] or [L11-C76], μM

0 0.2 0.4 0.6 0.8 1.0
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 1.0

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Figure 5

Graph A and B show the relationship between [KCl], M, and relative fluorescence intensity. The graphs depict different conditions or treatments, indicated by various line styles and data points.
Figure 6

A

Relative Fluorescence Intensity

0.088
0.089
0.090
0.091
0.092
0.093
0.094
0.095
0.096
0.097
0.098
0.099
0.100

Time, sec

0 500 1000 1500 2000

B

Normalized Fluorescence Intensity

0
0.2
0.4
0.6
0.8
1

[L11], μM

0.0001 0.001 0.01 0.1 1
Figure 7

(A) Graph showing the relationship between [L11-C76] concentration (μM) and relative fluorescence intensity.

(B) Graph showing the relationship between [yL11] concentration (μM) and relative fluorescence intensity.
Interactions of the N-terminal domain of ribosomal protein L11 with thiostrepton and rRNA
Sarae L. Bausch, Ekaterina Poliakova and David E. Draper

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