Cooperative Interactions of RNA and Thiostrepton Antibiotic with Two Domains of Ribosomal Protein L11†

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ABSTRACT: Ribosomal protein L11 interacts with a 58-nucleotide domain of large subunit ribosomal RNA; both the protein and its RNA target have been highly conserved. The antibiotic thiostrepton recognizes the same RNA domain, and binds to the ribosome cooperatively with L11. Experiments presented here show that RNA recognition and thiostrepton cooperativity can be attributed to C- and N-terminal domains of L11, respectively. Under trypsin digestion conditions that degrade *Bacillus stearothermophilus* L11 to small fragments, the target RNA protects the C-terminal 77 residues from digestion, and thiostrepton and RNA in combination protect the entire protein. A 76-residue C-terminal fragment of L11 was overexpressed and shown to fold into a stable structure binding ribosomal RNA with essentially the same properties as full-length L11. An L11·thiostrepton·RNA complex was 100−200-fold more stable than expected on the basis of L11−RNA and thiostrepton−RNA binding affinities; similar measurements with the C-terminal fragment detected no cooperativity with thiostrepton. L11 function is thus more complex than simple interaction with ribosomal RNA; we suggest that thiostrepton mimics some ribosomal component or factor that normally interacts with the L11 N-terminal domain.

The large number of ribosomal protein sequences now available has made it evident that many ribosomal proteins are conserved in all prokaryotic and eukaryotic organisms, even to the extent that some mutations at homologous positions of yeast and bacterial ribosomal proteins have similar phenotypes (Alksne *et al*., 1993). These conserved proteins presumably have important roles in the ribosome, but relatively little is known of their structures, interactions with RNA, or specific functions.

Ribosomal protein L11 binds to a limited domain of large subunit rRNA and belongs to this class of conserved proteins: homologs have been found in bacterial, archaeal, and eukaryotic ribosomes, and the L11 homolog from one organism invariably binds specifically to the large subunit rRNA from other organisms (Stark *et al*., 1980; Beauclerk *et al*., 1985; El-Baradi *et al*., 1987). L11 stimulates a set of ribosome activities associated with several protein synthesis factors that bind the large subunit. Ribosomes lacking L11 synthesize protein 2-fold more slowly than normal ribosomes and are correspondingly defective in the elongation factor G (EF-G)-dependent GTPase (Stark & Cundliffe, 1979). L11-deficient ribosomes are also defective in release factor 1 (RF1)-dependent termination (Tate *et al*., 1984) and completely inactive in binding stringent factor, which is responsible for the synthesis of ppGpp and pppGpp in the stringent response (L11 is the *relC* locus) (Parker *et al*., 1976; Smith *et al*., 1980). In addition, L11 forms part of the target site for members of the thiazole family of antibiotics (e.g., thiostrepton). These antibiotics bind the same rRNA domain as L11 (Ryan *et al*., 1991), inhibit ribosome binding of EF-G·GDP and the EF-Tu ·aminoacyl-tRNA·GTP complexes (Modolell *et al*., 1971), and bind cooperatively with L11 (Thompson *et al*., 1979). Some mutations in L11 (including complete deletion of L11) render ribosomes thiostrepton resistant (Stark & Cundliffe, 1979; Wienen *et al*., 1979; Spedding & Cundliffe, 1984).

The specificity of L11 for binding a limited, 58-nt rRNA fragment (nucleotides 1051−1108, *Escherichia coli* numbering) has been known for some time (Schmidt *et al*., 1981), and we have shown that this RNA contains a set of specific tertiary interactions that are specifically stabilized by the protein (Laing & Draper, 1994; Xing & Draper, 1995). There has been a suggestion that the role of L11 is to promote specific conformational changes in the rRNA (Cundliffe, 1986). In this study we show that a 75-amino acid domain of L11 is responsible for the ability of L11 to stabilize the rRNA tertiary structure; the remaining part of the protein is essential for cooperativity with thiostrepton in binding rRNA. It appears that L11 is separable into two domains, each with conserved sequences and capable of distinct interactions.

MATERIALS AND METHODS

Buffers and Reagents. All buffers were made from sterile, deionized water run through a Barnstead Nanopure system. The following buffers were used: for filter binding measurements, TMK (30 mM Tris, pH 7.6, 20 mM $MgCl_2$, 175 mM KCl); for UV melting experiments, M_3K_{100} (10 mM MOPS pH 7.0, 3 mM $MgCl_2$, 100 mM KCl), M_3N_{100} and M_3A_{100} (substituting NaCl or NH_4Cl for KCl in M_3K_{100}, respectively), $M_3K_{100}G$ (M_3K_{100} adjusted to pH 7.9 and with 15% glycerol added), and $M_3K_{100}GD$ ($M_3K_{100}G$ with 5% DMSO).

Overexpression of L11 Fragments. The gene for *Bacillus stearothermophilus* L11 was previously cloned into pET11a for overexpression (Studier *et al*., 1990; Xing & Draper, 1995). DNA oligomers were designed for PCR amplification of the region extending from codon 59 through the C-terminus, and included *Nde*I and *Bam*HI restriction sites at the N- and C-termini, respectively. Vent polymerase was

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used for PCR amplification of the gene fragment (New England Biolabs). The DNA product was purified, cleaved with the restriction enzymes, and cloned into pET11a cut by the same enzymes. BL21(DE3) was transformed with plasmid DNA, and candidates were screened for overproduction of a protein of the desired size upon induction. One of these candidates was found to carry the correct DNA sequence (sequencing carried out by the Johns Hopkins Core Genetics Facility), and was used for purification of the protein fragment, termed L11−C76. A similar procedure was used to produce an L11 protein fragment extending (after an initial methionine) from codon 42 to the C-terminus and was termed L11−C92.

Bacteria containing the overexpression plasmids were grown in broth, induced, and harvested as previously described. L11−C92 could only be extracted from cells with denaturing buffers and was purified by the same protocol as used for full-length L11 (Xing & Draper, 1995). L11−C76 appeared in the supernatant after cell lysis by a lysozyme freeze−thaw procedure. This supernatant was treated with DNase and loaded onto a Sephacryl S200HR column (5 × 50 cm, Pharmacia) equilibrated in 20 mM sodium phosphate buffer, pH 6.0. Fractions containing L11−C76 were applied to a sulfylpropyl ion exchange column (BioRad TSK-SP-5PW) and eluted with a gradient of 0−400 mM KCl in the same phosphate buffer. Alternatively, the gel filtration step could be substituted by centrifugation at $10^5 g$ for 2 h to pellet ribosomes, followed by extensive dialysis against 20 mM sodium phosphate buffer, pH 6.0, and ion exchange chromatography.

L11−C76 has only one aromatic residue, a phenylalanine, and thus has very weak absorbance at 280 nm. The extinction coefficient at 230 nm was estimated as 24.8×10^3 M^{-1} cm^{-1}, on the basis of quantitative amino acid analysis.

Physical Measurements. Filter binding assays were carried out as previously described for full-length L11 (Ryan & Draper, 1989; Xing & Draper, 1995), except that L11−C76 did not need to be renatured before use. RNAs used in these measurements were T7 RNA polymerase transcripts containing nucleotides 1029−1126 of the *E. coli* large subunit rRNA (Ryan *et al*., 1991). UV absorbance *vs* temperature curves were collected and analyzed as previously described (Laing & Draper, 1994). The data are displayed as the first derivative of the absorbance with respect to temperature, averaged over a 4-degree window and normalized to the absorbance at 15 °C. The RNA used in the melting experiments was a T7 RNA polymerase transcript corresponding to nucleotides 1051−1110 of the 23S rRNA; it was renatured before use as described (Laing & Draper, 1994). The RNA concentration in these experiments was 0.6−0.8 μM. Differential scanning calorimetery was performed in a DASM 4 instrument in the Johns Hopkins Biocalorimetry Center. The protein concentration was 3.5 mg/mL, and the buffer used was 100 mM NaCl, 20 mM sodium phosphate, pH 6.0. CD spectra were collected on a Jasco 700 instrument, using 20 μM protein. Spectra were collected at half-unit pH intervals from 4.0 to 7.0 and at pH 7.9. pH 4.0 and 4.5 were buffered with 50 mM sodium acetate, and the other pH values were buffered with 50 mM sodium phosphate. The spectrum of the buffer alone was subtracted from the protein spectrum at each pH. Addition of 100 mM NaCl at pH 6.0 did not alter the spectrum.

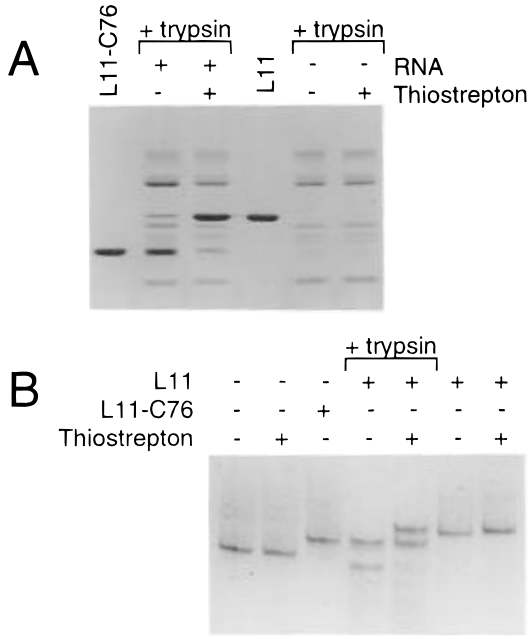

FIGURE 1: Gel analysis of trypsin digestion products. L11 was digested with trypsin at 0 °C in the presence or absence of excess ligands, as described in Materials and Methods. (A) SDS gel. Lanes marked L11 and L11−C76 are undigested controls. 1051−1110 RNA and/or thiostrepton was present in the lanes with trypsin, as indicated. With the exception of L11, proteins larger than L11−C76 in the trypsin digestion lanes are due to trypsin and trypsin inhibitor. (B) nondenaturing gel stained for RNA and showing RNA−protein complexes. The first three and last two lanes are controls showing the 1051−1110 RNA mobility with different ligands bound. Lanes 4 and 5 show the complexes remaining after trypsin digestion as in panel A.

Trypsin Digestion Experiments. Proteolysis of L11 was carried out by preparing 1.1 nmol of L11 in M_5K_{100} buffer, with various additions of 1.2 nmol of 1029−1127 RNA and 4.8 nmol thiostrepton (CalBiochem). A 5-μg amount of trypsin (Sigma) was added, and incubation continued for 16 h at 0 °C in a total volume of 15 μL. At the end of this time, 5 μg of trypsin inhibitor (Sigma) were added. Aliquots of the reactions were run either on 15% polyacrylamide SDS gels and stained for protein with Fast Stain (Zoion Research) or on 8% polyacrylamide gels in 4 mM $MgSO_4$, 100 mM potassium acetate, and 50 mM Tris (pH 8.0) and stained for RNA with methylene blue, as previously described (Xing & Draper, 1995). The protein fragment protected from trypsin digestion by rRNA was excised from an SDS gel, electroeluted onto PVDF membranes, and sequenced for nine residues from the N-terminus by the Edman method in the Johns Hopkins Core Genetics Facility.

RESULTS

Trypsin Digestion of L11−RNA Complexes. All experiments in the present work used an overexpressed homolog of *E. coli* ribosomal protein L11 cloned from *B. stearothermophilus*. We have previously shown that the RNA binding properties of this protein are closely similar to those of the *E. coli* protein in terms of binding affinity, specificity, and dependence on Mg^{2+} and NH_4^+ ions (Xing & Draper, 1995). For convenience, "L11" in this work refers to this overexpressed protein, unless otherwise specified. Trypsin digestions of L11 and its complexes with RNA and thiostrepton are shown in Figure 1. It was reported some time ago that *E. coli* L11 could be cleaved into two large fragments by

mild trypsin digestion (Choli, 1989); however, prolonged trypsin digestion of the *B. stearothermophilus* protein at 0 °C yields only small fragments. When an L11−RNA complex is digested under the same conditions, a fragment of ~75 residues is strongly protected. N-terminal sequencing of the protected fragment showed that it is the C-terminal region of the protein generated by cleavage after R56 (equivalent to R65 of the *E. coli* sequence). The same protein fragment was observed whether ribosomal RNA fragments spanning nucleotides 1051−1110 or 1029−1126 were used. After trypsin digestion, the protein−RNA complex still migrates more slowly than free RNA in non-denaturing gels, but not as slowly as the complex of full-length protein with RNA (Figure 1B).

Thiostrepton binds the same rRNA fragments as does L11, and there is substantial cooperativity between the protein and antibiotic. Evidence for a very stable thiostrepton·L11·rRNA complex is presented below. Thiostrepton by itself does not protect L11 from trypsin digestion, but thiostrepton and RNA together render the entire protein resistant to trypsin (Figure 1). Thus thiostrepton protects the L11 N-terminal domain from digestion, by direct or indirect means, while the C-terminal region is associated with RNA binding.

Preparation and Physical Characterization of L11−C76. In one preparation of L11 we discovered a protein fragment that bound rRNA fragments. It was purified and found to be a C-terminal L11 fragment beginning at T59, two residues shorter than the RNA-protected trypsin digestion fragment. To investigate the RNA binding properties of the C-terminal region, DNA encoding amino acids T59−D133 was cloned in an T7 polymerase-based expression system. The 76-amino acid protein was synthesized in an excellent yield of ~60 mg per liter of cell culture, and could be purified in one or two column steps without the use of denaturing buffers (see Materials and Methods). We have termed this fragment L11−C76. The mobility of this expressed protein in SDS gels is identical to that of the RNA-protected L11 fragment, and it slows RNA electrophoretic mobility by the same amount (Figure 1A,B).

Folding and stability of L11−C76 were assessed by circular dichroism, for comparison with the intact protein. The L11−C76 spectrum is pH-dependent, reaching a maximum intensity at about pH 6.3 (Figure 2A). The tendency of L11 to aggregate at lower pH has precluded measurement of the pH dependence of its spectrum, though at pH 7.9 it is nearly the same intensity (on a per residue basis) and shape as the spectrum of L11−C76 at the same pH [cf. Figure 3A of Xing and Draper (1995)]. Attempts to deconvolute the L11−C76 spectrum into contributions from different secondary structures did not result in satisfactory fits, though it is evident that the protein contains a high fraction of α-helix. The secondary structure derived from NMR studies, which will be reported elsewhere, confirms this.

Melting curves were obtained for L11−C76, following the CD intensity at 220 nm and pH 6.0. A single transition was observed with an inflection at 76 °C. A similar apparent T_m was obtained at pH 7.9 with 15% glycerol; under these identical conditions intact L11 has a T_m of at least 72 °C and melts somewhat more broadly (Xing & Draper, 1995). (Omission of the glycerol destabilizes L11−C76 by ~4 °C without changing its spectrum at low temperatures.) Since the experiment could not be taken to high enough temperature to obtain an apparent enthalpy for the unfolding (Figure 2B), differential scanning calorimetry was used to follow the unfolding to 105 °C, but even over that extended temperature range the higher temperature base line was not reliable enough to calculate an accurate enthalpy. The unfolding observed by calorimetry was reversible.

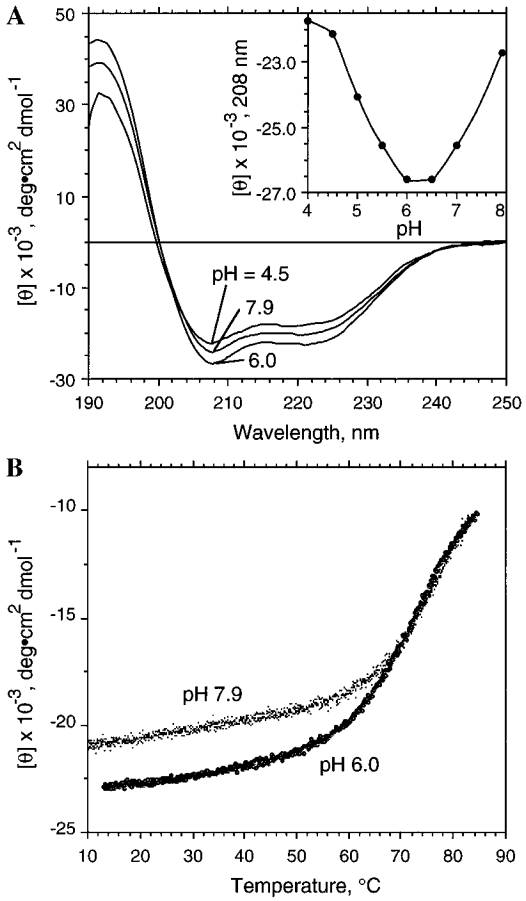

FIGURE 2: Physical properties of L11−C76. (A) CD spectra taken at the indicated pH values; ellipticity has been normalized per mole of amino acid. The inset shows the dependence of the ellipticity at 208 nm on pH. (B) Ellipticity at 220 nm as a function of temperature, at either pH 7.9 (15% glycerol included in buffer) or pH 6.0, as indicated.

RNA-Binding Properties of L11−C76. Experiments were done to compare the RNA binding properties of L11−C76 with those of intact L11. Gel mobility shift titrations at high RNA concentrations (~6 μM, as in control lanes of Figure 1B) gave a 1:1 stoichiometry for the L11−C76 complex with both 1029−1126 and 1051−1110 RNAs (not shown). At RNA concentrations comparable to the dissociation constant of the complex (~0.1 μM), dissociation of protein from the complex during electrophoresis is too extensive for equilibrium binding constants to be obtained. [Others have noted that dissociation during electrophoresis can cause serious errors in estimates of protein−nucleic acid affinities (Hall & Stumpf, 1992).] In nitrocellulose filter binding assays, the (L11−C76)−1029−1126 RNA complex was retained with a low efficiency of 21%−35% (Figure 3), compared to ~45% for similar L11−RNA complexes, but the apparent binding constant, $9.1 \pm 0.7\ \mu M^{-1}$ (average of three titrations), was nevertheless comparable to that of the full-length protein from either *E. coli* or *B. stearothermophilus*, 11−12 μM (Ryan & Draper, 1989; Xing & Draper, 1995). Several RNA sequence variants were also tried in the filter assay; these have previously been tested with the intact *E. coli* and *B.*

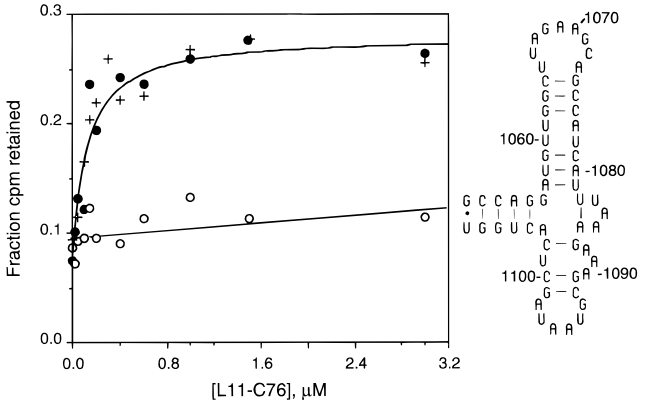

FIGURE 3: Filter binding assays of 1029−1126 RNA binding to L11−C76, measured in TMK buffer at 0 °C. (●) Wild-type RNA sequence; the curve is a least-squares fit with $K = 9.9\ \mu M^{-1}$, background retention of 0.07, and maximum retention 0.28. (+) 1094A → U variant; the least-squares best-fit binding affinity is $10.7\ \mu M^{-1}$, with background retention of 0.076 and maximum retention 0.28 (curve not shown). (○) A1085 → U variant; binding affinity is probably $<1\ \mu M^{-1}$, but is too weak to estimate reliably. The phylogenetically conserved secondary structure of the L11 rRNA binding domain (nucleotides 1051−1108) is shown to the right of the graph.

stearothermophilus L11 proteins (Ryan *et al*., 1991; Xing & Draper, 1995). A1085 → U and U1082 → C, which strongly affect *E. coli* L11 binding, also gave very weak binding ($<1\ \mu M^{-1}$) (Figure 3). L11−C76 binds as well as intact L11 to U1094 → A ($17 \pm 4.6\ \mu M^{-1}$, three titrations), which does not affect either *E. coli* L11 or thiostrepton binding significantly, and to 1082C/1086G ($10.2 \pm 3.4\ \mu M^{-1}$, two titrations), a compensatory base change found in eukaryotes (Figure 3).

Stabilization of rRNA Tertiary Structure by L11−C76. We have previously examined the unfolding of the 1051−1110 rRNA fragment by UV hyperchromicity and calorimetry (Laing & Draper, 1994). The RNA has a set of tertiary interactions, specifically stabilized by Mg^{2+} and NH_4^+ ions, that unfolds before the secondary structure (Wang *et al*., 1993; Laing *et al*., 1994) and requires a number of conserved bases (Lu & Draper, 1994). L11 stabilizes this tertiary structure by ∼25 °C (Xing & Draper, 1995). Similar behavior is seen with L11−C76; a set of melting profiles at different L11−C76:RNA ratios is shown in Figure 4A. The lowest-temperature unfolding transition is moved to higher temperatures with increasing concentrations of L11−C76. At saturating concentrations of protein, no unfolding of the RNA is seen up to ∼40 °C; by stabilizing the set of tertiary interactions, the entire RNA structure is prevented from unfolding. The melting profile seen in Figure 4A was reproduced almost exactly when the complex was cooled; there was only ∼9% loss of hyperchromicity, due to Mg^{2+}-induced RNA hydrolysis at the high temperatures. Thus the melting profile is reversible, and equilibrium is maintained during the melting experiment.

An unusual characteristic of the 1051−1110 RNA tertiary structure is its specificity for monovalent ions: NH_4^+ stabilizes the structure most effectively, K^+ is somewhat less effective, and all other ions (e.g., Na^+, Cs^+) have no specific effects. The stability of the L11−RNA complex accordingly increases when buffer ions are substituted in the order $Na^+ < K^+ < NH_4^+$ (Xing & Draper, 1995). Similar behavior is seen for the complex with L11−C76 (Figure 4B), demonstrating that the same tertiary structure (insofar as the tertiary structure is determined by the specific ion binding site) is stabilized by L11−C76 as by the full-length protein.

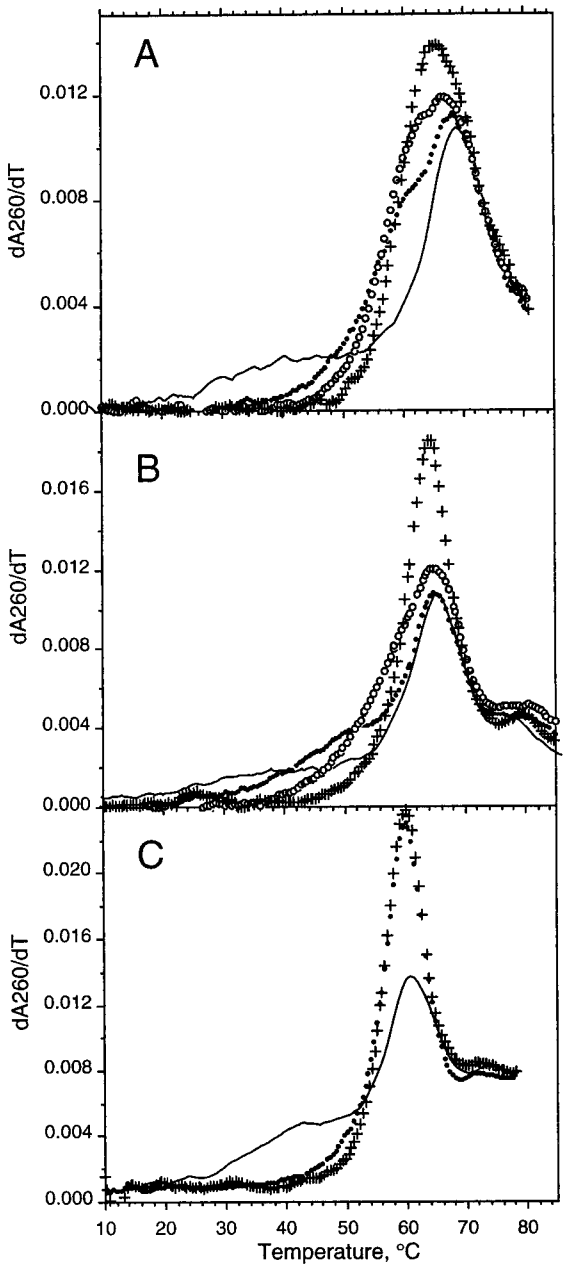

FIGURE 4: Melting profiles of 1051−1110 RNA with L11 and L11−C76. (A) Melting experiments at increasing concentrations of L11−C76 in M_3K_{100} buffer. Molar protein to RNA ratios were 0 (−), 0.5 (●), 1.0 (○), and 2.0 (+). (B) Comparison of melting experiments in Na^+, K^+, and NH_4^+ ions. Buffers used were M_3Na_{100} (●), M_3K_{100} (○), and M_3A_{100} (+) with 0.5 L11−C76 per RNA. For comparison, the solid line shows the melting profile of RNA alone in M_3K_{100} buffer. (C) Comparison of L11−C76 and L11 in $M_3K_{100}G$ buffer, using 0.5 equiv of protein per RNA: RNA alone (−), L11 (●), and L11−C76 (+).

The T_m of a melting transition is sensitive to small changes in free energy; thus a comparison of 1051−1110 RNA melting in the presence of L11 or L11−C76 is a stringent test for differences in the RNA binding thermodynamics of the two proteins. An example comparison is shown in Figure 4C; no significant difference between the melting profiles of the two protein−RNA complexes was observed in these experiments (<0.5-degree difference in T_ms). (At a 1:1 ratio of protein to RNA, small differences were seen at temper-

atures above 65 °C but were attributable to irreversible aggregation of L11.) From simulations of the L11−RNA melting curve [as described by Xing and Draper (1995)], we estimate that a factor of 2 difference in the binding constant would give a 1-degree difference in T_m under the buffer conditions of Figure 4C. L11 tends to aggregate under our standard conditions for RNA melting experiments, so 15% glycerol had to be included in the buffer for these experiments (Figure 4C). Glycerol stabilizes the tertiary structure by a small amount (Lu & Draper, 1994), as can be seen in a comparison of the melting profiles of RNA alone in Figure 4B,C. This extra stability accounts for the sharper (L11−C76)−RNA melting curve seen in Figure 4C compared to Figure 4B.

Cooperativity between L11 and Thiostrepton Detected by Melting Experiments. The second activity of L11 that can be assayed with RNA fragments is its ability to promote thiostrepton binding. Cooperativity between thiostrepton and L11 binding to ribosomes has been estimated by equilibrium dialysis and is on the order of 10^3 (Cundliffe, 1986). L11 has also been observed to stimulate thiostrepton binding to 23S rRNA, though binding constants were not estimated (Thompson *et al*., 1979). To observe L11−thiostrepton cooperativity with RNA fragments, RNA was melted in the presence of constant thiostrepton and increasing L11 concentrations (Figure 5A). L11 induces two new transitions: a peak at ∼61 °C appears at low protein concentrations, and then a transition at ∼71 °C becomes more prominent as more protein is added. The varying ratio of the peaks at 61° and 71 °C suggests that they correspond to melting of L11·RNA and L11·thiostrepton·RNA complexes, respectively; only the peak at 61 °C is observed when RNA is titrated with L11 in the absence of thiostrepton (Xing & Draper, 1995). The appearance of a separate peak corresponding to the ternary L11·thiostrepton·RNA complex is an argument for cooperativity between L11 and thiostrepton, based on the following reasoning. For the L11·RNA and ternary complexes to give separate melting transitions, the kinetics of thiostrepton dissociation must be slow relative to the heating rate. (RNA and RNA−ligand complex do not melt at different temperatures if the bound and free ligands exchange rapidly; the observed T_m is simply an average of the T_ms of the free and bound RNA T_ms.) Equilibrium is maintained during the melting of both the L11·RNA and thiostrepton·RNA complexes (Draper *et al*., 1995; Xing & Draper, 1995); therefore the L11·thiostrepton·RNA complex has additional interactions, not present in the L11·RNA or thiostrepton·RNA complexes, that slow thiostrepton dissociation.

A prediction of the above argument is that sufficiently slow heating rates should cause the ∼61 and ∼71 °C transitions to merge into a single peak of intermediate T_m. Figure 5B shows that the two transitions are indeed closer together when the heating rate is slowed. From estimates that the thiostrepton·L11·RNA complex is 10−11 degrees more stable than the L11·RNA complex and that the apparent van't Hoff enthalpy for the 61 °C transition is 110 kcal/mol, the two complexes are calculated to differ in stability by a factor of 100−200. Since thiostrepton alone does not bind tightly enough to stabilize RNA detectably at these temperatures, the estimated stability difference is entirely attributable to the cooperative free energy between thiostrepton and L11. A similar estimate was obtained by a more elaborate simulation of the RNA melting profile that uses a partition function for RNA unfolding previously deduced from RNA melting experiments in the presence of thiostrepton or L11 (Draper *et al.*, 1995; Xing & Draper, 1995). This estimate of the cooperativity is somewhat lower than the factor of 10^3 cited above for L11−thiostrepton binding to intact ribosomes, but it has been made at a much higher temperature. Whether L11−thiostrepton cooperativity is much different in ribosomes *vs* RNA fragments will require measurements under identical conditions to determine.

Melting of the thiostrepton·L11·RNA complex was repeated in buffers containing different monovalent cations (Figure 5C). As expected, the stability of the ternary complex increases in the order $Na^+ < K^+ < NH_4^+$, indicating that the NH_4^+-dependent RNA tertiary structure is present in the ternary complex. We have also observed a strong dependence of the complex stability on Mg^{2+} concentration (not shown), which was also expected from the stabilization of the RNA tertiary structure by Mg^{2+} (Laing *et al*., 1994).

Lastly, we used a gel band shift assay to demonstrate that thiostrepton binds the L11−RNA complex with the same 1:1 stoichiometry seen in thiostrepton complexes with RNA alone (Ryan *et al*., 1991). A thiostrepton·L11·RNA complex has a slightly slower electrophoretic mobility than L11·RNA and is quantitatively formed by the addition of one thiostrepton per L11·RNA (Figure 6). Addition of thiostrepton to 8 equiv per RNA did not induce any additional electrophoretic species (not shown). The thiostrepton·RNA complex either migrates no differently than RNA alone or (more likely) exchanges too rapidly to be detected by a gel assay.

L11−C76 Does Not Interact Cooperatively with Thiostrepton. The cooperative protein−thiostrepton interaction observed in melting experiments with L11 could not be detected with L11−C76. An example set of melting profiles is shown in Figure 7A. Thiostrepton alone stabilizes the RNA tertiary structure under the conditions of the experiment, but the antibiotic does not induce any additional stability at the higher melting temperature of the L11−C76 complex. To a first approximation, removal of the protein N-terminus has completely eliminated the protein−antibiotic cooperativity. As expected from this result, thiostrepton does not affect the mobility of a (L11−C76)−RNA complex (Figure 6, last two lanes).

Properties of an L11−C92 Fragment. In an attempt to define the L11 sequences required for thiostrepton binding cooperativity more closely, we prepared a 92-amino acid L11 fragment beginning with G43. This endpoint was chosen to include a conserved region from G43 to I50. It had similar solubility properties as L11, i.e., urea was required during purification and pH 7.9 and 15% glycerol were needed in buffers to slow aggregation. L11−C92 RNA binding properties were indistinguishable from those of L11: it bound rRNA fragments with 1:1 stoichiometry and similar binding constants and stabilized 1051−1110 RNA tertiary structure to the same degree (not shown). However, it did not show any cooperativity with thiostrepton in melting experiments (Figure 7B) or in gel mobility shift experiments (not shown).

DISCUSSION

Properties of Two L11 Domains. The results presented here demonstrate that the ability of L11 to bind and stabilize a tertiary structure within the large subunit rRNA is entirely confined to ∼75 residues at the protein C-terminus; the L11−

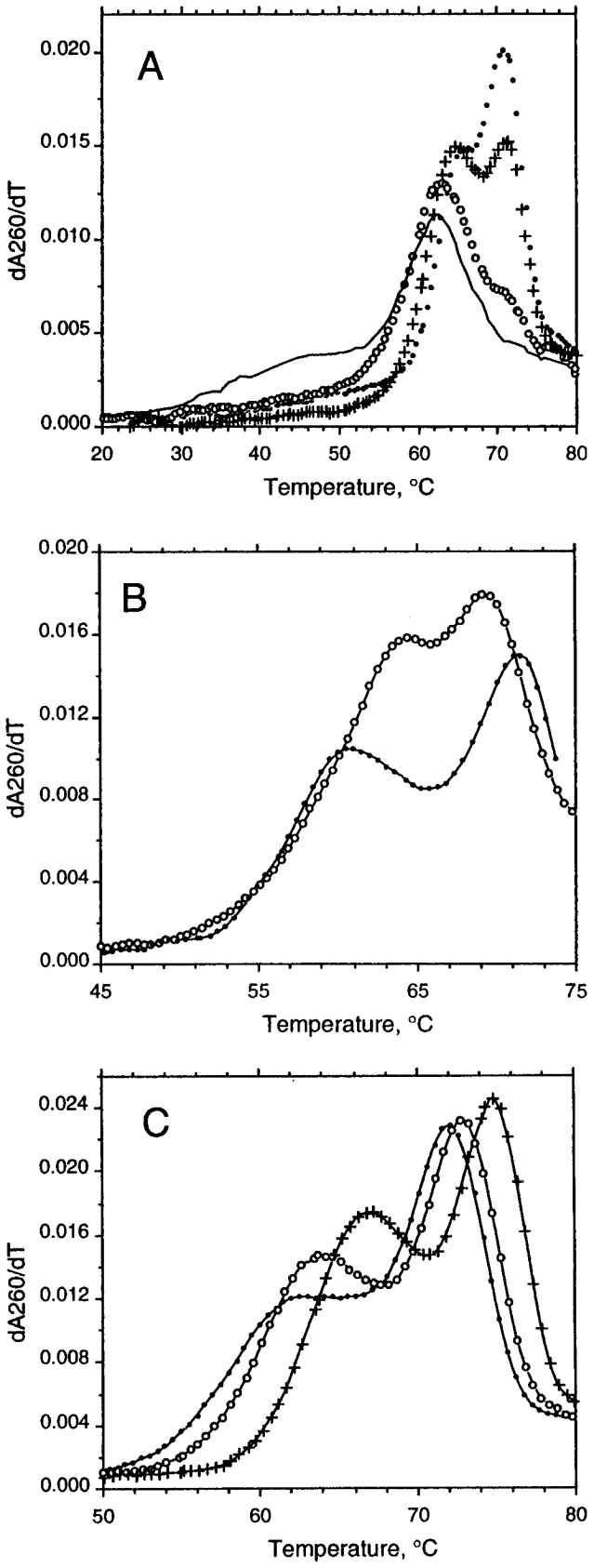

FIGURE 5: L11−thiostrepton cooperativity. (A) Melting profiles in M_3K_{100}DG buffer with 4 μM thiostrepton, 0.7 μM 1051−1110 RNA fragment, and various L11:RNA ratios: no L11 (—); 0.2 (○); 0.5 (+); 1.0 (●). (B) Melting profiles in M_3K_{100}DG buffer with 4 μM thiostrepton, 0.7 μM RNA fragment, and 0.35 μM L11, using heating rates of 0.8 (●) or 0.25 (○) deg/min. (C) Melting profiles of 0.7 μM RNA, 0.35 μM L11, and 4 μM thiostrepton in M_3Na_{100}-DG (●), M_3K_{100}DG (○), and M_3A_{100}DG (+).

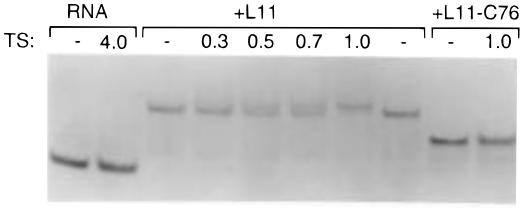

FIGURE 6: Stoichiometry of thiostrepton binding to L11−RNA complexes. 1051−1110 RNA (18 μM) was incubated with the same concentration of L11 (middle six lanes) or L11−C76 (last two lanes), and the indicated molar ratios of thiostrepton in electrophoresis running buffer with 10% DMSO. Samples were run on non-denaturing gels as described in Materials and Methods.

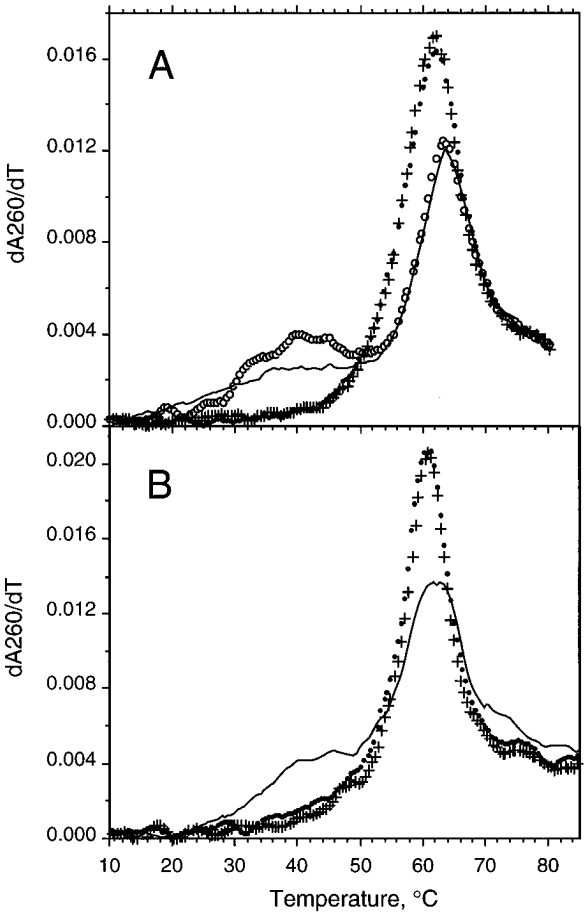

FIGURE 7: L11−C76 and L11−C92 do not bind cooperatively with thiostrepton. (A) Melting profiles of 1051−1110 RNA in M_3K_{100} buffer with 5% DMSO: RNA alone (—), 3.2 μM thiostrepton (○), 0.5 equiv of L11−C76 (●), 0.5 equiv of L11−C76 and 3.2 μM thiostrepton (+). (B) Same as panel A, but substituting L11−C92 for L11−C76 and using M_3K_{100}DG buffer.

C76 fragment and the full-length protein differ by less than a factor of two in their rRNA binding affinities. This RNA binding domain adopts a very stable structure, and probably is an independently folding domain within the L11 protein. A search of data bases for sequences similar to the C-terminal domain turned up only the 16 known L11 homologs derived from eukaryotes, archaea, and chloroplasts as well as other eubacteria. Evidently evolution has not taken advantage of the L11 framework to create other RNA binding proteins, at least not to the extent that the "RNP motif" has been used (Dreyfuss *et al*., 1988; Kenan *et al*., 1991). This is perhaps not surprising, since the L11 target site is highly structured and may demand a protein fold not suited for generic RNA recognition. It remains to be seen if homology with other

ribosomal or RNA binding proteins will appear at the secondary or tertiary structural level. The very stable and soluble L11−C76 fragment is well-suited for solution NMR studies, which will be presented elsewhere.

The N-terminal region of the protein can also be identified with a specific function, *viz*., stimulation of thiostrepton−RNA interactions. The simplest interpretation of our results is that the protein−antibiotic cooperativity is due to direct interactions between the two. It is unlikely that cooperativity derives from a protein-induced change in RNA conformation that promotes thiostrepton binding; if this were the case, then L11−C76 (which has apparently identical RNA binding properties as L11) would also be expected to induce thiostrepton cooperativity. Although it is difficult to rule out the possibility that L11 induces some RNA conformational change that L11−C76 does not, the similarity of the L11 and L11−C76 affinities for RNA argues against any induced conformational change that could enhance thiostrepton binding by a factor of 100.

Thiostrepton (MW = 1665) is much smaller than the N-terminal protein domain it protects. However, trypsin digestion sites are sparse within the first 55 residues (K2, R9, and K32, compared to nine basic residues between 59 and 133), so that thiostrepton might need to protect only a small part of the protein surface from trypsin.

A number of L11 homologs from different sources have been tested for binding to heterologous large subunit rRNAs, e.g., the yeast equivalent of L11 (YL15) with *E. coli* rRNA (El-Baradi *et al*., 1987). These experiments have all demonstrated specific binding (Stark *et al*., 1980; Beauclerk *et al*., 1985). It thus appears that the rRNA−L11 contacts have been conserved through all of evolution. [The same is not true of all ribosomal proteins binding rRNA; for instance, *E. coli* S4 does not recognize eukaryotic rRNA (Sapag *et al*., 1990) even though eukaryotic homologs of S4 have been identified (Mizuta *et al*., 1991).] The strong conservation of L11−RNA contacts contrasts with a more limited conservation of thiostrepton activity; eukaryotic and some archaebacterial translation systems are resistant to the drug (Cammarano *et al*., 1985). Resistance is not attributable to the ribosomal RNA, since substitution of the yeast homolog of the 1051−1108 region into *E. coli* ribosomes yields thiostrepton-sensitive ribosomes (Thompson *et al*., 1993). It is therefore possible that eukaryotic resistance to thiostrepton is due to weakened protein−antibiotic interactions in the N-terminus.

Implications for L11 Function. The L11 N-terminal region presumably has not evolved solely to promote thiostrepton binding; it is more likely that thiostrepton mimics some ribosomal component, either protein or RNA, normally interacting with the L11 N-terminus. L11 shows some cooperativity with L10 in ribosome assembly (Dijk *et al*., 1979), but there is no evidence that L10 binding is affected by thiostrepton. A more likely candidate for L11 interactions is EF-G, which has been cross-linked to A1067 within the L11 binding domain (Sköld, 1983). A GTP analog bound to EF-G has also been cross-linked to L11 (Maasen & Möller, 1978), and binding of a EF-G·GDP complex to ribosomes appears to be competitive with thiostrepton (Highland *et al*., 1971). Other protein synthesis factors may also interact with L11. Either iodination of Y7 within the *E. coli* L11 N-terminal domain or reaction of antibodies specific for *E. coli* residues 1−64 interferes with binding of release factor 1 (Tate *et al*., 1984, 1986), and stringent factor activity strongly depends on the presence of L11 (Stark *et al*., 1980). It is possible that the L11 N-terminal domain promotes protein synthesis factor binding to ribosomes by direct interactions and that thiostrepton competes for these interactions; experiments with intact ribosomes will be able to test this speculation.

L11 affinity for the 1051−1108 RNA fragment is comparable to its affinity for intact 23S rRNA (Thompson & Cundliffe, 1991), which would seem to rule against rRNA binding as a role for the N-terminus. However, *in vitro* selection experiments have shown that L11 is capable of binding its target domain in an alternative mode independent of the NH_4^+-dependent RNA tertiary structure (Lu & Draper, 1995), and our preliminary experiments suggest that the N-terminus is required for this activity. It is of course possible that rRNA and protein factors each interact with the N-terminus at different stages of the ribosome cycle.

The existence of L11−ligand interactions in addition to its affinity for the 1051−1108 rRNA tertiary structure provides a potential answer to a question raised by RNA binding studies. Both L11 and thiostrepton bind and stabilize the same NH_4^+-dependent rRNA structure (Draper *et al*., 1995; Xing & Draper, 1995), yet L11 stimulates protein synthesis while thiostrepton inhibits it, even in the absence of L11 (Stark & Cundliffe, 1979). If the function of L11 were merely to stabilize a conformation otherwise difficult for the rRNA to achieve, thiostrepton and L11 might be expected to have similar effects on ribosome activities. Potential interactions of the L11 N-terminal domain with other ribosome components add the possibility of more complex functions than simple RNA structure stabilization.

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