The Antibiotic Thiostrepton Inhibits a Functional Transition Within Protein L11 at the Ribosomal GTPase Centre

Bo T. Porse1, Ilia Leviev1, Alexander S. Mankin2 and Roger A. Garrett1*

1RNA Regulation Centre
Institute of Molecular Biology
University of Copenhagen
Sølvgade 83H
DK1307, Copenhagen K
Denmark

2Center for Pharmaceutical Biotechnology-m/c870
University of Illinois
900S Ashland Ave., Chicago
IL 60607, USA

A newly identified class of highly thiostrepton-resistant mutants of the archaeon Halobacterium halobium carry a missense mutation at codon 18 within the gene encoding ribosomal protein L11. In the mutant proteins, a proline, conserved in archaea and bacteria, is converted to either serine or threonine. The mutations do not impair either the assembly of the mutant L11 into 70 S ribosomes in vivo or the binding of thiostrepton to ribosomes in vitro. Moreover, the corresponding mutations at proline 22, in a fusion protein of L11 from Escherichia coli with glutathione-S-transferase, did not reduce the binding affinities of the mutated L11 fusion proteins for rRNA or of thiostrepton for the mutant L11-rRNA complexes at rRNA concentrations lower than those prevailing in vivo. Probing the structure of the fusion protein of wild-type L11, from E. coli, using a recently developed protein footprinting technique, demonstrated that a general tightening of the C-terminal domain occurred on rRNA binding, while thiostrepton produced a footprint centred on tyrosine 62 at the junction of the N and C-terminal domains of protein L11 complexed to rRNA. The intensity of this protein footprint was strongly reduced for the mutant L11-rRNA complexes. These results indicate that although, as shown earlier, thiostrepton binds primarily to 23 S rRNA, the drug probably inhibits peptide elongation by impeding a conformational change within protein L11 that is important for the function of the ribosomal GTPase centre. This putative inhibitory mechanism of thiostrepton is critically dependent on proline 18/22. Moreover, the absence of this proline from eukaryotic protein L11 sequences would account for the high thiostrepton resistance of eukaryotic ribosomes.

#1998 Academic Press Limited

Keywords: ribosomal GTPase centre; thiostrepton; drug inhibitory mechanism; ribosomal protein L11; protein footprinting

*Corresponding author

Introduction

The GTPase centre is an important functional site on the large ribosomal subunit that participates in GTP hydrolysis reactions involving ribosomal factors. It is centred on a double hairpin structure within domain II of 23 S-like rRNA that provides binding sites for protein L11 and the pentameric protein complex L10.(L12)5 and their homologues (Schmidt et al., 1981; Beauclerk et al., 1984; Egebjerg et al., 1990; Rosenbach & Douthwaite, 1993). RNA footprinting studies have shown that one elongation factor, EF-G/EF-2, protects nucleotides in this rRNA region (Moazed et al., 1988; Holmberg & Nygård, 1994) and EF-G has been chemically cross-linked there (Sköld, 1983).

The cyclic peptide antibiotic thiostrepton can inhibit several reactions at the ribosomal GTPase centre (Cundliffe, 1986). In vivo the drug probably acts by blocking access of the ternary complex (aminoacyl-tRNA-GTP-EF-Tu/EF-1) to this site (Cundliffe, 1971; Modolell et al., 1971; Hornig et al., 1987) whereas in vitro it can block all uncoupled factor-dependent GTP hydrolysis reactions (Cundliffe, 1986), except for the uncoupled GTP hydrolysis of the EF-Tu-GTP complex (Ballesta & Vázquez, 1972; Mesters et al., 1994). These multiple inhibitory mechanisms of thiostrepton in vitro led to the suggestion that the ribosomal GTPase centre undergoes functionally important conformational
transitions, one or more of which is blocked by thiostrepton (Cundliffe, 1986).

Several lines of evidence indicate that thiostrepton binds strongly, and primarily, to rRNA in this region. They include: (1) 2’-O-methylation of A1067 in Streptomyces aureus, the producer of thiostrepton, inhibits drug binding to the ribosome (Thompson et al., 1982); (2) rRNA footprinting results reveal drug protection effects around both A1067 and A1095 (Egebjerg et al., 1989; Rosendahl & Douthwaite, 1993, 1994); (3) mutation of these nucleotides in vivo in Halobacterium halobium, and in vitro in Escherichia coli, produces high drug-resistance levels (Hummel & Böck, 1987; Mankin et al., 1994; Rosendahl & Douthwaite, 1994), and (4) thiostrepton exhibits a high binding affinity for 23 S rRNA (Thompson et al., 1993; Xing & Draper, 1996). Given this evidence for drug-rRNA binding, it was argued that the functional transitions in the ribosomal GTPase centre occur at an rRNA level (Cundliffe, 1986).

L11 also binds to this rRNA region and, although little is currently known about its functional role, studies with L11-minus mutants of both E. coli and Bacillus megaterium, which are viable, but very sick (Cundliffe et al., 1979; Dabbs, 1979), have demonstrated that the protein is important, but not essential, for function. Insight into its function may be gained by examining the interplay of L11 with thiostrepton, first because they both bind cooperatively to free rRNA (Thompson et al., 1979; Ryan et al., 1991), probably via the N-terminal domain of L11 (50 to 60 amino acid residues) since the C-terminal domain (70 to 80 amino acid residues) is primarily involved in rRNA binding (Xing & Draper, 1996) and, secondly, because the abovementioned L11-minus mutants show greater resistance to thiostrepton than wild-type ribosomes (Cundliffe et al., 1979).

In this context, we examined a group of mutants that were shown to be highly resistant to thiostrepton, but lacked changes in their 23 S rRNA sequence (Mankin et al., 1994). It is demonstrated that these mutants carry single-site mutations at proline 18/22 in the L11 which accounts for the high drug-resistance. Evidence is also provided for this residue having a pivotal role in the mechanism of inhibition by thiostrepton at the ribosomal GTPase centre.

Results

Mutations at the conserved proline 18 within protein L11 render H. halobium cells highly resistant to thiostrepton

H. halobium cells were shown to acquire resistance towards thiostrepton by the single site mutations, A1159 → G (medium resistance) and A1187 → G (high resistance), within domain II of 23 S rRNA (A1067 and A1095 in E. coli 23 S rRNA) (Mankin et al., 1994). However, 23 S rRNA sequencing of approximately half of the highly resistant colonies failed to reveal any nucleotide changes within either this region or within domains IV to VI of 23 S rRNA; moreover for one of the mutants the whole 23 S rRNA gene was sequenced but no changes were detected (data not shown). Since L11 constitutes part of the GTPase site on 23 S rRNA, the L11 genes from five independent thiostrepton-resistant colonies, that did not carry A1159G or A1187G mutations, were amplified by PCR and cloned. DNA sequencing of the PCR products and the clones revealed two drug-resistant H. halobium colonies containing a C → T transition and three colonies containing a C → A transversion all at the first position of codon 18. The C → T and C → A mutations convert the highly conserved proline 18 to serine (P18S) and threonine (P18T), respectively (Figure 1A). The levels of thiostrepton-resistance of the mutants were estimated from growth curves, in liquid medium, at different drug concentrations. The results show that the P18T mutation conferred higher resistance than the P18S mutation (Figure 1B) and that both L11 mutants were more resistant than the rRNA mutant strains, A1159G and A1187G, characterized by Mankin et al. (1994).

RNA structural analyses of wild-type and thiostrepton-resistant H. halobium ribosomes: footprinting of thiostrepton on rRNA

To test whether the altered L11 proteins from the P18S and P18T mutants were incorporated into the H. halobium ribosome, ribosomes were isolated from wild-type, P18S and P18T cells and the GTPase region of the 23 S rRNA was subjected to chemical and enzymatic probing (Figure 2A). Protein L11 protects a set of nucleotides in this region of the E. coli ribosome from attack by chemical and ribonuclease probes (Egebjerg et al., 1989), such that if a mutant L11 bound more weakly to the ribosome the protection effects would diminish or disappear. Both the wild-type and mutant ribosomes from H. halobium displayed the same modification pattern towards the nucleotide-specific probes, dimethylsulphate (DMS), kethoxal, and RNase T1 (Figure 2A). Moreover, both displayed altered reactivities at positions G1139, A1142, G1160, G1163 and G1178 compared with the free wild-type rRNA (Figure 2A) and, since G1160, G1163 and G1178 all lie within the L11 binding site of the E. coli ribosome (Egebjerg et al., 1990), we infer that L11 was assembled stoichiometrically in both wild-type and mutant ribosomes. In addition, no differences were observed in the ribosomal protein contents of wild-type and mutant ribosomes as judged by analysis on polyacrylamide gels containing SDS (data not shown).

Finally, we tested whether the thiostrepton-resistance phenotype of the mutant ribosomes reflected their inability to bind the drug. Wild-type and mutant ribosomes were modified by DMS in the presence of increasing amounts of thiostrepton and the only reactivity changes displayed by the wild-type ribosomes were protection effects at
A1159 (A1067 in *E. coli*) and A1187 (A1095 in *E. coli*) and an enhancement at A1188 (Figure 2B). Thiostrepton also produced similar footprints on the ribosomes of both the mutants and bound with comparable affinities (see below), as judged by the response to increasing amounts of thiostrepton (Figure 2B and D). These *in vitro* experiments were performed at ribosome concentrations well above the *K*<sub>d</sub> value (*<10<sup>-9</sup>* M) for thiostrepton-ribosome complexes (Cundliffe, 1986), which means that thiostrepton will bind stoichiometrically. However, although it is impracticable to perform RNA footprinting experiments in the sub-nanomolar range, the ribosome concentration employed here (50 nM) is well below the estimated ribosomal concentration of 1 to 10 μM in *H. halobium* (and *E. coli*) cells (see Materials and Methods). Therefore, we conclude from our results that thiostrepton will bind stoichiometrically to the mutant and wild-type ribosomes, *in vivo*, and that the drug-resistant phenotype is not due to a decreased binding of thiostrepton to the mutant ribosomes.

**Protein footprinting of complexes of the *E. coli* fusion proteins HTG-L11 and GTH-L11 with L11-RNA1**

The structure and function of L11 was investigated further by protein footprinting (Jensen et al., 1995a) where the recombinant protein was <sup>32</sup>P labelled at the N or C terminus and subjected to limited proteinase treatment alone and complexed with rRNA. Since *H. halobium* ribosomes require very high salt concentrations, that are inhibitory for the proteinases, experiments were performed with L11 from *E. coli* ribosomes that are also thiostrepton-sensitive (Gale et al., 1981); this also allowed us to examine potential differences between the L11 proteins from the two organisms.

The L11 gene from *E. coli* was cloned into the expression vectors pET-HTG and pGEX-GTH, yielding plasmids pET-HTG-L11 and pGEX-GTH-L11. Fusion proteins expressed from these vectors contain a glutathione-S-transferase (GST) tag of 220 amino acid residues at the C terminus, HTG-L11, or N terminus, GTH-L11, as illustrated in Figure 3A and B. These constructions allow for a one-step purification procedure, and they include a heart muscle kinase site (sequence RRASV) at either the N terminus (HTG-L11) or C terminus (GTH-L11), that can be labelled using [γ<sup>32</sup>P]ATP (Figure 3A and B). Moreover, a cleavage site for the endoproteinase thrombin (of sequence LVPRGS), inserted between the GST tag and the fused protein, facilitates removal of the GST tag (Figure 3A and B). However, owing to problems with internal cleavage of L11 by thrombin, when incubated at 37°C, the GST-tag was not removed. Nevertheless, control experiments demonstrated that fusion proteins, HTG-L11 and GTH-L11, and the thrombin-released L11 bound equally strongly to L11-RNA1 as assayed by protein footprinting with endoproteinase Lys-C (data not shown).

<sup>32</sup>P end-labelled GTH-L11 and HTG-L11 were incubated in the absence and presence of L11-RNA1 encompassing nucleotides 1016 to 1146 of *E. coli* 23 S rRNA (corresponding to 1108 to 1238 of

---

**Figure 1.** Characterization of thiostrepton resistant mutants of *H. halobium*. A, Sequencing of the L11 gene (*rplK*) from wild-type *H. halobium* and two thiostrepton-resistant mutant strains P18S and P18T. Changes in the sequence of codon 18 are indicated by an asterisk and the altered codons are indicated below the sequence tracks. B, Growth curves for the wild-type and mutant strains of *H. halobium* cultured in the presence of thiostrepton where A1159G and A1187G are mutated in the 23 S rRNA and were described earlier (Mankin et al., 1994). Growth in the presence of drug was normalized to growth in the absence of drug. Exponentially growing cultures (*A<sub>550</sub>* were diluted 100-fold with fresh medium, containing different concentrations of thiostrepton, and growth was continued for two days at 37°C. *A<sub>550</sub>* values were measured and cell growth in the absence of drug was taken as 100%. The same relative positions of the curves were obtained in two independent experiments.
**Figure 2.** RNA footprinting of L11 and thiostrepton in the GTPase centre of *H. halobium* ribosomes. A, *H. halobium* ribosomes were probed with DMS, kethoxal (Keth) or RNase T1 followed by reverse transcription (see Materials and Methods). Symbols: −, control sample without modification; RNA, free rRNA; w.t., wild-type ribosomes; P18S and P18T, mutant ribosomes and RNA (G, A, U, C) sequencing reactions. Nucleotides that exhibit altered reactivities are numbered. The reverse transcriptase stops one nucleotide before the position of the corresponding dideoxy nucleotide. B, Thiostrepton (Thio) was footprinted on wild-type and mutant ribosomes using DMS: 5 pmol of ribosomes (0.05 mM) was modified in 100 µl of buffer in the absence (0), or presence, of increasing amounts of thiostrepton (0.001, 0.01, 0.1, 1, 10 and 100 µM, a precipitate was observed only at the highest concentration). Altered reactivities were observed at A1159, A1187 and A1188. C, Relative reactivity of A1187 to DMS on titrating wild-type (■) and mutant P18S (○) and P18T (▲) ribosomes with thiostrepton. The reactivity of A1187 was quantified relative to A1190 in an Instant Imager and plotted against the logarithm of the thiostrepton concentration. The data are averaged from three experiments. Standard deviations, at each data point, were between 4% and 11% and 5% and 18% for the lower (<0.1 mM) and higher thiostrepton concentrations, respectively. D, A summary of the RNA structural data and thiostrepton footprinting data superimposed on the secondary structure of the GTPase region of 23 S rRNA from *H. halobium*. (■) Sites within the L11 binding site that are protected on ribosomal assembly. (○) Protections and (▲) enhancement within the L10(L12)4 binding site that occur during ribosomal assembly. Thiostrepton-induced protection (□) and enhancement effects (○) are also shown.
The structure of protein L11 was then probed with ten proteinases of different specificities (Table 1). Protein fragments were separated by gel electrophoresis and autoradiographed (Figure 3C and D). The proteinase cuts were assigned to specific amino acids on the basis of band patterns generated by each site-specific proteinase. The L11 component of the free fusion proteins, HTG-L11 and GTH-L11, were highly accessible to most of the proteinases in the absence of L11-RNA1 and cuts were mainly observed in the C-terminal part of L11. This is evident from the figure.

**Figure 3.** Protein footprinting of L11-RNA1 on the L11 fusion proteins. A and B, An outline of the fusion proteins of *E. coli* L11, GTH-L11 and HTG-L11, showing the GST tags (striped box), the thrombin sites (filled arrow) and the heart muscle kinase sites (32P, cross-hatched box). C and D, Protein footprinting of L11-RNA1 on GTH-L11 (C, labelled at the C terminus) and HTG-L11 (D, labelled at the N terminus). Thrombin cleaved within the L11 protein, when incubated at 37°C, and it was not used to remove the GST-tag. End-labelled fusion proteins were digested in the presence (+) or absence (−) of L11-RNA1 with the following set of proteinases: Lys-C (K), Arg-C (R), chymotrypsin (Ch), Glu-C (E), Asp-N (D), trypsin (Tr), proteinase K (PK), pronase (Pn), thermolysin (Th) and bromelain (B). No indicates control samples without proteinase and T is the fusion protein treated with thrombin at 0°C. All of the cuts are assigned to the amino acid positions indicated. E, Summary of the protein footprinting data of L11-RNA1 on HTG-L11 (square) and GTH-L11 (circle) superimposed on the primary sequence of *E. coli* L11. The reactivity was enhanced for underlined proteinases.

*H. halobium* 23 S rRNA). The structure of protein L11 was then probed with ten proteinases of different specificities (Table 1). Protein fragments were separated by gel electrophoresis and autoradiographed (Figure 3C and D). The proteinase cuts were assigned to specific amino acids on the basis of band patterns generated by each site-specific proteinase. The L11 component of the free fusion proteins, HTG-L11 and GTH-L11, were highly accessible to most of the proteinases in the absence of L11-RNA1 and cuts were mainly observed in the C-terminal part of L11. This is evident from the
autoradiograms where only few cuts were present above L79 in GTH-L11 (Figure 3C) and below L79 in HTG-L11 (Figure 3D). In general, the same cuts were observed for both fusion proteins albeit with varying degrees of intensity (Figure 3E), implying that they are all primary cuts (i.e. they are not formed as a direct consequence of other cuts; such dependent cuts are classified as secondary (Douthwaite & Garrett, 1981)). Possible exceptions were the cuts near the C terminus (R134, L138, E141 and D142) where the fragments deriving from GTH-L11 would have been too small to be resolved. Therefore, only the chymotrypsin cuts at F38 and V98, observed for GTH-L11 and HTG-L11, respectively, were considered to be secondary cuts (Figure 3C and D). Small fragments that migrated abnormally in the gels were observed when GTH-L11 was cut with either endoproteinase Arg-C (around position 90), trypsin (around position 90), proteinase K (around position 100), thermolysin (around position 80) or bromelain (around position 95). The mobilities of these so-called ‘ghost bands’ varied from experiment to experiment (data not shown) and were considered by Jensen et al. (1995b) and Lykke-Andersen et al. (1996).

In the presence of L11-RNA1 the few reactive sites within the N-terminal domain, except for E37, remained accessible, whereas all proteinase sites within the highly reactive C-terminal domain of L11 were protected (Figure 3C and D). This indicates that a general tightening of the C-terminal domain occurred on binding to the rRNA and this correlates with the recent demonstration that this domain (76 amino acid residues) from B. stearothermophilus binds to its RNA target with about the same affinity as the whole protein (Xing & Draper, 1996).

Analysis of RNA binding of the L11 mutants P22S and P22T

In order to study the functional significance of proline 18 for H. halobium L11, and the molecular basis of thiostrepton resistance, the codon for the corresponding proline 22 of E. coli L11 was altered and the mutated genes were expressed in the pGEX system. The fusion proteins GTH-P22S and GTH-P22T were purified and their binding to increasing amounts of L11-RNA1 was assayed using endoproteinase Lys-C (Figure 4A). The quantified data revealed no significant differences for the wild-type protein and the proline 22 mutants (Figure 4B). This correlates with the similar modification patterns observed for the H. halobium L11 RNA site within wild-type and mutant L11 ribosomes (Figure 2A).

Since the $K_d$ value for L11-rRNA fragment complexes is approximately $10^{-7}$ M (Ryan & Draper, 1991), a further experiment was performed at a lower protein concentration (50 nM), in order to amplify any small binding differences. However, the similar binding curves obtained for the wild-type and mutant fusion proteins (Figure 4C) reinforce that the mutations at proline 18/22 do not alter the RNA binding properties of L11 and they support the above conclusion that the thiostrepton-resistance of the H. halobium mutants did not result from deficient assembly of L11 into ribosomes.

Protein footprinting of thiostrepton on fusion proteins HTG-L11 and GTH-L11 complexed with L11-RNA1

The possibility of a direct interaction of thiostrepton with L11 was investigated by the protein footprinting method. Fusion proteins HTG-L11 or GTH-L11, either alone or complexed with L11-RNA1, were treated with chymotrypsin, endoproteinase Glu-C, proteinase K or thermolysin, in the presence and absence of thiostrepton. The same altered reactivities were observed for both fusion proteins and, therefore, the results are only shown for HTG-L11 (Figure 5), except for the digestion

<table>
<thead>
<tr>
<th>Proteinase</th>
<th>Specificity</th>
<th>Final concentration (ng/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoproteinase Lys-C</td>
<td>K-</td>
<td>0.6</td>
</tr>
<tr>
<td>Endoproteinase Arg-C</td>
<td>R-</td>
<td>4.0</td>
</tr>
<tr>
<td>Chymotrypsin</td>
<td>F-, Y-, W- &gt; L-, M-, A-</td>
<td>2.0</td>
</tr>
<tr>
<td>Endoproteinase Glu-C (V8)</td>
<td>E- &gt; D-</td>
<td>0.2</td>
</tr>
<tr>
<td>Endoproteinase Asp-N</td>
<td>-D</td>
<td>5.0</td>
</tr>
<tr>
<td>Trypsin</td>
<td>R-, K-</td>
<td>0.2</td>
</tr>
<tr>
<td>Proteinase K</td>
<td>-Hydrophobic</td>
<td>0.1</td>
</tr>
<tr>
<td>Pronase</td>
<td>Unspecific</td>
<td>0.1</td>
</tr>
<tr>
<td>Thermolysin</td>
<td>-I-, -F-, -I-, -V-, -M-, -A</td>
<td>2.5</td>
</tr>
<tr>
<td>Bromelain</td>
<td>Unspecific</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* A dash indicates the position of cleavage relative to the adjacent amino acid.

Table 1. Specificity and concentration range of proteinases

The assignments of the proteinase cuts were verified by plotting the mobilities of protein fragments against the logarithm of their putative molecular mass (data not shown). This resulted in straight lines for fragments of both HTG-L11 and GTH-L11 with molecular mass above 5 kDa, in agreement with reports by Schägger & von Jagow (1987), Jensen et al. (1995a) and Lykke-Andersen et al. (1996).
with chymotrypsin (Figure 6A). Strong protection of the chymotrypsin cut at Y62 was induced by adding thiostrepton at a 1:1 molar ratio to either HTG-L11 or GTH-L11 complexed with L11-RNA1 (Figures 5 and 6A). The protection at Y62 was accompanied by minor protection effects at F38 and F67 (Ch) and a slight enhancement at E37 (E) (Figure 5).

Additional thiostrepton-dependent protections were also observed at L11 sites that were partially protected on complexing the protein with L11-RNA1. These effects at L79 (Ch and Th) with chymotrypsin (Figure 6A).

**Figure 5.** Protein footprinting of thiostrepton on HTG-L11 (wild-type, *E. coli*) in the presence (+) and absence (−) of L11-RNA1. HTG-L11 (60 nM) was complexed with L11-RNA1 (0.2 μM) as described in Materials and Methods and probed with chymotrypsin (Ch), Glu-C (E), proteinase K (PK) and thermolysin (Th). In the cleavage experiments with increasing amounts of thiostrepton (Ch cleavage), the concentrations were 60 pM, 0.6, 6 and 60 nM, 0.6 and 6 μM. A + indicates a thiostrepton concentration of 60 μM (1000-fold excess, where a precipitate was formed). Amino acid positions where altered levels of cleavage were observed are indicated (see Figure 3E).

**Figure 4.** rRNA binding properties of GTH-L11 (wild-type, *E. coli*) and the mutant proteins GTH-P22S (P22S) and GTH-P22T (P22T). A, 32P-end labelled fusion protein (0.3 μM) was cleaved by proteinase Lys-C in the presence of increasing amounts of L11-RNA1 (0, 7, 20, 70, 200 and 700 nM and 2 μM) and the level of RNA binding, as determined by cleavage at Lys113, was quantified in an Instant Imager (Packard). Amino acid positions are indicated where altered cleavage occurred (see Figure 3E). B, Relative Lys-C cleavage at K113, for wild-type (□), and L11 mutants P22S (○) and P22T (●), plotted against the logarithm of the concentration of L11-RNA1. Cleavage in the absence of RNA is set to 100%. The data are averaged from three different experiments. Standard deviations, for each data point, were between 4% and 10% and 10% and 23% for the lower (<0.2 μM) and higher concentrations of L11-RNA1, respectively. C, The protein-RNA binding experiment described in A and B was repeated at a lower concentration of 32P-end labelled fusion protein (50 nM) and the relative Lys-C cleavage at K113, was plotted for wild-type (□) and for L11 mutants P22S (○) and P22T (●) against the logarithm of the concentration of L11-RNA1 (0, 10, 20, 70, 200 and 700 nM and 2 μM).
Thiostrepton-resistant L11 Mutations

(Figures 5 and 6A) and E141 (PK and Th) (Figure 5), can be attributed to drug-induced tightening of the protein/RNA complex compatible with the cooperative binding of thiostrepton and L11 to the rRNA. We tested whether there was any direct effect of thiostrepton on the L11 proteins, by saturating the solution of fusion proteins with thiostrepton, in the absence of L11-RNA1, and no drug-induced protection of L11 was observed (Figures 5 and 6A); this correlates with the reported inability of thiostrepton to bind to free protein L11 (Highland et al., 1975).

The corresponding experiment was also performed on complexes of mutant fusion proteins GTH-P22S and GTH-P22T and L11-RNA1 where minor thiostrepton-dependent protection effects were observed at Y62 and F67 (Figure 6A). HTG-mutant proteins were not used in this experiment because they were less stable than their wild-type equivalents, as judged from the high levels of control bands. When the level of chymotrypsin cleavage at Y62 was quantified and plotted against the concentration of thiostrepton, for GTH-L11, GTH-P22S and GTH-P22T complexed with L11-RNA1, dose response curves displayed similar shapes (Figure 6B). However, whereas Y62 was almost fully protected by thiostrepton in the wild-type GTH-L11/L11-RNA1 complex (>90%), protection in the mutant complexes levelled off at about 30% and 40% protection, respectively (Figure 6B), and did not saturate. In order to establish that the plateaux effects reflect saturation of thiostrepton binding, the experiment was repeated at a six-fold reduced complex concentration of 50 nM. Similar curves were obtained with more pronounced plateaux (Figure 6C). Since the plateaux effects (Figure 6B) were reproducible for different protein preparations (data not shown), and since mutant fusion proteins bound to L11-RNA1 with the same affinity as wild-type fusion protein, partial denaturation is unlikely to explain the lower levels of thiostrepton protection in the mutant fusion proteins.

Figure 6. Protein footprinting of thiostrepton on wild-type and mutant L11 fusion proteins complexed with L11-RNA1. A, L11-RNA1 (1.2 μM) complexed with [32P]-end labelled GTH-L11, GTH-P22S or GTH-P22T (0.3 μM) was digested with chymotrypsin. The increasing thiostrepton concentrations are 0.6 nM, 6 nM, 60 nM, 0.6 μM and 6 μM, respectively, and (+) indicates a concentration of 60 μM (1000-fold excess, where a precipitate was formed). Lanes 1, 2 and 3 contain uncleaved samples of GTH-P22T, GTH-P22S and GTH-L11, respectively. Amino acid positions are indicated where altered proteinase cleavage was observed. (?) indicates that the assignments are uncertain. B, Relative chymotrypsin cleavage at Y62 for complexes of L11-RNA1 and GTH-L11 (□), GTH-P22S (○) or GTH-P22T (○). Cleavage in the sample with 0.6 nM thiostrepton was set to 100%. The chymotrypsin cleavage was quantified on an Instant Imager and plotted against the logarithm of the thiostrepton concentration. The data were averaged from two experiments. Standard deviations, for each data point, were between 7% and 12% and 6% and 17% for the lower (<0.06 μM) and higher concentrations of thiostrepton, respectively. C, The experiment described in A and B was repeated but at a lower concentration of [32P]-end labelled fusion protein (50 nM). The concentration of L11RNA-1 was 1 μM. The relative chymotrypsin cleavage at Y62 was plotted, for wild-type (□) and the L11 mutants P22S (○) and P22T (○), against the logarithm of the concentration of thiostrepton (0, 1, 10, 50 and 100 nM, 1 μM and 10 μM).
RNA footprinting of thiostrepton on L11-RNA1 complexed with wild-type or mutant L11 fusion proteins

The mutations at proline 18/22 of L11 do not affect the affinity of thiostrepton for the L11-RNA complexes, as judged by the data from both RNA footprinting of complexes of thiostrepton and H. halobium ribosomes and protein footprinting of thiostrepton complexed with E. coli fusion proteins and L11-RNA1. We attempted to confirm the latter result in the E. coli system by RNA footprinting of thiostrepton complexed with L11-RNA1 and GTH-L11, GTH-P22S or GTH-P22T. When these complexes were probed by DMS, at complex concentrations below the estimated in vivo ribosome concentration and above the \( K_d \) value for thiostrepton-ribosome complexes (see above), strong protections were observed at A1067 and A1095 in the presence of the drug (Figure 7A). Moreover, in agreement with the data presented above, the dose–response curves for both wild-type and mutant complexes had similar shapes (Figure 7B), suggesting that the drug showed similar binding to both wild-type and mutant L11-RNA complexes.

The wild-type protein appears to affect the L11RNA-1 structure more than the mutant protein, as judged by the DMS reactivity in the A1067 and A1095 loops (Figure 7A). This difference, which varied strongly with the experimental conditions and between experiments (sometimes no difference was observed), suggests that the wild-type protein stabilizes the RNA conformation more effectively than the mutant protein. The occurrence of conformational transitions in the L11RNA-1, under similar ionic conditions and temperature (37°C), was observed earlier (Xing & Draper, 1995; Draper et al., 1995).

Discussion

Structural properties of protein L11

L11 is a two-domain protein, where the C-terminal domain (70 to 80 amino acid residues) is involved in RNA binding and the N-terminal domain (50 to 60 amino acid residues) contributes to the cooperative binding of thiostrepton (Xing & Draper, 1996) and, possibly, L10.(L12)4 (Dijk et al., 1979; Rosendahl & Southwaite, 1995). A structural model for the former domain of B. stearothermophilus L11, deduced from NMR analyses, consists of two parallel \( \alpha \)-helices (I and III) with a third \( \alpha \)-helix (II) packed on top (Xing et al., 1997; Markus et al., 1997). The presence of \( \alpha \)-helices I and III is supported directly by our proteinase footprinting data for the E. coli fusion protein, since both dis-
play regularly spaced proteinase cuts (every three to four amino acid residues), consistent with one side of each helix being exposed to the solvent (Figure 8). In contrast, the high frequency of proteinase cuts occurring in the putative $\alpha$-helix II are incompatible with it being present in the free E. coli protein. Our data also reinforce earlier indications that the structure of the C-terminal domain tightens substantially on binding to rRNA (Choli, 1989; Markus et al., 1997) although the high resistance of the domain to proteinases, when bound to the rRNA, may also reflect that it binds in a cleft between two irregular double helices where it, nevertheless, would have limited access to the putative thiostrepton binding region on the rRNA (Egebjerg et al., 1990; Rosendahl & Douthwaite, 1993).

Three different classes of ribosomal thiostrepton-resistant mutants

Thiostrepton inhibits protein synthesis on most, if not all, bacterial and archaeal ribosomes (Cundliffe, 1986) and drug-resistance mutants fall into two main classes. In the first, mutations at nucleotides A1067 and/or A1095 (E. coli numbering), within the L11-rRNA site, produce high drug-resistance and result in weakened thiostrepton binding (Hummel & Böck, 1987; Thompson et al., 1988; Mankin et al., 1994; Rosendahl & Douthwaite, 1994; Aagaard et al., 1996). The nucleotide corresponding to A1067 is also modified (2'O-methylated) in S. azureus, the producer of thiostrepton (Thompson et al., 1982), such that no drug binding to ribosomes is detectable (Cundliffe, 1986). Moreover, both nucleotides are protected by thiostrepton, in wild-type E. coli ribosomes, against chemical and enzymatic probes and it was inferred that they are juxtapositioned in the rRNA structure (Egebjerg et al., 1990; Rosendahl & Douthwaite, 1995). The second class of mutants are L11-minus and they grow poorly and exhibit an unquantified level of thiostrepton resistance (Cundliffe et al., 1979; Wienen et al., 1979).

Earlier, some H. halobium mutants were shown to exhibit high drug-resistance although their 23S rRNA sequences were apparently unaltered (Mankin et al., 1994). Sequencing of their L11 genes has revealed missense mutations at proline 18 in the N-terminal domain for five individual colonies. This proline is conserved amongst all known archaeal and bacterial L11 sequences except for two mycoplasma strains which show a four amino acid gap around this position. The frequency of these mutations in the L11 gene was similar to the frequency of the single point rRNA mutations causing thiostrepton resistance (Mankin et al., 1994). Therefore, if one assumes that the mutation rate is the same throughout the bacterial genome, the occurrence of second site mutations elsewhere in the genome is very unlikely.

The capacity of the mutated proteins to assemble into ribosomes was established by demonstrating that the modification pattern of the L11-rRNA region in H. halobium ribosomes was indistinguishable from wild-type and mutant ribosomes, a result that is consistent with the C-terminal domain of E. coli L11-RNA and HTG-L11 or GTH-L11 (□) superimposed on the sequence alignment of L11 from E. coli (E.c.), B. stearothermophilus (B.s) and H. halobium (H.h). An enhanced reactivity at F67 is underlined. Amino acids protected by thiostrepton (thio) are indicated, as are the $\alpha$ and $\beta$ secondary structural elements deduced from NMR analyses (Xing et al., 1997; Markus et al., 1997). Identical amino acids are marked by asterisks and similar amino acids are denoted by dots below the H. halobium sequence. The vertical arrows indicate the position of the mutated proline.

Figure 8. Summary of the protein footprinting data for complexes of E. coli L11-RNA1 and HTG-L11 (□) or GTH-L11 (○) superimposed on the sequence alignment of L11 from E. coli (E.c.), B. stearothermophilus (B.s) and H. halobium (H.h). An enhanced reactivity at F67 is underlined. Amino acids protected by thiostrepton (thio) are indicated, as are the $\alpha$ and $\beta$ secondary structural elements deduced from NMR analyses (Xing et al., 1997; Markus et al., 1997). Identical amino acids are marked by asterisks and similar amino acids are denoted by dots below the H. halobium sequence. The vertical arrows indicate the position of the mutated proline.
of L11 carrying the primary rRNA binding site of L11 (Xing & Draper, 1996). Unexpectedly, no differences were detected in thiostrepton binding to wild-type and mutant ribosomes at lower ribosome concentrations than occur in vivo (Figure 2B). Therefore, we concluded that the P18S and P18T mutants belong to a novel class of thiostrepton-resistant mutants, where alterations in the L11 sequence have no detectable effect on either ribosomal assembly or drug binding to the ribosome.

Mutations were generated at the corresponding proline 22 of E. coli L11 in order to obtain protein footprinting data on the thiostrepton-L11-rRNA complex since, although E. coli cells are impermeable to thiostrepton, their ribosomes are highly sensitive (Gale et al., 1981). L11 was expressed as a fusion protein with GST and its binding affinity for the 138 nucleotide L11-rRNA fragment was indistinguishable for the wild-type GTH-L11 and the mutant fusion proteins GTH-P22S and GTH-P22T (Figure 4). Moreover, no changes in thiostrepton binding to the wild-type or mutant fusion protein-rRNA complexes were detected by RNA footprinting (Figure 7) and we inferred that the P18/P22 mutations affected neither L11 binding to the L11-rRNA nor thiostrepton binding to the complex, for either the H. halobium or the E. coli system, under the conditions tested. However, a major difference in the accessibility of Y62, and to some extent L79, to chymotrypsin was observed when thiostrepton was bound to complexes of L11 fusion proteins and rRNA; cleavage was protected by more than 90% in the wild-type GTH-L11/L11-rNA1 complex, but only by 40% and 30%, respectively, for the GTH-P22S/L11-rNA1 and GTH-P22T/L11-rNA1 complexes (Figure 6). These protection levels correlate inversely with the thiostrepton-resistance levels observed for the P22S and P22T mutants where the greater protection at Y62 corresponds to the mutant with the lower drug resistance (Figure 1B). In contrast to P18/22, Y62 is not highly conserved but it lies at the junction of the N and C-terminal domains of L11 (Figure 8).

The absence of proteinase cuts around proline 18/22 prevented a direct experimental testing of thiostrepton binding in this region. However, the capacity of the drug to bind with equal strength to each of the three protein-rRNA complexes (Figure 7), at a molar concentration below the ribosomal concentration occurring in vivo and above the $K_d$ value of thiostrepton-ribosome complexes, precludes a strong drug–L11 interaction in this region. Finally, the isolation of five independent thiostrepton-resistant mutants, all mutated at this amino acid, strongly argues for this site playing a key role in the inhibitory action of the drug.

**Thiostrepton acts by inhibiting a conformational transition within L11**

Thiostrepton can inhibit several processes on the ribosome in vitro, all of which involve the ribosomal GTPase centre. They include binding of the ternary complex to the ribosome in vitro (Modolell et al., 1971; Hornig et al., 1987) and in vivo (Cundliffe, 1971), translocation (Pestka, 1970; Rodnina et al., 1997), uncoupled hydrolysis of GTP by EF-G (Pestka, 1970), as well as recycling of IF-2, binding of release factors during peptide chain termination and formation of guanine tetra- and pentaphosphates catalysed by stringent factor (reviewed by Gale et al., 1981).

To explain this multiple inhibition by thiostrepton, it was proposed that the ribosomal GTPase site undergoes a series of conformational transitions during elongation and that thiostrepton blocks one (or more) of them, probably at an RNA level (Cundliffe, 1986). It is likely that functional rRNA transitions do occur in the GTPase centre and are facilitated, in particular, by protein L11. They may have survived from a primitive, protein-free, ribosome where they occurred with a lower efficiency; this could explain, for example, why L11-minus mutants are viable but very sick (Cundliffe et al., 1979; Dabbs, 1979; Wienen et al., 1979). Nevertheless, the present results provide a different insight into how thiostrepton acts at the GTPase centre. They indicate that mutations at P18/22 of L11 alleviate drug-related functional effects without affecting the interaction of the antibiotic with the rRNA under conditions compatible with those occurring in vivo. This indicates that although rRNA provides a binding site for thiostrepton, the drug-rRNA interaction, itself, does not directly cause drug inhibition.

Therefore, we propose a model in which thiostrepton binds to the rRNA, in the close vicinity of L11, and interferes, in some way, with conformational transitions in the L11 protein by, for example, impeding the movement of the N-terminal domain relative to the RNA-binding C-terminal domain. Tyrosine 62 is protected by thiostrepton (Figures 5 and 6A) and is located at the junction of the N and C-terminal domains of L11 (Figure 8).

Finally, the key role for proline 18/22 in the inhibitory mechanism of thiostrepton provides insight into the thiostrepton-resistance of eukaryotic ribosomes (Gale et al., 1981). Despite the yeast L11-rRNA region carrying several sequence differences from those of archaeal/bacterial ribosomes, including A1067→G, these changes only account for a small decrease in drug-resistance (Thompson et al., 1988, 1993). However, examination of the available eukaryotic L11 sequences reveals that proline 18/22, that is common to the archaeal/bacterial domains, is absent and it is likely that this difference is mainly responsible for the high drug-resistance of eukaryotic ribosomes.
Materials and Methods

Sequencing of the L11 gene from thiostrepton-resistant H. halobium cells

Thiostrepton-resistant H. halobium cells containing no sequence changes within the GTase centre of 23 S rRNA were isolated and grown in liquid medium as described (Mankin et al., 1994). Chromosomal DNA from five independent isolates of thiostrepton-resistant cells were prepared (Ng et al., 1995) and the rplK gene, encoding protein L11, was retrieved by PCR using the primers GGC AAA TCC CGC TCG AAA GAC and GAA AGT ACA A (underlined) or GTC CGG CTC TGG GTC TTA GCC CCG TTA CAT (underlined, 94°C, five minutes); 30 cycles of 94°C (30 seconds), 48°C (30 seconds), and 72°C (90 seconds). The resulting PCR product was cut with BamHI and EcoRI (partially due to an internal EcoRI site) and cloned into the expression vectors PET-HTG and pGEX-GTH (Jensen et al., 1995a,b), yielding pET-HTG-L11 and pGEX-GTH-L11.

In order to produce mutations at proline 22, two PCR fragments of L11 were generated employing PET-HTG-L11 as the template, Pfu DNA polymerase, and the oligonucleotides GA AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined) and GTC CGG CTC TGG TGC AAC A (5'-phosphorylated)/GA AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 5'-phosphorylated, site of mutation is underlined) or GTC CGG CTC TGG TGC AAC G (5'-phosphorylated)/GAG AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 5'-phosphorylated) (data not shown). The PCR products were gel-purified, ligated with T4 DNA ligase and used as templates in a second PCR reaction using the oligonucleotides GAG GAT CCA TGG CTA AGA AAG TAC AA (BamHI site is underlined) and GAG AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 5'-phosphorylated) (data not shown). The PCR products were gel-purified, ligated with T4 DNA ligase and used as templates in a second PCR reaction using the oligonucleotides GAG GAT CCA TGG CTA AGA AAG TAC AA (BamHI site is underlined) and GAG AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 5'-phosphorylated) (data not shown). The PCR products were gel-purified, ligated with T4 DNA ligase and used as templates in a second PCR reaction using the oligonucleotides GAG GAT CCA TGG CTA AGA AAG TAC AA (BamHI site is underlined) and GAG AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 5'-phosphorylated) (data not shown). The PCR products were gel-purified, ligated with T4 DNA ligase and used as templates in a second PCR reaction using the oligonucleotides GAG GAT CCA TGG CTA AGA AAG TAC AA (BamHI site is underlined) and GAG AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 5'-phosphorylated) (data not shown). The PCR products were gel-purified, ligated with T4 DNA ligase and used as templates in a second PCR reaction using the oligonucleotides GAG GAT CCA TGG CTA AGA AAG TAC AA (BamHI site is underlined) and GAG AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 5'-phosphorylated) (data not shown). The PCR products were gel-purified, ligated with T4 DNA ligase and used as templates in a second PCR reaction using the oligonucleotides GAG GAT CCA TGG CTA AGA AAG TAC AA (BamHI site is underlined) and GAG AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 5'-phosphorylated) (data not shown). The PCR products were gel-purified, ligated with T4 DNA ligase and used as templates in a second PCR reaction using the oligonucleotides GAG GAT CCA TGG CTA AGA AAG TAC AA (BamHI site is underlined) and GAG AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 5'-phosphorylated) (data not shown).

Construction and mutagenesis of vectors expressing GST-L11 fusion proteins

The rplK gene of E. coli, encoding L11, was retrieved by PCR from E. coli DNA using Pfu DNA polymerase, and the oligonucleotides GAG GAT CCA TGG CTA AGA AAG TAC AA (BamHI site is underlined) and GAG AAT TCC GTC CTC CAC TAC CAG GCC (EcoRI site is underlined, 94°C, five minutes); 30 cycles of 94°C (30 seconds), 48°C (30 seconds), and 72°C (90 seconds). The resulting PCR product was cut with BamHI and EcoRI and cloned into the expression vectors PET-HTG and pGEX-GTH (Jensen et al., 1995a,b), yielding pET-HTG-L11 and pGEX-GTH-L11.

Isolation, RNA structural analysis and RNA footprinting of thiostrepton on H. halobium ribosomes

Ribosomes from wild-type H. halobium and the mutants P18 S and P18 T were isolated as described earlier (Levie et al., 1994). The yield of ribosomes corresponded to a concentration in vivo ≥1 μM (100% preparative yield would correspond to 1250 ribosomes/cell; rod-shaped cell, 0.75 μm × 3 μm). For RNA structural analysis and RNA footprinting of thiostrepton, 5 to 10 pmol of ribosomes or rRNA, prepared by phenol extraction of ribosomes, were used. Ribosomes were renatured for 20 minutes at 37°C in 100 μl of 70 mM Hepes-KOH (pH 7.8), 60 mM magnesium acetate, 3.0 M KCl and 1 mM DTT, while rRNA was renatured, in the same buffer, by incubating at 95°C (30 seconds), 65°C (ten minutes) followed by slow cooling to 37°C.

In experiments where thiostrepton (Sigma, dissolved in 100% DMSO) was included, 0.1 to 1000 pmol of the drug was added, to a final concentration of 1% DMSO, and the incubation at 37°C was continued for 20 minutes. Chemical and enzymatic probing were performed at 37°C with DMS (Merck, 2 μl 1:30 dilution in ethanol, 20 minutes), Kethoxal (ICN, 2 μl 40 mg/ml in 20% (v/v) ethanol, 20 minutes) and RNase T1 (Sigma, ten units, ten minutes). Following modification, samples were treated as described earlier (Rodriguez-Fonseca et al., 1995; Christiansen et al., 1990). Primer extension and RNA sequencing was performed using AMV reverse transcriptase (Amersham) and the (5'-32P)-end-labelled oligonucleotide GCCCGAT-CATTTGGGC (complementary to nucleotides 1214 to 1231 of H. halobium 23 S rRNA; Mankin & Kagaranovna, 1986) as primer (Christiansen et al., 1990).

Complexes of thiostrepton with L11-rRNA1 (0.12 μM) and the L11 fusion proteins GTH-L11, GTH-P22S, GTH-P22T (1.2 μM), described below, were also examined by footprinting in a volume of 100 μl of footprinting buffer (20 mM Hepes-KOH (pH 7.8), 170 mM KCl, 2.5 mM MgCl2, 0.5 mM EDTA, 10% (v/v) glycerol, 0.3 μg/ml bovine serum albumin, 0.2 μg/ml yeast tRNA and 0.05% (v/v) Triton X-100). Increasing amounts of thiostrepton were added and samples were treated with DMS (2 μl 1:30 dilution in ethanol, 20 minutes) at 37°C and analysed as described above using the (5'-32P)-end-labelled oligonucleotide CGGTTA CATCTTCCGGGC (complementary to nucleotides 1120 to 1137 of E. coli 23 S rRNA) as primer.

Expression, purification and end-labelling of GST-L11 fusion proteins

Cultures (500 ml) of E. coli BL21/DE3 containing either pET-HTG-L11, pGEX-GTH-L11, pGEX-P22S or pGEX-P22T were grown at 37°C in LB medium containing ampicillin (100 μg/ml) to an OD600 of 0.8. Isopropyl-1-thio-β-D-galactopyranoside (0.5 mM) was added and the incubation was continued for two hours. Fusion proteins were purified in a one-step procedure using glutathione Sepharose (Pharmacia) and stored in aliquots as described earlier (Lykke-Andersen et al., 1996). End-labelling with [γ-32P]ATP and bovine heart muscle kinase (Sigma) has also been described (Lykke-Andersen et al., 1996). In the polyvinyl chloride-exchanging experiments, 32P-end-labelled fusion proteins, HTG-L11, GTH-L11, GTH-P22S and GTH-P22T, were employed because thrombin produced secondary cuts within the L11 part of the fusion proteins during the obligatory incubation at 37°C.

Protein footprinting

The L11 binding site on E. coli 23 S rRNA was amplified by PCR, using the Pfu polymerase and the oligonucleotides TTT AGT GCC AAA CGA TGT (5'-phosphorylated) and GGG GAA TCC GTG CAC GGT TTA GCC CCG TTA CAT (EcoRI site is underlined). The
PCR product was cut with EcoRI and cloned into the StuI and EcoRI sites of pUT719 (Leffers et al., 1988). The resulting plasmid pUT7-L11-RNA1 was used as template for the phage T7 RNA polymerase (Milligan & Uhlenbeck, 1989) and the 138 nucleotide RNA fragment, named L11-RNA1, (identical to nucleotides 1016 to 1146 of E. coli 23 S RNA + seven extra nucleotides) was purified on a 10% polyacrylamide gel containing 7 M urea, eluted with phenol and ethanol-precipitated.

L11-RNA1 was renatured in 20 mM Hepes-KOH (pH 7.8), 2 mM MgCl₂, 170 mM KCl by incubating at 95°C (30 seconds) and 65°C (ten minutes) followed by slow cooling to 37°C. Samples (2µl) of renatured L11 RNA-1 (8 pmol), or renaturation buffer, were added to 1 to 5 pmol (ca 300 dps) of either 32P-labelled HTG-L11 or GTH-L11, which had been incubated for 15 minutes at 37°C in 11 µl of 1.6 x footprinting buffer (see above). After incubating the mixture for 30 minutes at 37°C, 5 µl of proteinase (diluted in double-distilled water) was added, resulting in final concentrations of 20 mM Hepes-KOH (pH 7.8), 2.5 mM MgCl₂, 170 mM KCl, 0.5 mM EDTA, 10% glycerol, 0.3 µg/µl bovine serum albumin, 0.2 µg/µl yeast tRNA and 0.05% Triton X-100. Proteinase digestion was continued for 15 minutes and terminated by adding 6 µl of 100 mM Tris-HCl (pH 8.3), 4% (w/v) sodium dodecylsulfate (SDS), 50 mM DTT, 0.2% (w/v) bromophenol blue and 40% glycerol. The samples were denatured for two minutes at 95°C before loading on a 20 cm x 40 cm x 0.04 cm 7% stacking/20% separation polyacrylamide/SDS-Tricin gel (Schaëgger & von Jagow, 1987).

Protein footprinting of thiostrepton complexed with L11 fusion proteins and L11-RNA1

Complexes of fusion proteins (HTG-L11, GTH-L11, GTH-P22S or GTH-P22T) and L11-RNA1 were formed as described above. Increasing amounts of thiostrepton were added at decreasing concentrations in 20% DMSO, such that the final concentration of DMSO was 1%, and the mixtures were incubated for 30 minutes at 37°C and then treated with proteinase as described above.

Acknowledgements

We thank Hoa Phan Thi-Ngoc for excellent technical assistance, Jens Lykke-Andersen for advice on protein footprinting and for critically reading the manuscript, Jørgen Kjems for providing plasmids and Dr Eric Cundliffe for endless helpful discussions about thiostrepton. This research was supported by the RNA Regulation Centre, the Novo-Nordisk Foundation and by the Danish Natural Science Research Council that also provided a PhD fellowship to B. T. P., and by N.I.H. grant number RO1GM53762 to A. S. M.

References


Edited by D. E. Draper

(Received 20 August 1997; received in revised form 10 November 1997; accepted 15 November 1997)