Interaction of Thiostrepton and Elongation Factor-G with the Ribosomal Protein L11-binding Domain*

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Ribosomal protein L11 and the L11 binding region of ribosomal RNA constitute an important domain involved in active functions of the ribosome during translation. We studied the effects of L11 knock-out and truncation mutations on the structure of the rRNA in this region and on its interactions with a translation elongation factor and the antibiotic thiostrepton. The results indicated that the structure of the L11-binding rRNA becomes conformationally flexible when ribosomes lack the entire L11 protein, but not when the C-terminal domain is present on ribosomes. Probing wild type and mutant ribosomes in the presence of the antibiotic thiostrepton and elongation factor-G (EF-G) rigorously localized the binding cleft of thiostrepton and suggested a role for the rRNA in the L11-binding domain in modulating factor binding. Our results also provide evidence that the structure of the rRNA stabilized by the C-terminal domain of L11 is necessary to stabilize EF-G binding in the post-translocation state, and thiostrepton may modulate this structure in a manner that interferes with the ribosome-EF-G interaction. The implications for recent models of ribosome translocation activity and factor interactions are discussed.

The region of the prokaryotic 50S ribosomal subunit associated with interactions of ribosome-dependent GTPase proteins such as elongation factors-G and -Tu (EF-G and EF-Tu), initiation factor-2, as well as with interactions with release factors-1 and -2 (RF1 and RF2) during translation is referred to as the GTPase-associated center or region (1). It contains three structural domains that are proximal on the 50S subunit: A pentameric complex (L10-L7/L12)2 that forms a protein stalk on the right shoulder of the 50S subunit (2, 3); the highly conserved sarcin-ricin stem-loop domain (nucleotides 2646–2674 in Escherichia coli) (4); and ribosomal protein L11 and its binding site on 23S rRNA (L11-binding domain, nucleotides 1051–1102 in E. coli), adjacent to the binding site of the pentameric complex.

Ribosomal protein L11 and its binding domain on 23S rRNA (L11-rRNA complex) are involved in thiiazole peptide antibiotic binding (thiostrepton and micrococcin) (5, 6), have been implicated in binding of EF-G to the ribosome (7–9), and affect translation termination (10, 11). Ribosomes lacking L11 are resistant to thiostrepton, show severely reduced levels of protein synthesis activity in vitro, and bind thiostrepton poorly relative to wild type ribosomes (12, 13).

Thiostrepton has been found to inhibit most factor-dependent processes of GTPase proteins (e.g. see Refs. 14 and 15; reviewed in Ref. 16) and the functions of some non-GTPase factors (RF1 (17), RF2 (17), and stringent factor, RelA (18)) on the prokaryotic ribosome. Kinetic studies by Rodnina et al. (14) indicate that thiostrepton binding to the L11-rRNA complex does not appear to interfere with factor binding or coupled GTPase activity on the ribosome, but it inhibits EF-G turnover subsequent to GTP hydrolysis (14). However, recent biochemical analysis by Cameron et al. (15) indicated that thiostrepton and the related thiiazole antibiotic, micrococcin, interfere directly with EF-G binding. Previous studies indicated that the effect of thiostrepton may be to prevent conformational transitions in either the RNA (19, 20) or L11 (1, 21) that are important for ribosome function. However, as thiostrepton appears to interact with both L11 and its 23S rRNA-binding domain (21–23), it is not clear if a function of the protein or the rRNA is affected. Such apparently conflicting results emphasize a need for determining the structural basis and conformational requirements of the L11-rRNA domain that govern factor interactions during translation and its inhibition by thiostrepton.

With this in mind, we examined the effects on 23S rRNA structure, thiostrepton binding, and EF-G interactions in E. coli mutants that lack functional endogenous L11 (11), mutants lacking L11 that were supplemented with an inducible plasmid bearing the entire L11 coding sequence (11), mutants bearing plasmids containing the coding sequence for C-terminal residues 68–142 of L11 (24), or mutants bearing a control plasmid without the L11 coding sequence (Table I). Important structural changes were identified by using chemical modification techniques and by comparing the results from the mutant ribosomes in the presence and absence of thiostrepton with results from ribosomes from the isogenic parent strain (Table I) and from ribosomes from which the ribosomal proteins had been extracted. To analyze the effects of the mutations on EF-G interactions with the ribosomes, we probed both pre- and post-translocation complexes of EF-G on the ribosome and compared the results for wild type and mutant ribosomes.

Our results revealed, not surprisingly, that the loss of ribosomal proteins induces extensive structural destabilization of the entire thiostrepton domain. Also, when all proteins except L11 are present, the L11-binding domain on the 50S subunit becomes conformationally flexible, but not on those ribosomes
TABLE I

| Strain       | Designation Relevant genotype Reference |
|--------------|-----------------------------------------|
| NVD001       | Wild type (wt) L11 chromosomal gene (ΔCAT) control plasmid | 11 |
| NVD002       | L11N-Δ plasmid expressing 76-amino acid CTD of L11 | 24 |
| NVD003       | L11-Δ plasmid wt L11 | 11 |
| NVD005       | L11 plasmid | 11 |

Thiostrepton and EF-G Interactions

**Strains used for isolation of 70S ribosomes with ribosomal protein L11 mutations**

**RESULTS**

Chemical Probing of L11 Mutant Ribosomes—To determine whether the loss or truncation of L11 induced the loss of other ribosomal proteins, particularly the adjacent pentameric complex (L10-L7L12) (2, 25, 35) from ribosomes, we compared the reactivities of several nucleotides in the region associated with pentameric complex binding, including C1044, A1046, G1047, A1050, G1110, A1111, and G1112. These reactivities did not differ between the wild type and mutant (L11N) ribosomes. Interestingly, probing of L11N ribosomes revealed only two nucleotides A1046, G1110, that had altered reactivities on L11 mutant ribosomes compared to wild type. These residues included those residues possibly involved in direct interactions with the protein (A1088, U1082, U1061, and A1070) (1, 39) and those that had already been identified as stable with the protein (A1088, U1082, U1061, and A1070) (1, 39). Interestingly, probing of L11N ribosomes revealed only two residues whose reactivities were significantly altered.

**Experimental Procedures**

**L11 Mutant Strains**—Bacterial strains used in the study were derived from *E. coli* K-12 (Table 1). Chromosomal L11 gene (ΔR) knock-out mutants of *E. coli*-carrying plasmids for inducible (Ptac promoter) expression of the entire L11 protein from *E. coli* (L11-Δ) (11) or harboring a plasmid encoding the sequence for inducible expression of the C-terminal domain of L11 (residues 180-187) of L11 (L11ΔCTD) were described previously (24). The L11 knock-out mutant carrying a control plasmid without the L11 gene (L11Δ) was constructed as described (11) but in an ampicillin resistance background (pACYC177). The strain without the L11 knock-out, but harboring a control plasmid (ΔpCAT) (11), was utilized as wild type for these studies.

Isolation of Mutant Ribosomes and Extraction of rRNA—E. coli strains harboring wild type, L11N, and L11-Δ ribosomes were grown in 1 mM isopropyl 1-thio-β-D-galactopyranoside and 7 μg/ml tetracycline and the strain harboring L1 ribosomes in 1 mM isopropyl 1-thio-β-D-galactopyranoside and 100 μg/ml ampicillin (L1 Δ). Ribosomes from each strain were purified from frozen cells essentially as described previously (27, 28). After isolation, L11N, L11ΔCTD, and wild type ribosomes could not be distinguished upon sucrose gradient centrifugation or analytical ultracentrifugation (data not shown), indicating that mutant ribosomes assembled normally. Naked rRNAs were prepared by phenol-chloroform extraction as described (29). Prior to probing, ribosomes (25 pmol) were activated by incubation for 15 min at 37 °C in 20 mM Hepes (pH 7.6), 5 mM MgOAc, 100 mM KCl, and 1 mM dithiothreitol buffer (H4M6K3D1) with or without the addition of thiostrepton in MeSO to the desired final concentration (1% MeSO). Controls were made 1% in MeSO.

**Chemical Probing**—Chemical probing of dimethyl sulfite (DMS), 2-keto-3-ethoxybutyraldehyde (kethoxal), or 1-cyclohexy1-2-thioxo-1-benzothiazoline-3(2H)-oxazine (CMCT) were carried out in a manner adapted from Christensen et al. (30): 1 μl of 1.10 dilution of DMS in EtOH, 50 μl of 42 mg/ml CMCT in 1× H2M6K3D1 buffer, or 5 μl of 40 mg/ml kethoxal in 20% EtOH was added to samples followed by incubation for 5 min for DMS samples, 30 min for DMCT samples, or 10 min for kethoxal samples, respectively, at 37 °C. The samples were precipitated and phenol-chloroform was extracted in preparation for use as templates for reverse transcriptase primer extension and PAGE analysis (31, 32). Probing of thiostrepton titrations was carried out in the same manner as above with the addition of increasing amount of antibiotic in 100% MeSO (final concentration of MeSO was 2% in reactions to aid thiostrepton solubility at higher concentrations).

**Purification of EF-G**—From *E. coli*-His6-tagged EF-G on a pET24b-fusA plasmid was a generous gift of K. Lieberman and H. F. Noller and overexpressed in *E. coli* strain BL21(DE3) by growing in LB with 30 μg of kanamycin at 37 °C to late log phase, inducing expression with the addition of 1 mM isopropyl 1-thio-β-D-galactopyranoside, and growth for 4 more hours. Cells were harvested by centrifugation and frozen for storage. His6-EF-G was isolated as described (33, 34) with the following modifications: 1) cell lysis was performed by grinding 10 g of frozen cells with 20 g of baked aluminia; 2) EF-G bound to a nickel-nitriolithioc acid column was washed with 25 ml of buffer containing 10 mM imidazole (pH 8) before elution of protein.

**Binding of EF-G Complex to 70S Ribosomes**—Increasing concentration of ribosomes in 50 μl of 1× H2M6K3D1 buffer were incubated at 37 °C for 10 min with 0.5 μg/μl poly-U mRNA (Sigma) followed by the addition of mRNA25 to 1.5 μM and incubation at 37 °C for 10 min. To these pre-translocation complexes were added fusidic acid to 0.2 mM, [α-32P]GTP (Amersham Biosciences) to 0.5 mM, and EF-G to 2 μM, followed by incubation at 37 °C for 10 min. 45 μl of the reaction was filtered through 45-μM nitrocellulose filters and washed with 1 ml of ice-cold 1× H2M6K3D1 buffer. The percentage of ribosomes with bound EF-G complex was quantified by scintillation counting of washed filters and by calculating the concentration of retained radioactive signal from GDP in complex on ribosomes relative to controls incubated in the absence of ribosomes.

**Probing EF-G-ribosome Complexes**—Fusidic acid or GDPNP-stabilized EF-G-ribosome complexes were constructed in a manner similar to Moazed and Noller (8). Briefly, 0.5 μM 70S wild type or mutant ribosomes in 1× H2M6K3D1, buffer were incubated for 10 min at 25 °C with 5 μg of poly-U mRNA (Sigma), followed by 10 min at 25 °C with 1.5 μM deacylated tRNA25 from *E. coli* (Sigma). To these complexes were added either 0.5 mM GDP and 0.2 mM fusidic acid (Sigma) or 0.5 mM GDPNP (Sigma), followed by addition of EF-G to 2.5 μM and incubation at 25 °C for 10 min. The complexes were probed with DMS, kethoxal, and CMCT as above with minor deviations; probing temperature was 25 °C and no MeSO was added to the reactions. Preparation of rRNA and primer extension analysis for probing reactions was as above for thiostrepton experiments.
nucleotides for which changes in reactivity could be traced exclusively to truncation of the N-terminal domain of L11. Nucleotide U1061 became hyper-reactive to CMCT in the absence of the N-terminal domain of L11 but displayed reactivity only slightly greater than that of wild type ribosomes for L11\textsubscript{-/-} ribosomes (Fig. 1b). This could be explained if U1061 is exposed...
in the rRNA structure stabilized by the C-terminal domain of L11, but is partially buried when the structure shifts in response to the absence of L11. Nucleotide A1070 became more reactive to DMS both in L11N/H11002 and L11/H11002 ribosomes (Fig. 1a). Changes in reactivity on L11N/H11002 and L11N/H11002 ribosomes were not found outside of the L11-binding region of the rRNA.

**Thiostrepton Interactions with Mutant Ribosomes**—Chemical probing in the presence of thiostrepton on wild type ribosomes indicated protections at several residues previously associated with binding of the antibiotic (5, 25) and at several additional sites (Table II), including U1061 (CMCT), G1071 (Keth/DMS), and U1097 (CMCT). Probing of L11N ribosomes, L11/H11002 ribosomes, and the extracted rRNA from ribosomes (Fig. 1, lanes 11–13) revealed only partial protection for L11N ribosomes (40–50% protection relative to wild type at most residues) and L11− ribosomes (10–20%), and less than 10% protection for extracted rRNA relative to wild type in the presence of thiostrepton under the probing conditions. This is consistent with previous studies that showed a profound reduction in the affinity of thiostrepton for 23S rRNA versus intact ribosomes (6, 20). Therefore, we anticipated saturation of thiostrepton protection near wild type levels for mutant ribosomes at higher levels of antibiotic. However, protection levels on mutant ribosomes never reached the levels of wild type ribosomes. This may be explained if antibiotic binding on wild type ribosomes is dependent upon interactions with the N-terminal domain of L11 (21, 26), and, therefore, the binding interaction differs for mutant ribosomes.

Although many of the residues protected by thiostrepton on wild type ribosomes were at least partially protected on L11N− ribosomes and L11− ribosomes, several residues (U1061, U1097, and A1070) did not appear to be (Fig. 1, lanes 11 and 12). This may be attributable to an interaction between the
N-terminal domain of L11 and the rRNA upon thiostrepton binding, as is suggested in the crystal structure of the L11-rRNA complex (1) (see also Fig. 6B). To test this, we added increasing amounts of thiostrepton to wild type and mutant ribosomes and probed them with modifiers as above. The results from a representative experiment for protection at A1067 are shown in Fig. 3. In most cases, wild type ribosomes were protected more than mutant ribosomes at a given concentration of thiostrepton, saturating above 5 μM (Fig. 3). Both U1061 and A1070 are protected by thiostrepton on wild type and L11N- ribosomes. However, that protection is completely lost on L11- ribosomes. Nucleotide U1097 is the only residue that is protected by thiostrepton on wild type ribosomes, but that is not protected on either L11N- or L11- ribosomes. This indicates that tightening of the L11-rRNA junction probably occurs only near the proximal apices of the A1 and C stem-loops of the rRNA and the N-terminal domain of L11 (Fig. 6B).

### EF-G Interactions with Mutant Ribosomes

As the L11-binding region of the 70S ribosome is known to participate in binding of translation elongation factors, we sought to study the effects of the L11 deletion and truncation mutations on the interactions of EF-G with ribosomes. To do this, we utilized two

#### Table III

| rRNA nucleotide | Probing reagent | Protection* by EF-G-GDPNP | Protection by EF-G-GDP-FA |
|-----------------|-----------------|---------------------------|---------------------------|
|                 |                 | 70S WT | 70S L11N- | 70S L11- | 70S WT | 70S L11N- | 70S L11- |
| U1061           | CMCT            | 2 ± 6  | 5 ± 8     | 5 ± 9     | 27 ± 6 | 4 ± 11 | 6 ± 9  |
| A1067           | DMS             | 17 ± 5 | 23 ± 7    | 8 ± 6     | 41 ± 8 | 32 ± 9 | 4 ± 8  |
| A1069           | DMS             | 3 ± 6  | (7 ± 6)*  | 9 ± 12    | 4 ± 8  | 10 ± 9 | 8 ± 9  |
| U1097           | CMCT            | (4 ± 8)* | 2 ± 7 | 7 ± 7 | (15 ± 5)* | 4 ± 12 | 9 ± 7  |
| G2655           | Kethoxal        | 43 ± 9 | 37 ± 7    | 15 ± 10   | 57 ± 8 | 49 ± 8 | 3 ± 8  |
| A2660           | DMS             | 53 ± 7 | 24 ± 5    | 19 ± 7    | 46 ± 5 | 49 ± 5 | (3 ± 6)* |
| G2661           | Kethoxal        | 36 ± 10 | 44 ± 8 | 13 ± 8 | 52 ± 9 | 56 ± 6 | 1 ± 5  |

* Protection is relative to the reactivity of ribosomal residues in the absence of added EF-G (see "Experimental Procedures" for complex constituents). Values are an average of three experiments. Corrections for background and standard deviation are as described in the Fig. 3 legend.

Parentheses indicate an increase in reactivity relative to controls in response to complex binding. CMCT, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-p-toluenesulfonate; DMS, dimethyl sulfate; kethoxal, 2-keto-3-ethoxybutyraldehyde; GDPNP, guanylyl-5'-imidodiphosphate; WT, wild type; L11N-, 70S ribosomes with only the C-terminal domain residues 68–142 of ribosomal protein L11 present; L11-, ribosomes lacking the entire L11 protein; EF-G, elongation factor-G.
approaches. First, we used fusidic acid, an antibiotic that binds to the EF-G-GDP-ribosome complex and prevents dissociation of EF-G from the ribosome following GTP hydrolysis, stalling the complex in a post-translocation intermediate state (40). Second, we used guanosine 5'-[β,γ-imido]triphosphate (GDPNP), a non-hydrolyzable nucleotide analog that binds EF-G, prevents coupled GTP hydrolysis on the ribosome, and stalls the ribosome complex in a pre-translocation intermediate state (34, 41). By adding EF-G along with each of these components to translocation-competent wild type or mutant ribosomes (70S plus poly-U mRNA plus tRNA), we were able to compare the interactions of EF-G with the factor binding domains of the large ribosomal subunit in both the pre- and post-translocation states.

Several residues in the L11-binding region (Table III) showed changes in reactivity to chemical modifiers upon the addition of the EF-G complexes to wild type and mutant ribosomes. A previous report identified A1067 and A1069 as residues that are protected upon binding EF-G to wild type ribosomes (8). Our probing identified protection at A1067 for both fusidic acid-stabilized and GDPNP-stabilized complexes (Fig. 4b, Table III). The reactivity of U1061 was increased in the presence of the EF-G-GDP-fusidic acid complex (Fig. 4a), and U1097 showed a slight increase in reactivity on wild type ribosomes upon the addition of the fusidic acid, but not the GDPNP-stabilized complex (Fig. 4c).

L11N ribosomes showed protection at a level similar to wild type at A1067 for both the fusidic acid and GDPNP-stabilized complexes (Fig. 4b). However, L11N ribosomes were not protected at U1061 or U1097 in the presence of either complex (Fig. 4a and c), suggesting that the N-terminal domain of the protein is required to protect these residues on wild type ribo-
Our results for L11\textsuperscript{−} and L11N\textsuperscript{−} ribosomes indicate that the RNA in the L11-binding region becomes conformationally flexible only in the absence of the entire L11 protein, and not when the C-terminal domain is present. Therefore, the results do not support the model in which reversible dissociation of the N-terminal domain of L11 governs a functional transition in the structure of the associated rRNA (20). But, the results do favor a model in which the N-terminal domain of L11 modulates direct interactions of L11 with factors (9, 21), whereas the C-terminal domain stabilizes the conformation of the L11-binding region of the rRNA in the post-translocation state. Therefore, we utilized filter-binding analysis to determine the extent of binding of radiolabeled EF-G-GDP-fusidic acid complex on wild type and mutant ribosomes. Fig. 5 illustrates that wild type and L11N\textsuperscript{−} ribosomes bind the complex to similar extents, whereas binding to L11\textsuperscript{−} ribosomes is only slightly above background levels. Because the only difference between L11N\textsuperscript{−} and L11\textsuperscript{−} ribosomes is the presence of the C-terminal domain, the results further establish the importance of this domain of L11 for EF-G binding in the post-translocation state.

**Discussion**

**Modulation of rRNA Structure in the L11-binding Domain—** The results of this study suggest that L11 binding stabilizes key tertiary interactions in the rRNA structure around the L11-binding domain. For instance, nucleotides G1071 and A1089, involved in two important base triple interactions (G1071:A1089:C1100; A1089:A1090:U1101 (1, 39)), became reactive in the absence of L11. In addition, bases U1066, U1083, and U1094, each involved in a U-turn motif (42), became reactive on L11 ribosomes. Finally, G1068 and A1096 became reactive in ribosomes lacking L11. G1068, in the A1 stem-loop, normally interacts with the phosphate backbone of A1096 in the C stem-loop, stabilizing the juxtaposition of the two stem-loops (1).

L11 binding also protected A1088 and U1092 from chemical modification. The A1088:U1090 reverse-Hoogsteen base pair is a universally conserved feature of this RNA domain and probably stabilizes the long-range interactions of the A1 and C helices. The reverse-Watson-Crick U1082:A1086 pair closes the short junction loop and participates in a ribose zipper-minor groove interaction that stabilizes the interaction between the junction loop and the B helix (1). Taken together, these results indicate that L11 binding to the RNA combines stabilization of key tertiary interactions with stabilization of the rRNA backbone fold, a strategy that has also been documented for another ribosomal protein, S15 (43).

Our results for L11\textsuperscript{−} and L11N\textsuperscript{−} ribosomes indicate that the RNA in the L11-binding region becomes conformationally flexible only in the absence of the entire L11 protein, and not when the C-terminal domain is present. Therefore, the results do not support the model in which reversible dissociation of the N-terminal domain of L11 governs a functional transition in the structure of the associated rRNA (20). But, the results do favor a model in which the N-terminal domain of L11 modulates direct interactions of L11 with factors (9, 21), whereas the C-terminal domain stabilizes the conformation of the L11-binding rRNA. This would explain why nucleotides U1061 and A1070, which are packed against Gln-12 and Lys-10 at the junction of the L11 N-terminal domain and the L11-binding RNA (Fig. 6c), become hyper-reactive in L11N\textsuperscript{−} mutants.

**Thiostrepton Interactions with rRNA in the L11-binding Domain—** Many protections from thiostrepton binding occur in the binding cleft between the proximal apices of the A1 and C stem-loops of the RNA (Fig. 6a). However, the known thiostrepton protection at A1070 and novel protections found in this study (U1061 and U1097) suggested that thiostrepton binding may induce a tightening of the junction between the RNA and the N-terminal domain of L11. However, by titrating thiostrepton to saturating concentrations in the probing reaction, we discovered that only the protection of U1097 was specifically dependent upon the presence of the N-terminal domain of L11. As the concentration of thiostrepton was increased, U1061 and A1070 became protected on L11N\textsuperscript{−} ribo-

**Fig. 5. Binding of EF-G complex to wild type and L11-mutant ribosomes.** Binding of EF-G-GDP-fusidic acid (as percentage of ribosomes with bound complex relative to controls without added ribosomes) was quantified by filtering complexes of increasing concentrations of wild type or mutant ribosomes with poly-U mRNA (0.5 μg/μl), tRNA\textsuperscript{3H} (1.5 μM), EF-G (2 μM), [γ-\textsuperscript{32P}]GTP (0.5 μM), and fusidic acid (0.2 mM) through nitrocellulose filters and scintillation counting of radioactivity left on the filters (see “Experimental Procedures” for details). Results are an average of ≥2 experiments with standard deviations for experiments are shown. WT, wild type 70S ribosomes; L11N\textsuperscript{−}, 70S ribosomes with only the C-terminal domain (residues 68–142) of ribosomal protein L11 bound; L11\textsuperscript{−}, 70S ribosomes lacking L11.
somes, but not on L11 ribosomes. Therefore, protection of these two residues is dependent upon the presence of only the C-terminal domain of L11. Because the C-terminal domain of L11 is known to be responsible for binding and stabilization of the rRNA structure in the L11 binding region (44), we propose that only this stabilized structure of the L11-binding domain presents A1070 and U1061 in the orientation necessary for antibiotic interactions. Conversely, thiostrepton binding to the rRNA may induce a conformational change around U1061 and A1070 that protects these residues from modification. This protection does not occur in the absence of L11, or at least the C-terminal portion of L11.

Additional changes in reactivity induced by thiostrepton involve residues adjacent to, but probably outside of, the binding site. The changes in reactivity of G1071, part of a crucial base triple at the junction of the four helices (Fig. 2a) that stabilizes the rRNA fold (19), changes at U1061 and A1070, and several thiostrepton-induced reactivity changes on the rRNA surface opposite the L11 interaction (Fig. 6B) suggest that either thiostrepton spans the two surfaces of the rRNA, or, more likely, binding induces small structural changes on both faces of the A1 and C stem-loops. Recent cryo-electron microscopy and biochemical studies (9, 15) that suggested thiostrepton and EF-G may compete for binding to the same location (L11-rRNA) on the 50S ribosomal subunit would explain the modulation of reactivity by thiostrepton of A1067 on the opposite face of the

![Chemical modification protection of rRNA by thiostrepton shown on the crystal structure of the rRNA fold (Protein Data Bank: 1MMS (1)). A, protections on the top face of the L11-binding domain rRNA in the cleft between the A1 and C stem-loops. Blue, previously identified protections; green, novel protections from this study. (Residue numbers (E. coli numbering) and atom positions are labeled.) B, protections on the bottom face (opposite side of structure shown) of the L11-binding domain rRNA. C, potential interactions of rRNA bases with residues from the N-terminal domain of L11 (N-terminal residues shown in wireframe presentation for clarity, and the coloring and labeling are as in A and B). A mercury ion, present in the crystal structure, suggests a potential ion mediated interaction between U1097 and the L11. The graphics were made using RASMOL (49).](http://www.jbc.org/content/2941/45/2941/F6)
L11-rRNA complex (Fig. 6B), which is protected upon binding of EF-G (8). Interestingly, both thiostrepton and EF-G binding modulate the reactivity of U1061 near the junction of the rRNA and the N-terminal domain of L11 (Fig. 6C), several angstroms from the presumed binding site for both antibiotic and factor. A recent NMR study described docking of thiostrepton onto the crystal structure of the L11-binding rRNA (26). The authors postulated that the likeliest direct interactions of thiostrepton with 23S rRNA are with residues A1067, A1095, and A1096 at the apices of the A1 and C stem-loops (Fig. 6A). This provides further evidence that the distal surfaces of the rRNA must both be indirectly but significantly altered by antibiotic binding. These alterations could be substantial enough to account for the effect of the antibiotic on factor interactions.

**Interactions of EF-G with the L11-binding Domain**—The evidence outlined above provides important insight into the function of the L11-binding domain of large subunit rRNA. Although it has long been suspected that this domain forms part of the factor binding site or GTPase-associated domain on the large subunit, little is known about the structural requirements for this function. Previous reports suggested that EF-G may undergo GTPase-associated conformational changes on the ribosome in the process of stimulating translocation of tRNAs from the peptidyl (P) site to the exit (E) site and acceptor (A) site to the P site (45, 46). Such changes would probably require distinctive modes of interaction with the ribosome in the pre- and post-translocation states. Our results corroborate data from a recent report (34) that described increases in EF-G protections from hydroxyl radicals in the L11-binding domain rRNA following translocation and GTP hydrolysis (fusidic acid-stalled complex). In our study, protection of A1067 in this domain is 50% higher in the presence of this complex versus the pre-translocation (GDPNP-stalled) complex. This difference may be associated with a shift in domain V of EF-G toward the L11-binding domain following translocation seen in recent cryo-electron microscopic studies (45, 46). The present study also provides evidence that the N-terminal domain of L11 probably reversibly dissociates from the L11-binding rRNA in a step following translocation. Nucleotide U1061, a residue closely associated with the N-terminal domain of L11 in an x-ray crystal structure of the ribosomal fragment containing L11 and the L11-binding domain rRNA (1), becomes reactive to chemical probes both in L11N/H11002 mutants and when EF-G is stalled on the ribosome in complex with GDP and fusidic acid (Figs. 2 and 5). However, this reactivity is not evident on ribosomes lacking bound EF-G-GDP-fusidic acid, nor on ribosomes with EF-G bound in the pre-GTPase state (EF-G-GDPNP). A similar change is postulated from the results of a cryo-electron microscopic study of the same complex (9).

Our results with mutants of ribosomal protein L11 suggest that stabilization of the post-translocation state is acutely dependent upon a specific structure of the L11-binding domain rRNA that is stabilized by its interaction with the C-terminal domain of L11 (Fig. 7). Only ribosomes with the C-terminal domain present are able to form substantial post-translocation complexes. Protections of rRNA in the sarcin-ricin domain by the pre-translocation complex are also reduced significantly (Fig. 4; A2660, G2655, and G2661) on ribosomes lacking L11, suggesting that the structure of the L11-binding domain is involved in stabilization of the pre-translocation state as well.
Previously it was also suggested that the proximal L10-binding domain in the region around the A1050 internal loop (Fig. 2) may also be associated with pre-translocation complex formation (34). The significance of these structural transitions is not fully understood. Changes in the conformation of EF-G on the ribosome are postulated to induce changes in ribosome structure that drive the process of translocation (46). The structural basis of these conformational changes and concomitant structural changes at the molecular level will be the subject of future studies.

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Interaction of Thiostrepton and Elongation Factor-G with the Ribosomal Protein L11-binding Domain

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