A Base Substitution within the GTPase-associated Domain of Mammalian 28 S Ribosomal RNA Causes High Thiostrepton Accessibility*

Toshio Uchiumi, Akira Wada, and Ryo Kominami

From the Department of Biochemistry, Niigata University School of Medicine, Niigata 951 Japan and the Department of Physics, Faculty of Science, Kyoto University, Kyoto 606-01 Japan

A number of antibiotics bind to prokaryotic ribosomes and interfere with protein synthesis. These antibiotics have been used as powerful tools for dissecting the translational mechanism (1–3). Thiostrepton, one of such compounds, binds tightly to the 50 S ribosomal subunit with 1:1 stoichiometry (4) and is used as powerful tools for dissecting the translational mechanism. Thiostrepton, one of such compounds, binds tightly to the 50 S ribosomal subunit with 1:1 stoichiometry (4) and is used as powerful tools for dissecting the translational mechanism. Thiostrepton, one of such compounds, binds tightly to the 50 S ribosomal subunit with 1:1 stoichiometry (4) and is used as powerful tools for dissecting the translational mechanism.

A molecular basis for the insensitivity of eukaryotic ribosomes to the antibiotic thiostrepton was investigated using synthetic 100-nucleotide-long fragments covering the GTPase domain of 23/28 S rRNA. Filter binding assay showed no detectable binding of the rat RNA to thiostrepton, but the binding capacity was markedly increased by base substitution of G\textsuperscript{1067} to A at the position corresponding to 1067 of Escherichia coli 23 S rRNA. The association constant (K\textsubscript{a}) for the rat A\textsuperscript{1067} mutant was 0.60 \times 10\textsuperscript{10} M\textsuperscript{-1}, which was comparable with that of the E. coli RNA (K\textsubscript{a} = 1.1 \times 10\textsuperscript{10} M\textsuperscript{-1}). This suggests that the eukaryotic G\textsuperscript{1067} participates in the resistance for thiostrepton. On the other hand, the RNA fragments of the two species had a similar binding capacity for E. coli ribosomal protein L11 and its mammalian homologue L12. Gel electrophoresis under a high ionic condition, however, revealed a difference between the two proteins. E. coli L11 formed stable complexes with both the E. coli RNA and the rat A\textsuperscript{1067} mutant RNA in the presence of thiostrepton, while rat L12 failed to exhibit such complex formation. This suggests that the eukaryotic L12 protein may also be an element giving the resistance for thiostrepton. These results are discussed in terms of preserved three-dimensional conformation of the RNA backbone between prokaryotes and higher eukaryotes.

A number of antibiotics bind to prokaryotic ribosomes and interfere with protein synthesis. These antibiotics have been used as powerful tools for dissecting the translational mechanism (1–3). Thiostrepton, one of such compounds, binds tightly to the 50 S ribosomal subunit with 1:1 stoichiometry (4) and inhibits ribosome-associated GTPase events (2). The primary target of thiostrepton lies within a limited region comprising residues 1052–1112 in domain II of 23 S rRNA, termed the “GTPase domain” (5, 6). Binding of thiostrepton to this RNA domain is greatly enhanced by an association of Escherichia coli ribosomal protein L11 with a region encompassing the thiostrepton site (7), presumably by stabilizing and/or adjusting RNA structure (8). The residue A\textsuperscript{1067} in this domain plays an important role in the drug binding, i.e. methylation of the 2’-O of this residue renders ribosomes resistant to thiostrepton (9); base substitutions at this residue reduce the affinity for thiostrepton (6, 10–12); the antibiotic protects the N1 position of A\textsuperscript{1067} from chemical modification (5). On the other hand, the base A\textsuperscript{1067} is known as a plausible site of direct interaction with elongation factor EF-G (13, 14).

Eukaryotic ribosomes are totally resistant to thiostrepton despite a high level of conservation of primary and secondary structure at the target RNA region (15). It is noteworthy that the base at the position equivalent to E. coli A\textsuperscript{1067} is replaced to G in the eukaryotic GTPase domain. This G base is specifically protected from chemical modification by the binding of eukaryotic elongation factor EF-2 (16). Furthermore, the G base is the element required for recognition by anti-28 S autoantibody, which has a preference for the eukaryotic GTPase domain (16). These data imply that the G base at the 1067 equivalent position is an important identity element of the eukaryotic domain that is involved in the resistance for thiostrepton. To know whether this is the case, we tested an effect of substitution of A for the G base in the eukaryotic RNA domain on the thiostrepton binding. Here, we show that the binding affinity of the rat RNA for thiostrepton increases to a level comparable to that of E. coli wild type by a single G to A substitution. Also, effects of L11 and its mammalian homologue L12 on the drug binding are presented.

**MATERIALS AND METHODS**

Ribosomes and Ribosomal Proteins—Preparation of rat liver ribosomes (17) and purification of the L12 protein (18) was performed as described previously.

The prokaryotic 50 S ribosomal subunits were prepared from E. coli W3110 strain, as described previously (19). The total proteins (TP50) were extracted from the subunits with 66% acetic acid, 33 mM MgCl\textsubscript{2} (20) and recovered by precipitation with 7 volumes of cold acetone. The TP50 was fractionated by a stepwise elution from CM-cellulose (Whatman) column equilibrated with a buffer consisting of 6 M urea, 5 mM 2-mercaptoethanol, and 20 mM sodium acetate, pH 4.6. A protein fraction enriched with E. coli L11 was eluted with the same buffer containing 75 mM LiCl. L11 was further purified by high performance ion-exchange chromatography; the protein fraction was applied to a CM-SPW column (Tosoh) equilibrated with a buffer consisting of 6 M urea, 5 mM 2-mercaptoethanol, 40 mM LiCl, and 20 mM sodium phosphate, pH 6.5, and eluted with a linear gradient of 40–200 mM LiCl. Purity and identity of the final L11 sample was ascertained by two-dimensional polyacrylamide gel electrophoresis (19). The proteins were concentrated with Centriplus-10 (Amicon) and dialyzed against the renaturation buffer (300 mM KC\textsubscript{1}, 5 mM 2-mercaptoethanol, and 20 mM Tris-HCl, pH 7.5).

Plasmid Construction and Site-directed Mutagenesis—The DNA fragment containing residues 1841–1939 of rat 28 S rRNA and the fragment corresponding to residues 1029–1127 of E. coli 23 S rRNA were amplified by the polymerase chain reaction (21) and inserted into HindIII and XbaI sites of an expression vector, pSP78 (Boehringer Mannheim). Base substitution was performed by oligonucleotide-directed mutagenesis using polymerase chain reaction (22). Oligonucleotides used were synthesized on an Applied Biosystems 380B synthesizer. All of the cloned DNA sequences were verified by dideoxy sequencing (23).

In Vitro Transcription—The plasmid DNA were linearized with XbaI and transcribed with SP-6 RNA polymerase in the presence of [\textsuperscript{32P}]UTP.
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RESULTS

Synthetic small RNA fragments covering the GTPase domain of E. coli 23 S rRNA bind to thiostrepton with almost the same affinity as full-length 23 S rRNA (6, 11). An RNA fragment containing residues 1841–1939 of rat 28 S rRNA (Fig. 1A) and one containing residues 1029–1127 of E. coli 23 S rRNA (Fig. 1B) were synthesized using a SP-6 transcription system, and the two RNAs were subjected to a filter binding assay. Fig. 2A shows a titration of the E. coli wild-type RNA with thiostrepton. The apparent association constant was 1.1 \times 10^{6} M^{-1} as determined by double reciprocal plot of the binding data (Fig. 2C). This is comparable to values previously estimated by other workers using either the equilibrium dialysis (2) or the filtration technique (11). Rat wild-type RNA, however, showed no detectable binding at concentrations up to 2 \mu M of thiostrepton (Fig. 2B) and even at 16 \mu M (not shown). The binding could not be tested at concentrations higher than 16 \mu M because of low solubility of thiostrepton. Hence, we failed to determine the binding constant.

Data from footprinting (5) and site-directed mutagenesis (11) suggest that bases A_{1067} G_{1878}, A_{1095}, and A_{1098} within the E. coli domain interact with thiostrepton directly. Among these bases only A_{1067} is unique to prokaryotic RNA domain; the equivalent position in eukaryotic 28 S rRNA has G (G_{1878} in rat). The other bases are preserved between prokaryotes and eukaryotes (see Fig. 1). We therefore reasoned that the eukaryotic G_{1878} at the equivalent position of E. coli 1067 site may be a key element giving the resistance for the thiostrepton binding. To test this, we synthesized RNAs with base substitutions of A_{1067} to G in E. coli RNA and G_{1878} to A in rat RNA and examined their effects on thiostrepton binding. The A_{1067} to G substitution in E. coli RNA significantly weakened the binding affinity; no detectable binding was observed in the filter binding analysis used here (Fig. 2A). The reciprocal substitution, G_{1878} to A in rat RNA, greatly increased the binding capacity (Fig. 2B). The estimated K_{s} value for the rat A_{1878} variant was 0.6 \times 10^{6} M^{-1} (Fig. 2C), which was close to the value for the E. coli wild type (1.1 \times 10^{6} M^{-1}). The results indicate that this substitution results in the up-mutation of thiostrepton binding and suggest that the A_{1067} base participates in the drug binding.

The RNA fragments were also tested for their binding capacity to E. coli ribosomal protein L11 (Fig. 3A) and its rat homologue L12 (Fig. 3B) by filter binding assay. E. coli L11 bound not only to the E. coli RNA (K_s = 1.0 \times 10^{7} M^{-1}) but also to the rat RNA (K_s = 0.44 \times 10^{7} M^{-1}). Similarly, rat L12 bound to both the rat RNA (K_s = 1.4 \times 10^{7} M^{-1}) and the E. coli RNA (K_s = 1.5 \times 10^{7} M^{-1}) with almost the same affinity. Either of the base substitutions, E. coli A_{1067} to G and rat G_{1878} to A, gave no significant effect on the binding of E. coli L11 and rat L12 (not shown). In these protein binding experiments, we used rat S1 RNA variant (18) as a negative control. This RNA contains a mutation of U_{1871} to A, which leads to disruption of rat L12 binding (18), probably due to perturbation of the conserved bulge structure.

It has been shown that the binding of E. coli L11 protein to
the GTPase domain greatly enhances the accessibility to thiostrepton and that thiostrepton is also able to stabilize the binding of L11 (7). To analyze such cooperativity of the RNA

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FIG. 2. Binding of ribosomal proteins to the RNA fragments. Increasing concentrations of E. coli L11 protein (A) and rat L12 protein (B) were incubated with the E. coli wild-type RNA (●), the rat wild-type RNA (○), and rat S-1 variant (▲). The S-1 variant has A in the position of U1871 and used as a negative binding probe (see text). RNA-protein binding was analyzed as described in the legend for Fig. 2.

FIG. 3. Effect of base substitutions on the thiostrepton binding: A1067 to G of the E. coli RNA (A) and G1876 to A of the rat RNA. A and B, thiostrepton binding curves for the E. coli RNA fragment and its G1067 variant (A) and the rat RNA fragment and its A1876 variant (B). Increasing concentrations of thiostrepton were incubated with 0.1 μM of each 32P-labeled RNA: the E. coli wild-type RNA fragment (E. coli-WT, ●), the E. coli G1067 variant (E. coli-G, □), the rat wild-type RNA fragment (Rat-WT, □), and the rat A1876 variant (Rat-A, ●). Each reaction mixture was applied to a nitrocellulose filter. Binding is expressed as retention, corresponding to a ratio between the radioactivity retained on the filter and the input (25). C, double reciprocal plot of RNA-thiostrepton binding data. The reciprocal of retention given in A and B (the mean of three experiments) was plotted as a function of the reciprocal of the thiostrepton concentration. The apparent association constants (K_a) were estimated from slopes of lines for the E. coli wild-type RNA (E. coli-WT) as 1.1 × 10^6 M^-1 and for the rat A1876 RNA (rat-A, □) as 0.6 × 10^5 M^-1, based on a relation of slope = 1/K_a (35).

FIG. 4. Gel retardation analysis of the RNA-protein complex stabilized with thiostrepton. The E. coli wild-type 32P-labeled RNA was incubated at various combinations of the ribosomal proteins and thiostrepton, as indicated above gel lanes: without protein (lanes 1 and 2), with 0.25 μg of E. coli L11 (lanes 3 and 4), or 0.3 μg of rat L12 (lanes 5 and 6). The incubation was done in the presence of 20 pmol of thiostrepton (lanes 2, 4, and 6) or in the absence (lanes 1, 3, and 5). The complexes were analyzed on 6% nondenaturing polyacrylamide gels containing 0.3 M KCl, 10 mM MgCl2, and 50 mM Tris-HCl, pH 8.0.

the GTPase domain greatly enhances the accessibility to thiostrepton and that thiostrepton is also able to stabilize the binding of L11 (7). To analyze such cooperativity of the RNA-L11-thiostrepton complex, we used a gel mobility shift analysis in an electrophoresis buffer containing 0.3 M KCl. Despite this high ionic condition, a clear band of the complex was observed in a gel by mixing the three components (Fig. 4, lane 4). In the absence of the antibiotic, however, the RNA-L11 complex itself was not detected as a discrete band, presumably due to dissociation of the complex during electrophoresis (Fig. 4, lane 3). Lane 6 shows that rat L12 protein was not able to substitute for E. coli L11 in the formation of such a stable complex, indicating that the rat protein may not cooperate with the RNA and thiostrepton despite its high affinity for the E. coli RNA (Fig. 3B).

The rat RNA was also tested for forming the complex with E. coli L11 and thiostrepton. As shown in Fig. 5, rat wild-type RNA formed no stable complex in the gel (Fig. 5, lane 4). However, the substitution of A for G1876 gave a discrete complex surviving gel electrophoresis (Fig. 5, lane 8). These results
show that the G to A substitution at the 1067 equivalent position makes the eukaryotic RNA highly accessible to thiostrepton and E. coli L11 in a cooperative manner.

**DISCUSSION**

The secondary structure of the GTPase domain has been deduced by comparing 23 S-type RNA sequences from diverse organisms (5, 11, 15). Fig. 1 shows models for the domain of rat 28 S rRNA (A) and E. coli 23 S rRNA (B), both of which represent a conserved four-loop/four-helix structure. The minimal binding site for thiostrepton comprises residues 1052–1112 sequence of E. coli 23 S rRNA (6) to which ribosomal protein L11 also binds (26). Among these 61 residues, there are 26 bases different between the E. coli and the rat domain (Fig. 1). Some of the different bases are assumed to be responsible for the low affinity of the rat RNA to the drug. The present data demonstrate that an exchange of the eukaryote-specific base G to A at the single position equivalent to 1067 of rat 28 S rRNA increases the affinity for thiostrepton to a level comparable to that of E. coli wild-type RNA.

The significance of A1067 residue in the thiostrepton binding has been shown by several approaches using E. coli ribosomes. A footprinting study by Egebjerg et al. (5) showed that 10 bases including A1067 are protected from chemical attacks. An extensive mutational analysis by Ryan et al. (11) showed that among these 10 protected bases, substitutions at the following 5 positions gave considerable reduction of the drug binding: A1067, G1068, G1071, A1095, and A1098. These results lead to an implication that the 5 bases are major sites of direct contacts with thiostrepton. Among these 5 bases, only A1067 base is different from G of the eukaryotic domain; the others are preserved between E. coli and rat at respective positions, implying that A1067 is an important element for the specificity of thiostrepton-RNA binding. Reduction of thiostrepton binding by base substitution at A1067 (6, 10–12) obviously supports this view. In contrast to disruption analyses of this kind exhibiting “down-mutation,” we have used here the reverse approach making the eukaryotic RNA highly accessible to thiostrepton, i.e. “up-mutation” analysis. By the G to A substitution at the 1067 equivalent position of rat RNA, the thiostrepton binding is markedly enhanced (Fig. 2B). This result is more straightforward to explain a key role of A1067 in thiostrepton binding.

Cundliffe provided a hypothesis that the GTPase RNA domain has multiple functional conformations and that thiostrepton may lock the RNA in a single conformation (2). From the data by footprinting that the bases protected by thiostrepton are concentrated in two loop regions of residues 1065–1073 and of residues 1093–1098, Egebjerg et al. (27) proposed a model of the three-dimensional conformation of the GTPase domain, in which the two loops are folded close together to construct the drug binding site. By this binding, a single conformation may be rigidly locked, as suggested by Cundliffe (2). In a previous study (16), we showed this three-dimensional model is also available for the eukaryotic RNA domain using another ligand, anti-28 S autoantibody specific for the eukaryotic GTPase domain (24). Anti-28 S antibody protects 4 bases within the two loop regions of the eukaryotic domain corresponding to positions 1066, 1067, 1068, and 1098 of E. coli 23 S rRNA (16).

Unlike thiostrepton, anti-28 S antibody shows an affinity for the prokaryotic GTPase domain much lower than that for eukaryotic one. However, the binding affinity for E. coli RNA is greatly enhanced by substitution of G for A1067 (16). From such interchangeability of the ligand specificity by a single base exchange, it is highly probable that these bases are important identity elements discriminated by anti-28 S and thiostrepton and that the basal structure of the ligand binding site is fairly preserved between prokaryotes and higher eukaryotes as far as protein-free RNA state is concerned.

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**Fig. 5.** Effect of the rat base G1067 to A substitution on stabilization of the RNA L11 complex with thiostrepton. The rat wild-type RNA (lanes 1–4) and its A1067 variant (lanes 5–8) were incubated at various combinations of thiostrepton and E. coli L11, as indicated above gel lanes: without protein (lanes 1, 2, 5, and 6), with E. coli L11 (lanes 3, 4, 7, and 8) in the presence of 20 pmol of thiostrepton (lanes 2, 4, 6, and 8), or its absence (lanes 1, 3, 5, and 7). The complex was analyzed on gels, as described in the legend of Fig. 4.

**Fig. 6.** Schematic representation of the contrasting properties of thiostrepton and anti-28 S autoantibody. The prokaryotic A base at the position 1067 of E. coli 23 S rRNA and eukaryotic G base at the equivalent position are indicated with large letters. An importance of the prokaryotic A base for the binding specificity of thiostrepton (this study) and the eukaryotic G base for that of anti-28 S (our previous study, see Ref. 16) has been verified by experiments using reciprocal base substitution mutants. Bindings of E. coli L11 and rat L12, which are involved in the specificity of ligand bindings, are represented.
E. coli ribosomal protein L11 has been suggested to recognize the backbone of the GTPase domain in E. coli 23 S rRNA (11, 28) and stabilize the tertiary structure. The protein also binds to the RNA from archaeabacteria (29) and yeast and mouse (30). We used this E. coli protein and its mammalian homologue L12 (31) as probe for study of the domain structure. The observed close \( K_a \) values of both proteins for E. coli and rat RNAs (Fig. 3) indicate the extreme conservation of backbone structure between prokaryotes and higher eukaryotes. This may explain the extreme conservation of backbone structure between prokaryotes and higher eukaryotes (32, 33).

The binding affinity of thiostrepton for the intact E. coli 50 S subunit is extremely higher than that for 23 S rRNA. This is attributed to the cooperativity between thiostrepton and E. coli protein L11 (7). It is suggested that L11 conducts a fine tuning of the RNA tertiary structure for the cooperativity. This high affinity of thiostrepton for the intact ribosome is reduced only modestly, when A1067 is changed to the eukaryotic base G (10) and when whole domain containing residues 1056–1103 is exchanged with the yeast equivalent domain (33). These data imply that the total resistance of eukaryotic ribosomes to thiostrepton is not solely due to eukaryote-specific RNA elements including G base at the A1067 equivalent position but also to other protein components of the eukaryotic ribosome. Eukaryotic homologues of E. coli L11 are most likely to be related to the resistance, although no direct evidence has been reported.

To study the cooperativity between the L11 homologues and thiostrepton in the RNA binding, we used a gel electrophoretic analysis under high ionic condition. This gel system clearly discriminated a rigidly formed RNA-L11-thiostrepton complex from the RNA-L11 complex (Fig. 4). In the gel analysis, rat L12 failed to form the stable RNA-L12-thiostrepton complex (Fig. 4) despite its high affinity for the E. coli GTPase domain (Fig. 3B). This suggests that RNA binding required for the cooperativity may not be provided by rat L12. It is noteworthy that rat A1067 is not changed to the eukaryotic base G (34). As for the eukaryotic domain-specific ligand anti-28 S, a similar but contrasting result was obtained; rat L12 enhanced the binding of anti-28 S to the rat GTPase domain (3). This may be ascribed to its ability to adjust the structure to eukaryotic type. Therefore, we think it is possible that the ligand affinity strongly depends on the kind of associated protein, in addition to the base at the 1067 position (Fig. 6). The RNA structure itself can be rather flexible, and its binding to prokaryotic L11 and eukaryotic L12 may make up slightly different RNA conformations that have preferences for thiostrepton and anti-28 S, respectively.

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