Effects of Tetracycline and Spectinomycin on the Tertiary Structure of Ribosomal RNA in the Escherichia coli 30 S Ribosomal Subunit*

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Structural analysis of the 16S rRNA in the 30S subunit and 70S ribosome in the presence of ribosome-specific antibiotics was performed to determine whether they produced rRNA structural changes that might provide further insight into their action. An UV cross-linking procedure that determines the pattern and frequency of intramolecular 16S RNA cross-links was used to detect differences reflecting structural changes. Tetracycline and spectinomycin have specific effects detected by this assay. The presence of tetracycline inhibits the cross-link C967×U1400 completely, increases the frequency of cross-link C1402×U1501 twofold, and decreases the cross-link G894×U244 by one-half without affecting other cross-links. Spectinomycin reduces the frequency of the cross-link C934×U1345 by 60% without affecting cross-linking at other sites. The structural changes occur at concentrations at which the antibiotics exert their inhibitory effects. For spectinomycin, the apparent binding site and the affected cross-linking site are distant in the secondary structure but are close in tertiary structure in several recent models, indicating a localized effect. For tetracycline, the apparent binding sites are significantly separated in both the secondary and the three-dimensional structures, suggesting a more regional effect.

A variety of antibiotics interact with the ribosome to inhibit protein synthesis within bacterial and eukaryotic cells (1). The point of interruption in the translation cycle has been determined for some antibiotics (2), making them useful in vitro to investigate the nature of elongation in protein synthesis. Some of these antibiotics have been footprinted on the ribosomal RNAs in 30S and 50S subunits and in the 70S ribosome (3) as well as on model oligomers designed to mimic local regions of 16S rRNA (4, 5). Sites of action have also been established by affinity and photoaffinity experiments (6, 7). The binding sites frequently correspond to regions of the ribosome that are implicated by other experiments in ribosome function. In many cases, binding site assignments are supported by resistance-conferring mutations in 16S and 23S rRNAs (2, 8). Antibiotics, either directly by binding or indirectly through conformation alterations, must result in the inability of tRNA or translation factors to bind the ribosome or else inhibit some processes needed during translation. However, little is known regarding whether conformational perturbations accompany binding, even in instances in which detailed information supporting binding sites and the nature of translation interruption are known.

UV cross-linking of the rRNA in the ribosome provides an opportunity to monitor changes in rRNA conformation and, consequently, the ribosomal global structure. We have previously determined the identity of 14 UV-induced cross-links in 16S rRNA within the 30S subunit (9) and 15 cross-links in the 70S ribosome (10). Because of the gel electrophoresis method used in the detection, all of these cross-links occur between nucleotides that are distant in the primary sequence. These cross-links occur because the partner nucleotides possess a suitable distance and geometry during the lifetime of the excited state (on the order of 1 μs; Ref. 11); therefore, their frequency provides a method to screen substrates and other agents for their ability to affect ribosomal conformation.

In this report, UV irradiation was repeated in the presence of 13 antibiotics to determine whether they produce measurable changes in the ribosome structure that might be related to their activity. These 13 antibiotics were known to bind either the 30S or 50S subunit. Some of these antibiotics have localized binding sites on 16S rRNA, as determined by chemical probing, that are adjacent in the 16S rRNA secondary structure to nucleotides that participate in cross-links and may affect the frequency of such contacts. Two of these antibiotics, spectinomycin and tetracycline, have discernible effects on the frequency of specific UV cross-linking sites in 16S rRNA. The implications of these effects with respect to the antibiotic action are discussed.

MATERIALS AND METHODS

Preparation of Ribosomes and UV Cross-linking Procedures—Escherichia coli 70S ribosomes and ribosomal subunits were prepared according to Makhno et al. (12) and dissolved in CMN2 buffer. In some experiments, CMN buffer was used with Mg2+ concentrations from 0.5 to 10 mM. 70S ribosomes were prepared by reassociating equimolar amounts of 30S and 50S subunits and were free of mRNA or tRNA. Samples were incubated with the following concentrations of antibiotics (Sigma) that were previously determined to produce specific footprints in the 16S rRNA or 23S rRNA by chemical probing (2, 3): (a) 10−4 M neomycin, (b) 5 × 10−6 M paromomycin, (c) 5 × 10−6 M streptomycin, (d) 1 × 10−4 M gentamycin, (e) 1 × 10−4 M kanamycin, (f) 1 × 10−4 M spectinomycin, (g) 2.5 × 10−2 M tetracycline, (h) 5 × 10−6 M erythromycin, (i) 5 × 10−6 M thioestrept, (j) 5 × 10−6 M fusidic acid, (k) 5 × 10−6 M chloramphenicol, (l) 5 × 10−6 M vioycin, and (m) 1 × 10−4 M hygromycin. Concentration series have also been investigated for tetracycline and spectinomycin. Samples were incubated for 30 min at 37 °C, placed on ice for 10 min, and irradiated at 4 °C for 20 min in a quartz cuvette with continuous stirring. Irradiation was performed with a 312 nm trans-illuminator (Fotodyne Corp.) as described previously (9). Sample concentrations were usually 1 μg RNA/μL, with the exception of samples for preparative separation, which were irradiated

1 The abbreviations used are: CMN, 80 mM cacodylic acid, pH 7.5, 20 mM MgCl2, 100 mM NH4Cl, and 4 mM β-mercaptoethanol; BTBE, 30 mM bis(2-hydroxyethyl)iminotris(hydroxymethyl)methane, 30 mM boric acid, and 2.5 mM EDTA, pH 6.8.

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at 6 μg RNA/μl. RNA was recovered from the samples by protease K digestion, phenol extraction, and ethanol precipitation. The RNA was dephosphorylated with shrimp intestinal phosphatase and purified by protease K digestion, phenol extraction, and ethanol precipitation. 16 S rRNA was then isolated on a 1% agarose gel before end-labeling with [γ-32P]ATP by T4 polynucleotide kinase.

Cross-linked 16 S rRNA was separated by gel electrophoresis on gels made with 3.6% acrylamide:bisacrylamide (70:1), 8.3M urea, and BTBE buffer as described previously (9). For analysis of cross-linking sites, the location of the bands containing un-cross-linked and cross-linked 16 S rRNA were detected with a PhosphorImager, and bands were cut out before further analysis. 

**Determination of the Identity and Frequency of Cross-linked Sites**

The cross-linking sites in separated 16 S rRNA were found by primer extension analysis using 11 DNA primers complementary to regions throughout 16 S rRNA (9). The frequency of cross-linking was determined from PhosphorImager data (ImageQuant; Molecular Dynamics Inc.) of duplicate independent experiments. To normalize for RNA loading, cross-link band intensity was referenced to the same cross-link band (C54 Inc.) of duplicate independent experiments. To normalize for RNA load, the concentration used for chemical footprinting experiments (3), spectinomycin decreased the intensity of the cross-link involving U534. The third band from the top of the gel, which differences were seen in the tetracycline sample. In lane 5 of each panel, the stops indicating cross-links at U244 and G894 decreased in the tetracycline sample relative to the control sample. C and D show the results of reverse transcription reactions in the nucleotide intervals 952–983 and 1387–1409. Lane 7 in each panel, the stops indicating cross-links at U244 and G894 decreased in the tetracycline sample relative to the control sample. C and D show the results of reverse transcription reactions in the nucleotide intervals 952–983 and 1387–1409. 

**Results**

Six classes of antibiotics were examined in these experiments: (a) aminoglycoside (neomycin, paromomycin, streptomycin, gentamicin, kanamycin, spectinomycin, and hygromycin), (b) tetracycline, (c) macrolide (erythromycin), (d) peptide (thiostrepton and viomycin), (e) fusidic acid, and (f) chloramphenicol. The seven aminoglycosides, tetracycline, and the two peptide antibiotics are thought to bind primarily to the 30 S subunit, and the two peptide antibiotics also bind to the 50 S subunit (2). Erythromycin and chloramphenicol bind exclusively to the 50 S subunit, and fusidic acid prevents the release of elongation factor G-GDP from the ribosome (2). These last three antibiotics were included to test the possibility that an alteration in the 50 S subunit structure may induce changes in the structure of the 30 S subunit, within the 70 S ribosome. Both 30 S and 70 S ribosomes were irradiated in the presence of the listed antibiotics, and, with exceptions noted below, the effects were identical. In all instances in these experiments, empty ribosomes were used to avoid heterogeneity in the ribosomal state and to avoid the complication of possible substrate-mediated structure changes. In addition, previous characterizations of antibiotic binding sites (3, 7) and our identification of UV cross-links in 16 S rRNA (9) have been performed on empty ribosomes.

Tetracycline and spectinomycin were the only compounds that affected the cross-linking pattern of 16 S rRNA within 30 S subunits or 70 S ribosomes (Fig. 1). At $2.5 \times 10^{-4}$ M, the concentration used for chemical footprinting experiments (3), tetracycline affected three identified cross-links and one incompletely identified cross-link. The third band from the top of the pattern, which was verified as C967×C1400, was completely inhibited. In this and other gels, there was some decrease in the intensity of the top-most band in the pattern, which was shown to contain U244×G894. The third band from the bottom of the gel, which was shown to contain the cross-link C1402×C1501, increased twofold in intensity in both 30 S subunits and 70 S ribosomes. The fourth band from the top also decreased in the presence of tetracycline. U534 is one part of this cross-link, but we have been unable to find a partner for it. At $1 \times 10^{-4}$ M, the concentration used for chemical footprinting experiments (3), spectinomycin decreased the intensity of the cross-link found in the second band from the top (Fig. 1), which was shown to contain the cross-link C934×U1345, by 60%.

Reverse transcription analyses of the regions of 16 S rRNA containing the cross-links affected by tetracycline are shown in Fig. 2. A and B show the results of reverse transcription in the nucleotide intervals 212–265 and 866–914, respectively, in which differences were seen in the tetracycline sample. In lane 7 in each panel, the stops indicating cross-links at U244 and G894 decreased in the tetracycline sample relative to the control sample. C and D show the results of reverse transcription reactions in the nucleotide intervals 952–983 and 1387–1409. Lane 5 of each panel contains stops indicating that the cross-link C967×C1400 disappeared. E and F show the results of reverse transcription reactions in the nucleotide intervals 1390–1411 and 1491–1504. Lane 2 of each panel contains stops indicating that the cross-link C1402×C1501 increased in the presence of tetracycline relative to the control. The effects of tetracycline on cross-link intensity are the same in both 30 S subunits and 70 S ribosomes.

Reverse transcription analysis of the cross-linked band affected by spectinomycin is shown in Fig. 3. The only reverse transcription stops affected by spectinomycin were in intervals 907–953 (A) and 1330–1365 (B). A decrease in the stops at 935 and 1346 in lane 6 of each panel indicates that the cross-link C934×U1345 is affected. The decreases in intensity are consistent with the difference seen in the analytical gel pattern (Fig. 1).

Titration experiments were performed with tetracycline to
determine the concentration threshold on the 16 S rRNA tertiary structure (Fig. 4). At concentrations between $2.5 \times 10^{-4}$ M and $1 \times 10^{-5}$ M, tetracycline specifically affects the cross-links noted above. There was no change in the frequency of any of the cross-links before the low concentration limit was reached. In the sample irradiated in the highest concentration of tetracycline, several cross-links were partially or completely inhibited; this is attributed to the nonspecific binding of tetracycline to the ribosome and/or a decrease in cross-linking due to the high tetracycline concentration. The effects of tetracycline and spectinomycin were determined in 30 S subunits and 70 S ribosomes at Mg$^{2+}$ concentrations from 0.5 to 50 mM. Mg$^{2+}$ has been shown to govern the frequency of cross-linking of several cross-links, including C967×C1400 and C1402×C1501. C967×C1400 was inhibited at all Mg$^{2+}$ concentrations, except that in 0.5 mM Mg$^{2+}$ in 30 S ribosomes, the frequency of the cross-link, even in the control sample, was too low to detect. The increase in the frequency of C1402×C1501 due to tetracycline was seen in 30 S subunits and 70 S ribosomes at all Mg$^{2+}$ concentrations above 5 mM. Below 5 mM, no increase in C1402×C1501 could be seen (data not shown). The decrease in frequency of C934×U1345 due to spectinomycin did not change over the stated Mg$^{2+}$ concentration range (data not shown).

Several cross-links, C1400×C1501, C1402×C1501, and C1397×C1497, identified in the decoding region of 16 S rRNA nearly co-migrate in gel electrophoresis (10). There was a possibility that neomycin and streptomycin would affect the distribution of conformations in that region. However, no detectable changes in the analytical gel electrophoresis experiments involving these antibiotics were seen, nor were any changes seen in primer extension experiments (data not shown).

**DISCUSSION**

Of the 13 antibiotics examined in this study, tetracycline and spectinomycin showed specific and different structural effects in 16 S rRNA that were detectable by the UV cross-linking assay. The inhibition of cross-links by these antibiotics correlates well with their known effective concentrations (14, 15), indicating that the 16 S rRNA tertiary structure effects are linked to the loss of small subunit function during translation. For the tetracycline response, all three of the cross-links are affected at the same tetracycline concentration.

Tetracycline has been shown to inhibit protein synthesis by interfering with the binding of aminoacyl-tRNA to the ribosomal A-site (2, 14), but it does not prevent the binding of tRNA$^{Met}$ to the P-site (16). However, tetracycline has been reported to interfere with initiation factor-dependent tRNA$^{Met}$ binding (2, 17). In a study in which the binding of a fluorescent analogue of tetracycline, demeclocycline, was monitored, displacement of demeclocycline by tRNA$^{Met}$ and by A-site...
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tRNA\textsuperscript{3\text{\textlt}} binding was confirmed (18). Tetracycline did have an inhibitory effect on the P-site when the determination was done at high tRNA:70 S stoichiometries, and this was attributed to a fraction of ribosomes that bind tRNA in the P-site with lower affinity (19).

Photochemical cross-linking experiments have shown that tetracycline cross-links primarily to protein S7 and, to a lesser extent, S18 and S4 (20). S7 has been footprinted by both base cleavage experiments to the P-site with lower than 4\textsuperscript{\textlt} M, additional cross-linking sites, including one at G890, are seen (7). In experiments done at 1 \times 10\textsuperscript{\textlt} M, tetracycline produced a strong chemical protection footprint in 16 S rRNA at nucleotide G893 (near helix 23b), G1300, and G1338 (both of them near helix 42) (7). At concentrations of \textgeq 1.2 \times 10\textsuperscript{\textlt} M, additional cross-linking sites, including one at G890, are seen (7). In experiments done at 1 \times 10\textsuperscript{\textlt} M, tetracycline produced a strong chemical protection footprint in 16 S rRNA at nucleotide A892 and a weak reactivity enhancement of nucleotides U1052 and C1054 (3) (see Fig. 5). Recently, a C\textrightarrow G substitution at position 1058 granting resistance to tetracycline was reported (22), which also indicates that this region is connected to tetracycline function.

Footprints for tRNA in the P-site include C1400, G1338, C967, and G693 (23). Because two of the three affected UV cross-links and all three tetracycline-RNA cross-links are associated with the tRNA P binding site, it is worthwhile to consider how tetracycline might interfere with A-site tRNA binding. Rodnina et al. (24) have investigated EF\textsubscript{Tu}-aminocyl tRNA-GTP binding and found that the rate and strength of the initial complex and the codon recognition complex were both enhanced by cognate tRNA bound to the P-site. This suggests an effect by the P-site-bound tRNA or some change in the ribosome that depends on P-site occupancy that influences the ternary complex-ribosome interaction. Therefore, alteration of the P-site by tetracycline may not affect the binding of tRNA in the P-site itself but may inhibit some conformational adjustment needed for tRNA binding to the A-site.

The reported antibiotic binding sites (3, 7) and the affected RNA cross-linking sites are compared in a 16 S RNA threedimensional model (Fig. 6),\textsuperscript{2} which shows the locations of the helices containing the tetracycline and spectinomycin binding sites and the RNA-RNA cross-links affected by their binding. This model is different from recent models (26–28) in several regions because it incorporates new information including the UV cross-links C967\times C1400 (9, 10), U793\times G1517, and G976\times G1361\textsuperscript{3} and site-specific psoralen cross-links (25).\textsuperscript{4}

Approximate distances from G1300, G1338, and G693 to C1400 (the P-site) are 45, 35, and 25 Å, respectively, and distances from G1300, G1338, and G693 to A1408 (associated with the A-site) are 57, 50, and 36 Å, respectively. In another recently proposed model (26), the distances to C1400 are 67, 40, and 81Å, and the distances to A1408 are 95, 70, and 82 Å. In both models, the distances between the sites of tetracycline-RNA cross-linking (7) and the P-site nucleotide C1400 (3) are less than those to the A-site nucleotide A1408 (23). The molecular dimensions of tetracycline measure approximately 8 \times 12 Å.

Spectinomycin causes a structural change in domain III that results in a decrease in the frequency of cross-link C934\times U1345. Spectinomycin causes a strong protection from chemical reactivity at C1063 and G1064 (see Fig. 5) and a weak enhancement of reactivity at G973 (3). In addition, spectinomycin resistance is conferred by a C\textrightarrow A, G, U mutation at position 1192 in 16 S rRNA. Because overexpression of a fragment of helix 34 corresponding to nucleotides 1047–1067 and 1189–1210 (linked by a hairpin loop) also confers spectinomycin resistance (2), the binding site is most likely located in helix 34. In addition, it was shown that susceptibility to spectinomycin could be restored in C1192 mutants by an additional U1351\textrightarrow C mutation in the top of helix 43 (15). The partial inhibition of cross-link C934\times U1345 by spectinomycin supports evidence for an interplay between helix 34b and 43. The helix that contains the spectinomycin footprint at C1063 (helix 34b) and the nucleotides that form the C934\times U1345 cross-link at the end of helix 28 are highlighted in the model (Fig. 6). The distance between these two regions is 12 Å in the model shown (Fig. 6), compared with 20 Å in the Mueller and Brimacombe (26) model. A surprising result is that spectinomycin has no effect on the U1052\times C1200 cross-link, which is in close proximity to both the resistance mutation site (C1192) (3) and the proposed binding site in 16 S rRNA. In addition, there are no changes in the cross-links U1052\times C1200, A1093\times G1182, or U1126\times C1281, all of which are located in domain III. This indicates that spectinomycin is not producing a global rearrangement of the domain III tertiary structure but rather some specific alteration of the structure near the junctions of helices 28, 29, and 43. It is known that spectinomycin interferes with elongation factor G binding during early rounds of protein synthesis (2). It has recently been shown that elongation factor G interacts with the 30 S subunit near the 1338 region (29); therefore, it is also possible that the structural perturbation caused by spectinomycin alters elongation factor G association.

The neomycin-type aminoglycosides (hygromycin, gentamycin, neomycin, and paromomycin) protect 16 S rRNA nucleo-

\textsuperscript{2}M. A. Dolan, P. Babin, and P. Wollenzien, unpublished data.

\textsuperscript{3}T. Shapkina, unpublished data.

\textsuperscript{4}D. Mundus, unpublished data.
tides A790, A791, A909, A1394, A1413, and G1487 and enhance reactivity at C525 (3). These antibiotics are generally thought to induce miscoding by inhibiting A-site occupation and to inhibit translocation (2). Streptomycin causes miscoding (2) and protects 16S rRNA nucleotides 911–915, nucleotides in the 1408–1418 and 1482–1494 regions, and nucleotide 1468 in 30S subunits in 20 mM Mg\(^{2+}\) (3, 30) and binds to naked 16S rRNA fragments of the decoding region in 20 mM Mg\(^{2+}\). Vio- mycin and thiostrepton induce protections in both 16S and 23S rRNA (for viomycin, these are known to be protections at nucleotides 912–915), and both antibiotics also inhibit A-site occupation and cause miscoding (2). It is thought that their binding restricts the ribosome conformation, thereby preventing A-site occupation or translocation (2). None of those antibiotics produces an observable effect in this experiment, so it is possible that these antibiotics interact with only a local region of the ribosome. Alternatively, the tertiary structural effects for the other antibiotics may be too subtle to detect by the present cross-linking, may be elicited only in the ribosome under working conditions, or may affect parts of
the ribosome not monitored by the cross-links that are made by UV irradiation.

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