Sequence-Specific Recognition of the Major Groove of RNA by Deoxystreptamine†

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ABSTRACT: Aminoglycoside antibiotics specifically interact with a variety of RNA sequences, and in particular with the decoding region of 16S ribosomal RNA in the aminoacyl tRNA acceptor site (A-site). Ring II of aminoglycosides (2-deoxystreptamine) is the most conserved element among aminoglycoside antibiotics that bind to the A-site. NMR structures of aminoglycoside−A-site RNA complexes suggested that the 2-deoxystreptamine core of aminoglycosides specifically recognizes (5′)G-U(3′) and potentially (5′)G-G(3′) or (5′)U-G(3′) steps in the major groove of RNA. Here, we show that isolated deoxystreptamine specifically interacts with G-U steps within the major groove of the A-site RNA. The bulge residue of A-site RNA is required to open the major groove for accommodation of deoxystreptamine. The chemical groups of deoxystreptamine presented to the RNA by the framework of the 6-carbon ring modulate RNA recognition.

Aminoglycosides are the best characterized RNA-binding antibiotics. Aminoglycosides containing a 2-deoxystreptamine ring such as neomycin, paromomycin, and gentamicins (Figure 1) were shown to target the A-site ribosomal RNA (*1*) to induce codon misreading and inhibit translocation (*2*, *3*). The NMR[1] structures of a fragment of 16S rRNA complexed to paromomycin or gentamicin C1a revealed the detailed interactions of aminoglycoside with the A-site rRNA (Figure 2)(*4*, *5*). These results are in good agreement with the more recent crystal structure of the bacterial 30S subunit with paromomycin and hygromycin (*6*, *7*) and a high-resolution crystal structure of an oligonucleotide−paromomycin complex (*8*). Aminoglycosides containing a 2-deoxystreptamine ring bind in the major groove of the long penultimate stem H44, flipping out two universally conserved adenine residues in the minor groove (*6*, *9*). Comparison of the two NMR structures (paromomycin and gentamicin−RNA complexes) explained how chemically distinct aminoglycosides sharing common structural elements can specifically bind to ribosomal A-site (*5*). Neomycin (4,5-disubstituted antibiotic) and gentamicin (4,6-disubstituted antibiotic) classes of aminoglycosides (Figure 1) have different ring II−ring III linkages and a different number of rings, even though in both structures, common chemical groups of rings I and II (Figure 2) are making similar contacts with the RNA (*5*). Comparative NMR and biochemical study of the interaction of the aminoglycosides paromomycin, neomycin, ribostamycin, and neamine with the A-site RNA showed that rings I and II of neomycin class aminoglycosides are sufficient for specific binding to A-site RNA (*10*). Crystallographic studies showed that water molecules mediate some paromomycin−A-site RNA contacts (*8*).

Ring II of aminoglycosides is the most conserved ring among aminoglycosides that bind to the A-site of 16S rRNA. Ring II (2-deoxystreptamine) makes sequence-specific contacts with a (5′)G-U(3′) step of the RNA in the major groove. These contacts are reminiscent of DNA−drug interactions, in which hydrogen bond donor and acceptors read sequence information along the edge of a DNA minor groove (*11*, *12*). The ring II contacts appear somewhat independent of their structural context, and more dependent on helical sequence. A C1407U-G1494A double mutant does not bind paromomycin (*13*). This result indicates that the 2-deoxystreptamine ring of paromomycin strictly recognizes 5′G(N7)-U(O4)3′ but not 5′A(N7)-U(O4)3′ probably due to a steric clash between the bulky amino group of A and the deoxystreptamine moiety. Based on the NMR structures, we propose that isolated 2-deoxystreptamine may be an element to target specifically 5′G(N7)-U(O4)3′, 5′G(N7)-G(O6)3′, 5′G(O6)-G(N7)3′, or 5′U(O4)-G(N7)3′ sequences in the major groove of RNA molecules (Figure 3).

To test this hypothesis, binding properties of deoxystreptamine to wild-type and mutant A-site RNA oligonucleotides were examined by NMR. Both the RNA sequence and the structural requirements for deoxystreptamine binding were determined. The nature of the chemical groups of deoxystreptamine at the interface of the ligand−RNA contact area was studied with a closely related compound, streptamine (Figure 1). The results clearly demonstrate that isolated deoxystreptamine is a structural motif for recognition of G-U or G-G RNA sequences in a widened major groove. The bulged adenosine of the asymmetric internal loop of the A-site RNA is required for deoxystreptamine accessibility in the major groove. Addition of a bulky functional group at position 2 of deoxystreptamine disrupts specific binding to G-U steps of the RNA.

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[1] Abbreviations: NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; NOESY, nuclear Overhauser effect spectroscopy; HIV, human immune deficiency virus; RRE, Rev responsive element; TAR, trans-activating region.

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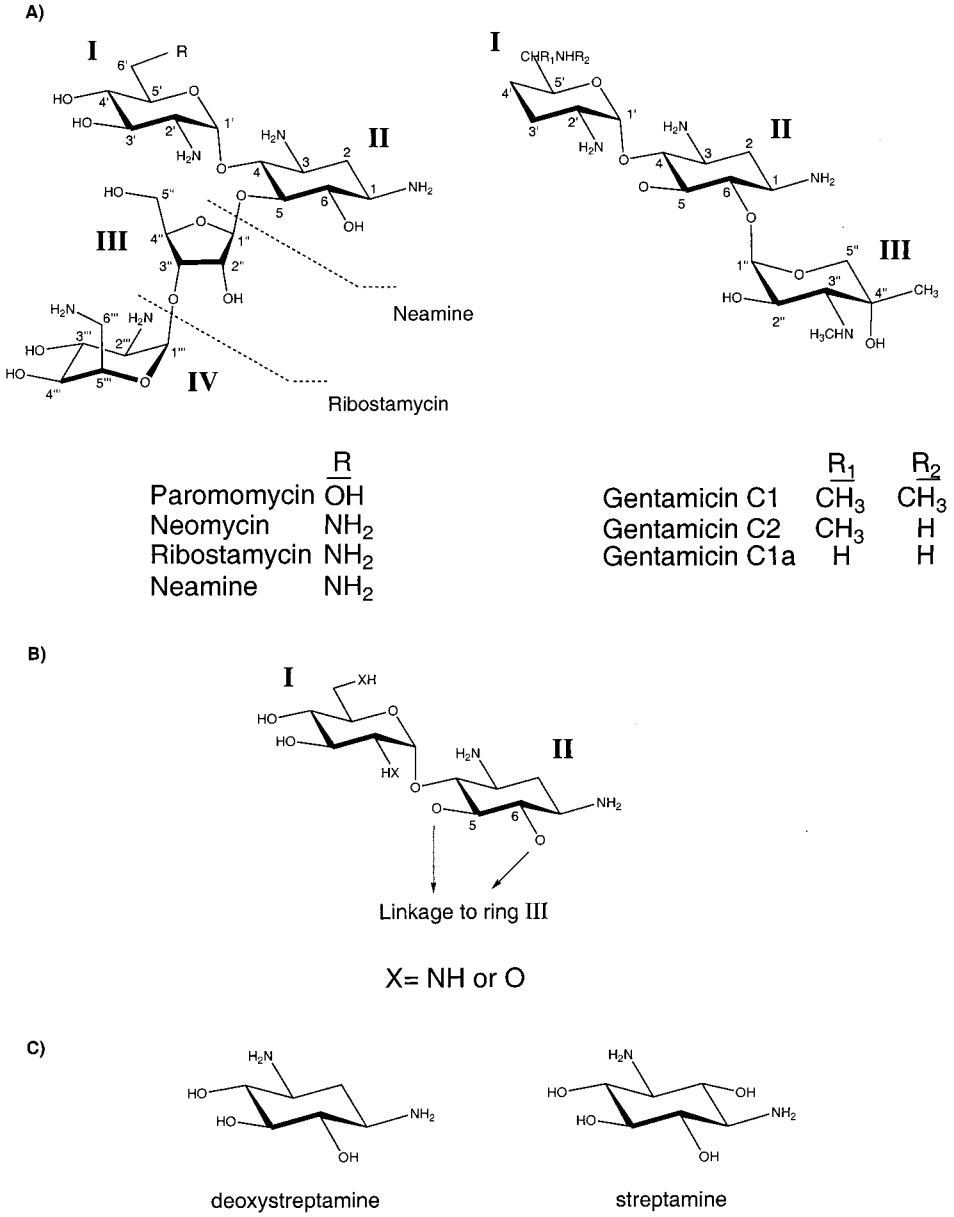

FIGURE 1: (A) Structures of the aminoglycoside antibiotics that bind in the A-site of the decoding region. The A-site binding aminoglycosides all contain rings I and II. The neomycin group of aminoglycosides includes paromomycin, neomycin, ribostamycin, and neamine. Ribostamycin contains all rings except ring IV, whereas neamine lacks both rings III and IV. (B) The common structural elements among all aminoglycoside antibiotics that bind to the ribosomal A site are in boldface type. (C) Structures of deoxystreptamine and streptamine.

MATERIALS AND METHODS

Preparation of 2-Deoxystreptamine and Streptamine. General Methods. Neomycin sulfate was obtained from Sigma. Streptomycin sulfate was purchased from Fluka. Other materials were obtained from Fluka or Fisher. TLC analyses were conducted on Analtech Silica Gel GF plates with 250 μm coating. Plates were developed with $CHCl_3$/CH_3OH/concentrated NH_4OH/water, 1:4:2:1. TLC spots were visualized by *p*-anisaldehyde or ninhydrin. Cation exchange chromatography employed Whatman P11 fibrous resin (cellulose phosphate). Free-base forms of compounds were obtained by treating solutions of the salt form with Amberlite IRA-400 resin (OH form).

Compounds were characterized by ^{1}H NMR and ^{13}C NMR on a Bruker AC 250. Chemical shifts are relative to TMS (δ 0.0) using the residual solvent signal as internal reference. Electrospray ionization mass spectra (ESI-MS) were obtained on a PE Sciex AP1 instrument with an accelerating potential of +4600 V and an oriface potential of 85−110 V.

(A) 2-Deoxystreptamine. 2-Deoxystreptamine was readily obtained by hydrolysis of neomycin following a published procedure (*14*). Neomycin sulfate (10 g, 11 mmol) was dissolved in 48% HBr (50 mL) in a large flask and stirred vigorously. After initial foaming subsided, the mixture was heated at reflux for 3 days. HBr was removed; then the residue was dissolved/resuspended in water and filtered through diatomaceous earth and activated carbon. The faint yellow filtrate was lyophilized; then the crude product was purified by cation exchange using acetic acid (1−2% in water) as eluant to give 1.71 g (55%) of 2-deoxystreptamine as the acetic acid salt. A portion was converted to the free-base for analysis. TLC: R_f = 0.56. ^{1}H NMR (250 MHz, D_2O): δ 3.23 (1H, t, J = 7 Hz, H5), 3.08 (2H, t, J = 7 Hz, H4,6), 2.66 (2H, dt, J = 10, 4 Hz, H1,3), 1.92 (1H, dt, J =

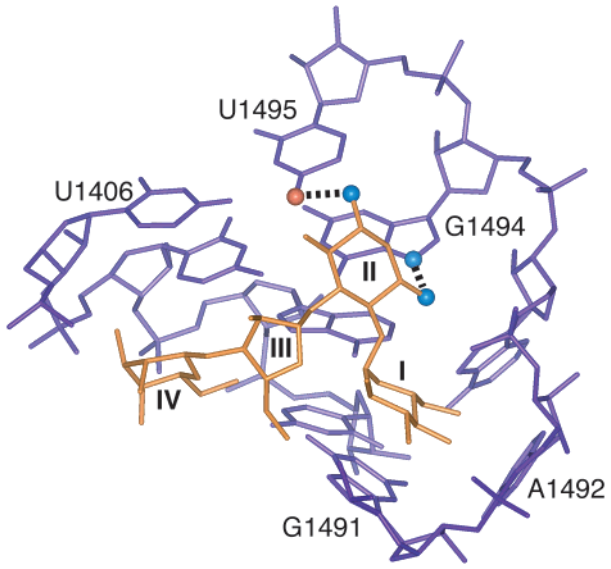

FIGURE 2: Specific contacts made between ring II of paromomycin and the A-site RNA (*4*). The view is into the major groove of the RNA core. The RNA is in blue; paromomycin is in tan. Important chemical groups are shown explicitly. Dashed lines indicate hydrogen bonding contacts between ring II and the G1494 and U1495.

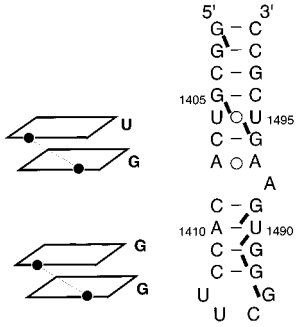

FIGURE 3: Schematic of the geometry of the hydrogen bond acceptor displayed by U(O4)-G(N7) and G(O6)-G(N7) steps (left). Secondary structure of the A-site RNA as determined from NMR data (*13*). All G-U and G-G steps, which represent possible binding sites for deoxystreptamine, are indicated with a boldface line (right).

14, 2 Hz, $H2_{eq}$), 1.11 (1H, q, $J = 10$ Hz, $H2_{ax}$). ^{13}H NMR: δ 80.2 (C4,6), 78.2 (C5), 52.9 (C1,3), 38.4 (C2). ESI-MS: $m/z = 162$ (MH^+, 60%), 638 ($(M + H_2O)^+$, 100%).

(B) Streptidine. Streptidine, the amidinated aminocyclitol component of streptomycin, was prepared by the HCl hydrolysis of streptomycin under more mild conditions (*15*), the product separating out of acid solution after standing 3 days at room temperature. Streptomycin sulfate (10.7 g, 14.7 mmol) in water (20 mL) was treated with a solution of H_2SO_4 (3 mL) in methanol (80 mL). After standing for 3 days (ambient temperature), product precipitated and was collected, giving 2.89 g (55%) of the sulfate of streptidine as an amorphous white powder. A portion was recrystallized from 2% H_2SO_4 to give white needles. TLC: $R_f = 0.56$. ESI-MS: $m/z = 263$ (MH^+, 100%).

(C) Streptamine. Streptamine was obtained by barium hydroxide hydrolysis of streptidine (*16*). Streptidine sulfate (1.0 g, 3.6 mmol) was refluxed in a saturated solution of $Ba(OH)_2$ for 2 days and then cooled, and barium salts were filtered off. The filtrate was treated with 10% H_2SO_4 to pH 1−2, precipitating $BaSO_4$. This was removed, and then the filtrate was dried to give a dark brown syrup, which was then converted to the free-base form by treatment with IRA-400 resin (OH form). TLC: $R_f = 0.62$. ESI-MS: $m/z = 263$ (MH^+, 100%).

NMR Sample Preparation. The preparation of milligram quantities of the A-site oligonucleotide RNA (27 nucleotides) for NMR was performed according to (*17*). After electroelution and ethanol precipitation, the RNA was dissolved in 10 mM sodium phosphate buffer (pH 6.4) and dialyzed against the buffer used for the NMR experiments in a microdialysis apparatus with a 3500 MW cutoff membrane.

NMR Spectroscopy. All the NMR experiments were recorded on either a Varian Unity+ 500 MHz spectrometer or a Varian Inova 500 MHz spectrometer equipped with triple-resonance, z-gradient probes. NMR data were processed using Varian software. NMR experiments were performed in 10 mM sodium phosphate (pH 6.4) at 5 or 10 °C. The concentrations of A-site RNA wild-type and mutant RNAs were 3mM. Sample volumes were 270 μL in Shigemi NMR tubes. The exchangeable and nonexchangeable protons of the A-site RNA oligonucleotides containing the studied mutations were assigned using standard homonuclear NMR experiments. Solvent suppression for samples in 90% H_2O/10% D_2O was achieved using the WATERGATE sequence (*18*). Two-dimensional NOESY spectra in 95% H_2O/5% D_2O were acquired with mixing times of 75, 150, and 300 ms. NOESY spectra with mixing times of 250 and 400 ms in D_2O were measured at 5 and 10 °C. The residual HDO resonance in D_2O was suppressed using low-power presaturation.

RESULTS

G(N7)-U(O4) and G(N7)-G(O6) Sequences as Potential Deoxystreptamine Binding Sites. Prior structural work suggested that 2-deoxystreptamine is a sequence recognition element to bind 5′G(N7)-U(O4)3′ step in the major groove of RNA. In the A-site RNA, a U1495G substitution would place a hydrogen bond acceptor (O6) atom of the G residue in a similar position as the O4 atom of the U residue. Biochemical data showed that a U1495G mutant A-site RNA binds paromomycin with the same affinity as the wild-type RNA (*13*). We performed 2D NOESY experiments on a U1495G RNA−paromomycin complex (mixing time of 250 ms) and observed intermolecular NOEs indicating that paromomycin binds to the same site in a similar conformation as in the wild-type RNA−paromomycin complex (data not shown). In U1495G RNA−paromomycin complex, ring II of the antibiotic recognizes the G1494(N7)-G1495(O6) RNA segment, indicating that a 5′G(N7)-G(O6)3′ sequence could also be a potential receptor site for isolated 2-deoxystreptamine. Likewise, 5′U(O4)-G(N7)3′ and 5′G(O6)-G(N7)3′ steps could also bind deoxystreptamine.

The potential recognition of hydrogen bond acceptors in the major groove by deoxystreptamine can be analyzed structurally. The nitrogen atoms of amino groups 1 and 3 in deoxystreptamine are spaced by 4.95 Å, which allows hydrogen bonding with the G1494(N7)-U1495(O4) step of the free A-site RNA [average distance of 5.3 Å measured within the collection of the 20 final structures of the free A-site RNA (*9*)]. In the RNA complexed with paromomycin,

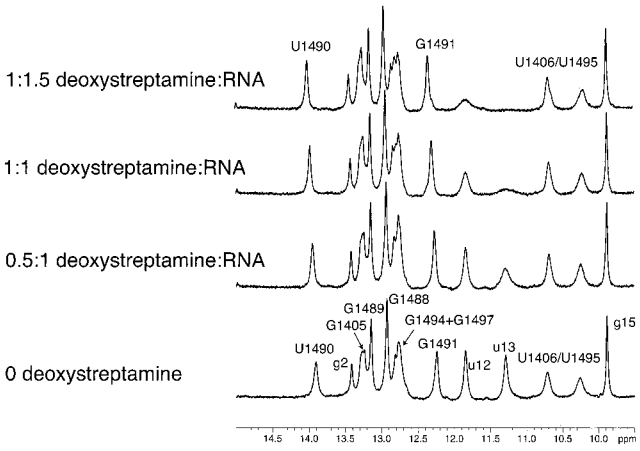

FIGURE 4: Titration of A-site oligonucleotide with deoxystreptamine. Imino proton resonances that change significantly upon binding of the ligand are indicated in the 1:1.5 RNA/deoxystreptamine spectrum. Titration was performed at 5 °C at pH 6.4 in 10 mM sodium phosphate.

the average distance for the G1494(N7)-U1495(O4) step was 5.2 ± 0.3 Å (*9*) and 5.8 ± 0.1 Å in the complex with gentamicin C1a (*5*). Standard values in A-form RNA for 5′purine(N7)-U(O4)3′, 5′purine(N7)-G(O6)3′, 5′G(O6)-purine(N7)3′, and 5′U(O4)-purine(N7)3′ steps in A-form RNA are 4.10, 5.10, 4.20, and 4.25 Å, respectively (*19*). Other hydrogen bond acceptor pairs such as 5′G(O6)-U(O4)3′, 5′U(O4)-G(O6)3′, 5′G(O6)-G(O6)3′, and 5′U(O4)-U(O4)3′ are shorter than 4.0 Å (3.1, 3.9, 3.3, and 3.6 Å, respectively). Distances for interstrand pairs G(O6)-U(O4) are 4.9 or 3.3 Å and 4.2 or 2.9 Å for a G(O6)-G(O6) pair where each acceptor is on a different strand. Pairs of hydrogen bond acceptor atoms in the major groove involving phosphate were not characterized. Electrostatic contribution to deoxystreptamine affinity to the major groove with the neighboring phosphates was not characterized in this work, but clearly contributes to recognition.

From all possible pairs of hydrogen bond acceptor, only 5′purine(N7)-U(O4)3′, 5′purine(N7)-G(O6)3′, 5′G(O6)-purine(N7)3′, and 5′U(O4)-purine(N7)3′ have a distance between acceptor atoms suitable to bind deoxystreptamine. Biochemical data showed that the C1407U-G1494A double mutant does not bind paromomycin (*13*), which may exclude the 5′A(N7)-U(O4)3′ sequence from possible binding sites. These hypotheses were investigated by studying binding of isolated 2-deoxystreptamine to the A-site model oligonucleotides.

Deoxystreptamine Binding to the A-Site RNA. Formation of the complex between the A-site RNA and deoxystreptamine was characterized by monitoring the chemical shifts of imino proton RNA resonances as a function of deoxystreptamine concentration. Specific binding of aminoglycoside antibiotics such as paromomycin, neomycin, gentamicin, ribostamycin, and neamine to the A-site RNA at 25 °C results in a downfield shift (up to 0.2 ppm at 1/1 stoichiometry in a slow exchange regime) of two imino proton resonances, U1490 and G1491 (*13*). The same two imino proton resonances are increasingly shifted downfield upon addition of deoxystreptamine up to a drug:RNA ratio of 1.5 (Figure 4) at 5 °C. No new imino proton resonances are observed through the titration. This is characteristic of a fast exchange regime, indicating a low affinity of deoxystreptamine for the RNA oligonucleotide (K_d is ca. mM). A linear correlation between the chemical shift change and deoxystreptamine concentration was observed; thus, precise K_d could not be measured (data not shown).

The NMR titration data do not yield the stoichiometry of deoxystreptamine−RNA binding. Ring II of aminoglycosides is an element that can bind specifically to 5′G(N7)-U(O4)3′, 5′U(O4)-G(N7)3′, 5′G(N7)-G(O6)3′, or 5′G(O6)-G(N7)3′ steps of the major groove of A-form RNA; seven sites should be available for deoxystreptamine binding on the A-site RNA molecule as represented in Figure 3. Even in a fast exchange regime, intermolecular NOEs can be observed between the RNA and the ligand (*20*). For ratios of 1.5:1 and 3:1 deoxystreptamine/RNA, 2D NOESY experiments (mixing times of 250 and 400 ms) were performed in D_2O to detect any intermolecular RNA−drug NOEs. Intermolecular deoxystreptamine/RNA NOEs (Figure 5) between the two protons at position 2 of deoxystreptamine and U1495(H5) are observed at 5 and 10 °C, which are characteristic of the ring II contacts in the paromomycin or gentamicin C1a−RNA complexes (*4*, *5*, *10*). At a ratio of 3:1, intermolecular NOEs were observed between U1495 (H6) and U1490 (H6) and deoxystreptamine protons at a 400 ms mixing time. Very weak intermolecular NOEs could also be detected between the G1489(H8) and deoxystreptamine. Additional NOEs to U1406(H5) and U1490(H5) were observed that indicate multiple deoxystreptamine interaction sites on the RNA as expected in Figure 3. These multiple binding sites were further characterized.

A mutation of U1495A is deleterious for paromomycin binding by preventing specific hydrogen bonding of ring II (deoxystreptamine) to the RNA (*13*). Binding of deoxystreptamine to this RNA mutant was studied to distinguish among the G1494(N7)-U1495(O4) and G1405(N7)-U1406(O4) possible binding sites that would account for the observed NOEs with (H5)U1406 and (H5)U1495 protons. A 2D NOESY experiment was performed in the same experimental conditions as for wild-type RNA with a ratio of 1.5:1 deoxystreptamine/RNA. RNA−deoxystreptamine intermolecular NOEs could be detected with U1406(H5) and U1490(H5-H6). This demonstrates that the intermolecular NOEs between U1406-(H5) and deoxystreptamine observed with the wild-type RNA result from binding of deoxystreptamine to the G1405(N7)-U14O6(O4) sequence and not from weakened binding of deoxystreptamine to U1495-G1494.

A similar study was done to distinguish between two possible binding sites of deoxystreptamine in the lower stem of the A-site RNA (G1489-U1490 and U1490-G1491) that could account for the observed NOEs between deoxystreptamine and the H5 of U1490. A C1409U-G1489A mutant complexed to deoxystreptamine (ratio of 1.5:1 deoxystreptamine/RNA) was studied. Titration of this mutant RNA with deoxystreptamine lead to a downfield shift (0.1 ppm at 1.5/1 deoxystreptamine/RNA stoichiometry) of the imino proton resonance of U1490 but left unaffected the neighboring imino proton of U1409. Analysis of a 2D NOESY experiment performed in the same conditions as for the wild-type (at 5 °C with mixing times of 250 and 400 ms) revealed intermolecular NOEs between the protons of deoxystreptamine and the G-U binding sequence in the upper stem and U1490-(H5). This result indicates binding of deoxystreptamine to the U1490-G1491 binding site.

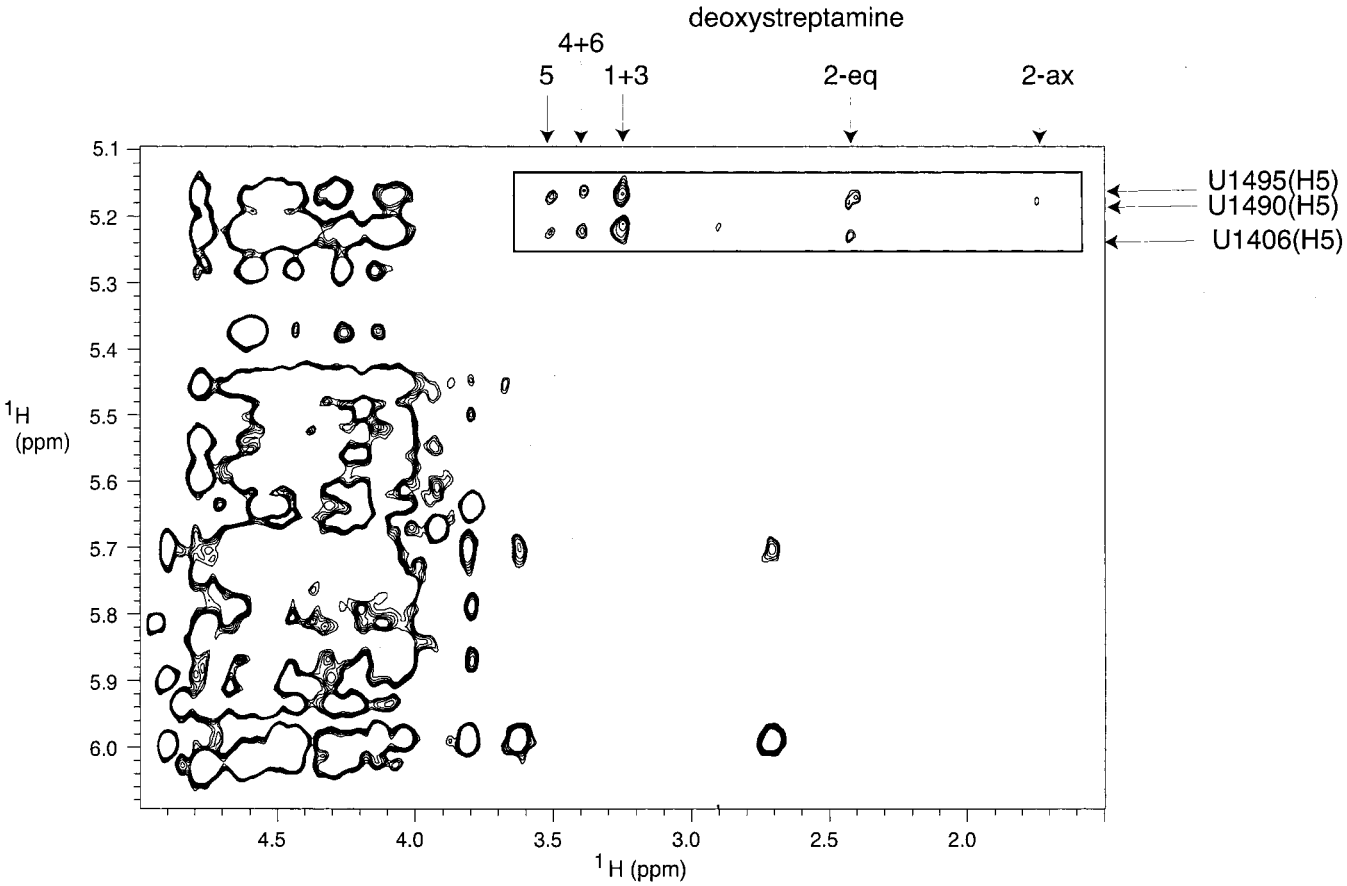

FIGURE 5: 2D NOESY experiment performed at 5 °C with a mixing time of 400 ms on the deoxystreptamine−wild-type RNA complex. This region displays the NOE cross-peaks between the H5 of U1406, U1490, and U1495 with the protons of the deoxystreptamine ring. The intermolecular NOE cross-peaks between the A-site RNA and deoxystreptamine are boxed.

Major Groove Accessibility. The width of the major groove may also be important for deoxystreptamine binding. An A-site RNA with a deletion of the A1492 bulge nucleotide was prepared and titrated with deoxystreptamine. By increasing deoxystreptamine up to a ratio of 1:1.5 RNA/deoxystreptamine, no shift of any imino proton resonances was observed (data not shown). A 2D NOESY experiment performed in the same conditions as for the wild-type (at 5 °C with mixing times of 250 and 400 ms) revealed the absence of deoxystreptamine−RNA intermolecular NOEs and confirmed that deoxystreptamine at this concentration is not binding specifically to this A-site mutant RNA. Intramolecular NOE cross-peaks between deoxystreptamine protons have the same sign as intramolecular NOE cross-peaks between RNA protons, supporting a weak interaction of the ligand with the RNA.

The A1492 deletion mutant RNA was further changed to form an A-form stem where the A1408·A1493 base pair was substituted with an A1408·U1493 base pair. Except for the U1406·U1495 base pair, this RNA contains only canonical base pairs along the stem. An NMR sample of this mutant RNA was prepared and titrated with deoxystreptamine. Again, no shift of any imino proton resonances was observed (data not shown). The absence of deoxystreptamine−RNA intermolecular NOEs in a 2D NOESY experiment (at 5 °C with mixing times of 250 and 400 ms) confirmed that deoxystreptamine at this concentration is not binding to this A-site mutant RNA. These results, together with the NMR data for the A1492 deletion mutant, indicate that deoxystreptamine affinity for the A-site RNA is strongly reduced when the bulged nucleotide (A1492) is removed.

Deoxystreptamine Analogue. Ring II of paromomycin or gentamicin C1a in the A-site RNA−aminoglycoside complexes is contacting G1494 and U1495 through a tight surface complementarity. To test the role of ligand shape on RNA affinity, the binding of a deoxystreptamine analogue, streptamine, to wild-type A-site RNA was investigated. Streptamine contains a hydroxyl group at position 2 (Figure 1C). The A-site RNA was titrated with streptamine as described above for deoxystreptamine. At 5 °C, no shift of any imino protons of the RNA was observed (data not shown). No streptamine−RNA intermolecular NOEs were detected in 2D NOESY experiments (at mixing times of 250 and 400 ms). This indicates that the presence of a bulky hydroxyl group at position 2 prevents tight binding of streptamine to the A-site RNA.

DISCUSSION

Prior structural studies have suggested the importance of the deoxystreptamine moiety in directing sequence-specific RNA recognition. In the RNA−antibiotic complexes formed with paromomycin, neomycin, gentamicins, or ribostamycin, intermolecular NOEs from U1495(H5) were exclusively observed with protons of ring II of aminoglycosides (*4*, *5*, *10*). Ring II, deoxystreptamine, directs binding to the G1494-(N7)-U1495(O4) step of the A-site RNA. Here we have used NMR to detect specific interactions between isolated deoxy-

streptamine and RNA. We propose that ring II of aminoglycosides is a sequence-specific element to target 5′G(N7)-U(O4)3′, 5′G(N7)-G(O6)3′, 5′G(O6)-G(N7)3′, or 5′U(O4)-G(N7)3′ sequences in the major groove of RNA molecules. The data presented here indicate that indeed 2-deoxystreptamine is a structural motif for specific recognition of G(N7)-U(O4) steps in major groove of RNA.

Aminoglycosides can bind to alternate sites within the major groove of an RNA helix. For neamine, which binds weakly to the A-site RNA, intermolecular NOEs from ring II and proton H5 of residues U1495 and U1406 were both observed (*10*). This was interpreted as alternate binding modes of neamine within the binding pocket or dynamics within this site. We observed the same pattern of NOEs upon binding of 2-deoxystreptamine to the A-site RNA. Our study of deoxystreptamine interaction with a mutant U1495A A-site RNA unambiguously assigned intermolecular NOEs [U1406-(H5)/deoxystreptamine] to binding of 2-deoxystreptamine to the G1405(N7)-U1406(O4) sequence. Multiple binding sites for aminoglycosides to the A-site oligonucleotide have been detected using titration calorimetry (R. G. Eason, unpublished data).

In addition to binding to two G-U steps in the upper stem of the A-site RNA, deoxystreptamine binds in the vicinity of U1490 in the lower stem of the A-site RNA. This binding site is located far apart from the normal aminoglycoside-binding site in the upper stem. Two G-U steps can account for the observed intermolecular NOEs: G1489(N7)-U1490-(O4) and U1490(O4)-G1491(N7). Intermolecular NOEs to H8 of G1489 indicate that the G1489(N7)-U1490(O4) sequence is occupied. Deoxystreptamine binds to the U1490-(O4)-G1491(N7) step in a C1409U-G1489A mutant RNA where the G1489(N7)-U1490(O4) binding site is disrupted.

The major groove needs to be opened by a bulge nucleotide to allow deoxystreptamine hydrogen bonding with 5′G-(N7)-U(O4)3′ sequences. From the seven putative deoxystreptamine binding sites (Figure 3), only four G-U steps close to the bulged A1492 residue were found to be occupied. Deletion of the bulged nucleotide disrupts deoxystreptamine binding to all four sites in the major groove.

Steric hindrance may prevent binding of deoxystreptamine in the major groove of a canonical A-form stem RNA. A deoxystreptamine molecule was docked to a G-U step in the major groove of an A-form RNA (according to the hydrogen bonding network found for binding of ring II of paromomycin and gentamicin to the A-site RNA). In an A-form RNA, a steric clash may occur between the N3 amino group of deoxystreptamine and the aromatic moiety of the base of the preceding nucleotide, preventing binding of deoxystreptamine. In the A-site RNA, the major groove is opened by the bulged A1492 residue (*5*). The A1493 aromatic moiety is displaced toward the minor groove of the RNA (*9*) upon paromomycin or gentamicin binding, with the 2-deoxystreptamine moiety interacting with the G1494-U1495 step. Opening of the major groove by the presence of A1492 allows accessibility of the N7 position of G1494, as well as G1491(N7) and G1405(N7), to the N3 amino group of deoxystreptamine. An increased accessibility of the N7 of G1491 and G1405 can explain binding of deoxystreptamine to G1491-U1490 and G1405-U1406 sequences. In a similar way, an increase of G1489(N7) accessibility in the vicinity of the tetraloop sequence can account for the binding of

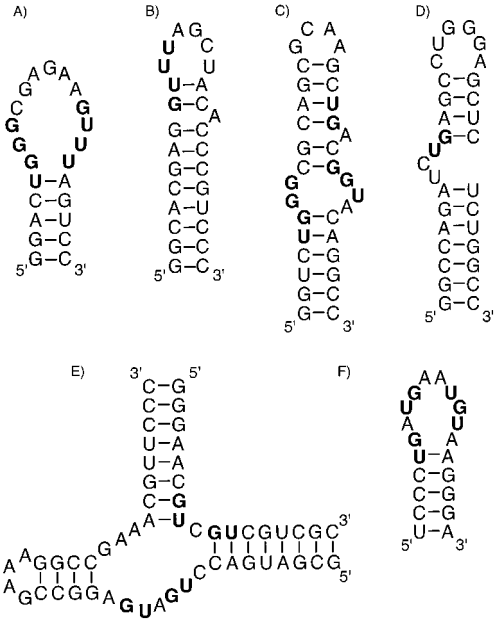

FIGURE 6: Secondary structures of RNA sequences known to bind specifically aminoglycosides containing 2-deoxystreptamine. G-U and G-G steps that are potential binding sites for deoxystreptamine are indicated in boldface type. (A) Neomycin aptamer, (B) tobramycin aptamer, (C) HIV-1 RRE, (D) HIV-1 TAR, (E) hammerhead ribozyme, and (F) group I intron p7.1.

deoxystreptamine to the G1489-U1490 sequence. Our NMR results clearly demonstrate that the bulged nucleotide is strictly required for accessibility of deoxystreptamine to the N7 position of G1494.

Aminoglycosides can bind a wide variety of RNA molecules with a similar affinity such as A-site rRNA, ribozymes (*21*−*23*), HIV-1 RRE, and TAR RNA (*24*−*26*). All these RNA sequences have in common G(N7)-U(O4), G(N7)-G(O6) steps next to bulge nucleotides or in noncanonical stem structures (Figure 6).

The structures of aminoglycoside−RNA aptamer complexes support the role of deoxystreptamine in sequence-specific recognition (*27*−*29*). A neomycin B−RNA aptamer complex structure was characterized by NMR (*29*) where the binding pocket for neomycin is formed of U-G and G-G steps. In the structure of the complex, the amino 1 of deoxystreptamine ring II can form potential hydrogen bonds to the acceptor O4 atoms of U8 and U21 and the O6 atom of G9 whereas the amino group 3 can form potential hydrogen bonds to the acceptor N7 atom of G10. The recognition of 5′G(O6)-G(N7)3′ in the major groove by the deoxystreptamine ring of neomycin agrees with our results. These hydrogen bonds between amino groups 1 and 3 of deoxystreptamine and U(O4)/G(O6)-G(N7) are common to the A-site 16S rRNA−paromomycin (and gentamicin) (*4*, *5*) complexes and the neomycin B−RNA aptamer complex.

NMR structures of tobramycin−RNA aptamers revealed a different mode of interaction of aminoglycosides with the RNA that involves partial recognition of U(O4)/G(O6)-G(N7) sequences. For tobramycin aptamer selection, the drug was connected to the support of the column using ring I. This explains that in this complex, rings I and II only partially interact with the RNA whereas ring III is completely buried in the binding pocket, conferring tight binding affinity and specificity for tobramycin versus gentamicin C (*30*). In the

tobramycin aptamer structure, the mode of interaction of ring II with the RNA involves only the recognition of the N7 guanine residue of the G-U step and not the O4 of the uracil. Recognition of the G-U step as in the A-site−aminoglycoside complexes requires ring II to access the uracil O4 position deep in the major groove of the RNA and brings ring I inside the groove.

The binding affinity of deoxystreptamine to the A-site RNA is similar to that observed for other simple ligand−RNA interactions. The dissociation constant for deoxystreptamine to one of the sites in the RNA is approximately millimolar, based on the NMR titration data. The amino acid arginine binds in a sequence-specific manner to the HIV TAR hairpin stem−loop near a distorted bulge region (*20*, *31*). The arginine binds in the major groove, making base-specific and electrostatic contacts; the dissociation constant for this interaction is also millimolar. Specific arginine binding requires the formation of a base triple to stabilize the amino acid binding pocket. Mutations that disrupt this pocket lead to arginine binding at alternate sites in the major groove.

Paromomycin binds to the eukaryotic ribosomes with decreased affinity compared to that for the prokaryotic ribosomes (*32*). A single mutation in the decoding region A-site (A1408G) results in a different mode of binding of aminoglycoside to the RNA mediated through partial recognition of the G1494-U1495 sequence by deoxystreptamine. Ring II interacts with the RNA in a different manner compared to the prokaryotic complex (*33*). The amino group at position 1 of ring II interacts with G1494 N7 and U1495 O4, and the hydroxyl group at position 6 interacts with U1406 O4. The difference in the geometry of ring II−RNA interaction results in a different positioning of ring I, which is located more toward outside of the major groove of the RNA compared to the prokaryotic complex. The eukaryotic decoding region has a shallow binding pocket for aminoglycosides that does not accommodate the drugs into deep inside the major groove. The complete recognition of the G1494-(N7)-U1495(O4) sequence by deoxystreptamine is not possible, resulting in a weak affinity for the aminoglycosides and exclusion of ring I from the binding pocket.

The structure of the tau exon 10 splicing regulatory element complexed with neomycin was determined by NMR spectroscopy (*34*). The drug binds with lower affinity and specificity to this target than to aptamers or the ribosomal A-site. Again, a different mode of ring II interaction with the RNA is observed. The N3 amine hydrogen from ring II is in hydrogen bonding contact with the phosphate backbone, while the N3 nitrogen is hydrogen bonded to the exocyclic amino group of C. The N1 nitrogen of ring II is in contact with the exocyclic amino group of A. The geometry of the ring II interaction brings the ring I toward outside of the major groove. In fact, in the complex ring I is in close proximity to the phosphate backbone, but there are no RNA base functional groups within 5 Å of ring I (*34*). Thus, the structural context of the deoxystreptamine binding site can pervert the simple rules of RNA recognition outlined in this paper.

The nature of the chemical groups presented to the RNA by the framework of this six carbon ring is also important. Two hydrogen bond donors (amino 1 and amino 3) are spaced by the structural frame of the ring at an adequate distance to two hydrogen bond acceptors, G(N7)-U(O4) or G(N7)-G(O6), of the major groove. The size of chemical groups at position 2 of this six carbon ring was also shown to be important, and substitution of the equatorial hydrogen for a hydroxyl group is deleterious for deoxystreptamine binding. Modeling of the presence of a hydroxyl group at position 2 of ring II in the paromomycin−RNA and gentamicin−RNA complexes showed possible steric hindrance with the base moieties of G1494 and U1495 and the hydroxyl group in the equatorial position.

Ring II of aminoglycosides (2-deoxystreptamine) is the most conserved element among aminoglycoside antibiotics that bind to the A-site. The present work shows that isolated deoxystreptamine specifically interacts with G-U steps within the major groove of the A-site RNA. Additional rings of aminoglycosides increase the affinity and specificity of the antibiotic for the A-site of the ribosome by creating additional drug−RNA and drug−drug contacts (*5*, *10*). Thus, deoxystreptamine can be used as a building block to construct high-affinity RNA binding compounds in similar ways as are used for creating new high-affinity protein ligands (*35*, *36*). In summary, deoxystreptamine plays a role as a molecular ruler to recognize G-U steps in the widened major groove. This explains why aminoglycosides can bind specifically to a wide variety of RNA molecules.

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