Specificity of Aminoglycoside Binding to RNA Constructs Derived from the 16S rRNA Decoding Region and the HIV-RRE Activator Region†

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ABSTRACT: Aminoglycoside antibiotics can bind to many different types of RNA molecules. It was of interest to determine the nature of the selectivity of binding of aminoglycosides to important, biologically relevant RNA targets. Fluorescence anisotropy methods were developed to quantitatively measure aminoglycoside affinities to constructs of the HIV-1 RRE transcriptional activation region and the prokaryotic rRNA decoding region which is the natural antibacterial target of the aminoglycosides. A fluorescent analog of Rev_{34-50} (Fl-Rev_{34-50}) was prepared and shown by fluorescence anisotropy measurements to bind to the HIV-1 RRE region with a stoichiometry of 1 and a dissociation constant of 7.6 nM. RRE RNA is a target for the arginine rich Rev protein, and the binding is known to be mimicked by Rev_{34-50}. The binding is driven by a strongly negative enthalpic term. Aminoglycosides compete with Fl-Rev_{34-50} binding and competition experiments with semisynthetic aminoglycosides and neomycin B and tobramycin show binding affinities in the 1−2 μM range. The binding of aminoglycosides to this construct is thus not highly selective. A prokaryotic rRNA construct was also prepared and shown to bind a fluorescent dye labeled derivative of the antibiotic paromomycin (CRP) stoichiometrically with a dissociation constant of 0.16 μM. Competition experiments with other aminoglycosides showed binding in the micromolar range, with limited specificity for aminoglycoside type, suggesting that much of the aminoglycoside molecule is not involved in binding. The relatively modest specificity in the binding of aminoglycoside described above is to be contrasted to the subnanomolar affinities and specificity of aminoglycoside binding found using *in vitro* selected RNA molecules (Wang et al., 1996).

Aminoglycoside antibiotics are thought to function by binding to sensitive prokaryotic ribosomes, causing misreading of mRNAs (Cundliffe, 1990; Gale et al., 1981). Often, resistance to these antibiotics develops when mutations arise in prokaryotic 16S ribosomal RNA, suggesting that RNA molecules, rather than ribosomal proteins, are the targets for aminoglycosides (Gale et al., 1981; Cundliffe, 1989). Moreover, chemical protection experiments on intact ribosomes generally show that the same regions of 16S RNA (the decoding region) where aminoglycoside resistance mutations are found is also protected from reaction with electrophilic reagents in the presence of aminoglycosides (Woodcock et al., 1991; Noller, 1991). Along these lines, chemical protection experiments with aminoglycosides on a truncated decoding region RNA construct demonstrate that this region can bind aminoglycosides and mRNAs (Purohit & Stern, 1994). These and similar experiments strongly suggest that the 16S rRNA is the target for aminoglycoside action (Hutchin et al., 1993). While the prokaryotic ribosome may be considered the natural target for many aminoglycoside antibiotics, aminoglycoside binding to RNA is not limited to binding to rRNA. In addition to binding to prokaryotic 16S RNA, aminoglycosides have also been found to bind to the HIV-1 RRE transcriptional activator region (Zapp et al., 1993), interfere with intron splicing (von Ahsen et al., 1991), and bind to hammerhead ribozymes (Stage et al., 1995). One is led to ask what the specificity is of aminoglycoside binding to these various RNA structures, particularly given the chemical structures of aminoglycosides; they are basic, H-bonding molecules that would be expected to possess a high inherent affinity for RNA molecules. Along these lines, families of aminoglycosides are generally active as antibiotics, suggesting that the inherent specificity for a particular aminoglycoside might be limited (Chambers & Sande, 1996). Furthermore, chemical protection experiments in which RNA molecules are reacted with group-selective reagents in the presence and absence of aminoglycosides suggest that the binding affinities of the aminoglycosides for the RNA molecules are in the micromolar range (Woodcock et al., 1991; Purohit & Stern, 1994; Zapp et al., 1993). This level of affinity would not suggest a high degree of specificity in the binding process.

Recently, we have developed sensitive fluorescence methods that enable direct, quantitative binding measurements on aminoglycoside−RNA interactions (Wang & Rando, 1995; Wang et al., 1996). In these studies, fluorescent dye tagged aminoglycosides are used to measure binding affinities to RNA molecules selected to bind to particular aminoglycosides (Wang & Rando, 1995; Wang et al., 1996). Fluorescence measurements are performed by both fluorescence quenching and fluorescence anisotropy methods (Wang & Rando, 1995; Wang et al., 1996). High affinity and specific subnanomolar binding to a particular aminoglycoside are observed (Wang & Rando, 1995; Wang et al., 1996).

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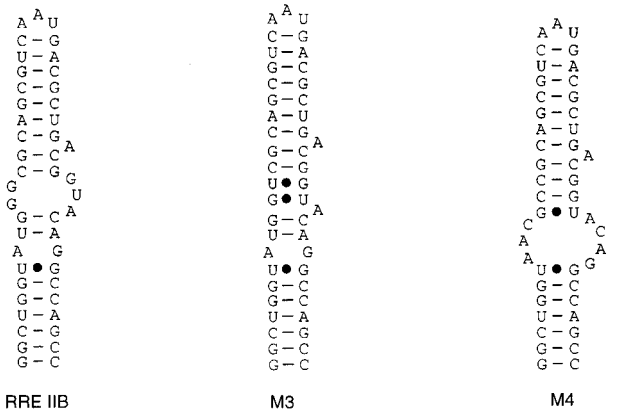
Chart 1: Structure of HIV-1 RRE IIB RNA, Its Mutants, and Rev Peptides Used in These Studies



H_2N-TRQARRNRRRRWRERQR-COOH

REV_{34-50}

Fluorescein-HN-TRQARRNRRRRWRERQR-COOH

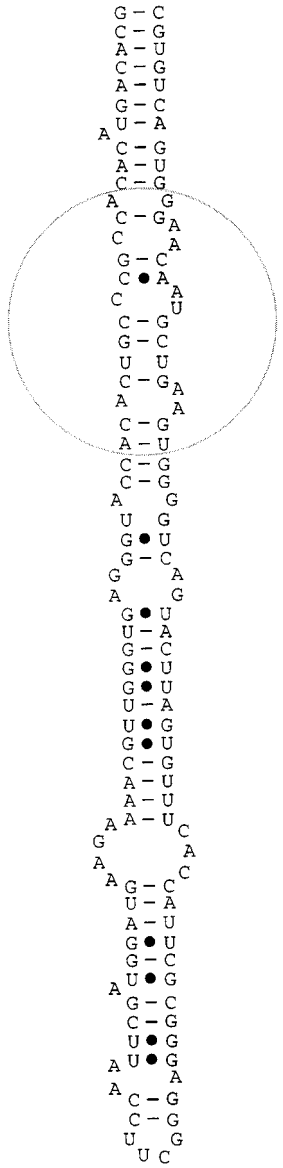
$Fl\text{-}REV_{34-50}$

These selected structures can differentiate between aminoglycosides by factors of 10^3 and greater (Wang et al., 1996). It is of interest to apply these binding techniques to naturally occurring RNA molecules which bind aminoglycosides, in order to determine the affinities and specificities of this binding, and to compare the specificity of aminoglycoside binding to the biologically relevant constructs to that of the *in vitro* selected constructs. Studies are reported here on the binding of aminoglycosides to RNA constructs derived both from the HIV-1 RRE transcriptional activator region (Chart 1) and the 16S rRNA decoding region (Chart 2).

The HIV-1 RRE region of RNA is important for the successful transcription of the HIV-1 genome (Mann & Karn, 1994; Gait & Karn, 1993; Fischer et al., 1995; Sodroski et al., 1986). RRE RNA is a target for the arginine rich Rev protein, whose initial binding is as a monomer, followed by the recruitment of additional molecules of Rev (Mann & Karn, 1994; Daly et al., 1995). The function of Rev binding to RRE appears, at least in part, to be to transport RNA through the nuclear membrane into the cytoplasm, where the RNA is translated into viral proteins (Fischer et al., 1995). One reason Rev−RRE interactions and recognition have been intensively studied is that inhibitors of Rev−RRE binding could lead to the development of drugs effective in the treatment of AIDS (Zapp et al., 1993). Most interestingly, Rev−RRE recognition appears to be modular, in the sense that manageable fragments of the two constructs appear to contain the relevant information underpinning recognition in the full constructs (Kjems et al., 1992; Symensma et al., 1996). This allows for the possibility of the structural analysis of RNA and peptide fragments which may mimic essentials of the normal recognition process (Battiste et al., 1994, 1995; Jensen et al., 1995; Pritchard et al., 1994; Scanlon et al., 1995; Tan et al., 1993; Tan & Frankel, 1994).

The RRE IIB region construct, shown in Chart 1, specifically binds both Rev protein and fragments derived from it (Tan et al., 1993). A particularly useful fragment is Rev_{34-50} (Chart 1),[1] which has been shown by chemical protection experiments to interact with RRE IIB in a manner quite similar to the way full Rev protein interacts with RRE IIB (Kjems et al., 1992). Moreover, Rev_{34-50} binds weakly to those mutant RRE constructs which weakly bind Rev protein, suggesting that RRE recognition by Rev is determined by the Rev_{34-50} fragment (Kjems et al., 1992). The K_D value for fragment binding to RRE IIB is in the 1 nM range, a value not too dissimilar from that obtained with the full Rev protein (Kjems et al., 1992). Circular dichroism studies on the Rev_{34-50} fragment show that it exists partially in an α-helical conformation (Tan et al., 1993). Moreover, modifications of the peptide which increase the α-helical content lead to the generation of higher affinity peptides, suggesting that the Rev protein contains an α-helical domain which interacts with RRE (Harada et al., 1996; Tan et al., 1993).

Chart 2: Secondary Structure of the 16S rRNA Analog Which Includes the Decoding Region Indicated by the Circle



[1] Abbreviations: Rev_{34-50}, 16 mer peptide derived from the Rev protein; $Fl\text{-}Rev_{34-50}$, fluorescein labeled 17 mer peptide derived from the Rev protein; ART, arginine modified tobramycin; C1-PYT, 1-pyreneacetyl labeled tobramycin; C4-PYT, 1-pyrenebutanoyl labeled tobramycin; CRT, 5-carboxytetramethylrhodamine labeled tobramycin; CRP, 5-carboxytetramethylrhodamine labeled paromomycin; CRG, 5-carboxytetramethylrhodamine labeled gentamycin C1; CRK, 5-carboxytetramethylrhodamine labeled kanamycin B.

Small molecules able to block RRE−Rev interactions could be useful as anti-AIDS therapies. Peptide libraries have been explored to discover new peptides able to interfere with RRE−Rev binding (Harada et al., 1996). Moreover, certain aminoglycosides have been shown to inhibit RRE−Rev binding, and also to interfere with HIV replication (Tan et al., 1993; Werstuck et al., 1996). Aminoglycosides and similar molecules would be useful alternatives to basic peptides as RRE antagonists for *in vivo* use, because the latter molecules are unlikely to penetrate cells and are likely to be metabolically unstable.

The second construct studied here is derived from the prokaryotic 16S rRNA (Chart 2). As mentioned above, 16S rRNA is thought to be the locus of action of the aminoglycoside antibiotics. While this region is extensive, resistance mutational and chemical protection experiments have localized the site of action of the antibiotics to a discrete region (the decoding region: Chart 2) near the 3′ terminus of the rRNA (Purohit & Stern, 1994). This decoding region contains the A and P sites for tRNA binding, as well as an important aminoglycoside binding region (Cundliffe, 1989; Woodcock et al., 1991). The binding of aminoglycosides to this region is thought to cause misreading of the mRNA, which constitutes a potentially lethal event for the bacterium (Chambers & Sande, 1996). While 16S rRNA is too large to consider studying at the molecular level at this time, recent studies have suggested that the activities of this RNA can be treated in a modular fashion (Purohit & Stern, 1994). For example, a simple decoding region analog of 49 nts, which includes the circled sequences shown in Chart 2, has been shown by chemical protection experiments to be capable of binding to aminoglycosides, tRNA, and mRNA (Purohit & Stern, 1994). The specificity of aminoglycoside binding to this construct is unclear. In this article, an extensive construct designed to include the decoding region is prepared and studied with respect to aminoglycoside binding specificities (Chart 2). As with the RRE region, aminoglycoside binding occurs with affinities in the micromolar range, without a great deal of specificity with respect to the binding of various aminoglycosides.

MATERIALS AND METHODS

Materials. Paromomycin and kanamycin B were from Sigma. Neomycin B, tobramycin, gentamycin C, and streptomycin were purchased from Fluka. 5-Carboxytetramethylrhodamine succinimidyl ester and 1-pyrenebutanoate succinimidyl ester were purchased from Molecular Probes. Template DNA was purchased from Oligo etc., IDT, or Biosynthesis. The transcription kit (T7-MEGA-shortscript) was from Ambion. Restriction enzymes, *Nar*I and *Bam*HI, and T4 DNA ligase were from Promega. Vector (pGEM 3z) was also purchased from Promega. Cell (DH5a) was purchased from Life Technologies. DNA isolation kit (Genie prep) was from Ambion. DNA extraction kit (QIA quick gel extraction kit) was from QUIAGEN. Boc-Arg-$(Boc)_2$-OH was from BACHEM Bioscience, Inc. Rev_{34-50} peptide and fluorescein labeled Rev_{34-50} peptide (Fl-Rev_{34-50}) were purchased from QCB Inc. All other chemicals were purchased from either Aldrich Inc., Fluka Inc., or Sigma Inc. and were of the highest purity available.

RRE IIB RNA, and the M3 and M4 mutants, were prepared by enzymatic transcription from the synthetic DNA template using the T7-MEGA-shortscript kit (Ambion) and were purified by polyacrylamide electrophoresis. The sequence of 47-mer RNA of RRE IIB was the same as that reported in the literature (Tan et al., 1993). Mutants M3 (G_{48} to U) and M4 (UG_{46} to AC) were also prepared by the literature procedure (Kjems et al., 1992).

For the preparation of the decoding region construct, a 157-mer double strand DNA template was linked with pGEM-3z vector by T4 DNA ligase and cloned. The DNA template contains the decoding region construct, a T7 promoter region, and restriction sites (*Nar*I and *Bam*HI). The vector was digested by restriction enzymes, *Nar*I and *Bam*HI, and then purified by agarose gel electrophoresis before ligation. Linearized plasmid pGEM-3z was amplified and purified by agarose gel electrophoresis and ethanol precipitation. The RNA of the decoding region construct (129 mer) was prepared by transcription of the PCR product (150-mer) using T7-MEGA-shortscript. The RNA was purified by gel electrophoresis. RNA concentrations were determined spectrophotometrically at 260 nm. C1-PYT, C4-PYT, and CRT were prepared as previously reported (Wang & Rando, 1995).

Syntheses. Synthesis of Arginine Modified Tobramycin (ART). Boc-Arg$(Boc)_2$-NHS was synthesized by coupling Boc-Arg-$(Boc)_2$-OH with *N*-hydroxysuccinimide (NHS) in the presence of dicyclohexylcarbodiimide (DCC). Boc-Arg-$(Boc)_2$-OH (1 g, 2.11 mmol), *N*-hydroxysuccinimide (0.243 g, 2.11 mmol), and DCC (0.435 g, 2.11 mmol) were dissolved in 4 mL of *p*-dioxane. The reaction mixture was stirred at room temperature for 2 h. The mixture was filtered and the filtrate was evaporated, leaving the product, which was dried overnight in vacuo (1.1 g).

Boc-Arg(Boc)$_2$-O-Tob. Boc-Arg$(Boc)_2$-NHS (117 mg, 0.21 mmol) and tobramycin (100 mg, 0.21 mmol) were dissolved in 10 mL of *N*,*N*-dimethylformamide (DMF) and 1.5 mL of water. This mixture was stirred at room temperature for 2 h. The solvent was evaporated in vacuo, and the product was dried overnight. This product was used without further purification.

Arginine Modified Tobramycin (ART). Trifluoroacetic acid (TFA; 4 mL) was added to Boc-Arg$(Boc)_2$-*O*-Tob in a flask equipped with a drying tube ($CaCl_2$); the flask was then cooled in an ice bath. The flask was kept on ice for 2 h, shaking occasionally. The TFA was removed by evaporation. The product was dried in vacuo and was purified by cation exchange column chromatography (CG-50, Sigma, 5 × 30 mm). ^{1}H-NMR (in D_2O, 500 MHz) δ = 1.121 (q, 1H), 1.514 (m, 5H), 1.894 (m, 2H), 2.721−2.835 (m, 3H), 2.904 (t, 3H), 3.080−3.431 (m, majority), 3.518 (t, 1H), 3.818 (d, 1H), 3.604−3.672 (m, 3H), 4.945 (d, 1H), 5.008 (d, 1H). FABMS: 624 $(M + H)^+$. FABMS: 624 (M + H)+.

5-Carboxytetramethylrhodamine Labeled Paromomycin (CRP). Paromomycin sulfate (55 mg, ca. 40 mmol) was dissolved in a 4 mL of sodium bicarbonate solution (0.1 M) with 2 mL of DMF. 5-Carboxytetramethylrhodamine succinimidyl ester (5 mg, ca. 10 mmol) in DMF (1 mL) was added to the paromomycin solution. The solution was stirred at room temperature for 10 h. Water (100 mL) was added to the reaction mixture. This solution was passed through a anion exchange column (CG50, Sigma) and washed with 150 mL of water and 150 mL of 0.025 M of ammonium hydroxide. Crude CRP was eluted with 1.25 M ammonium hydroxide. This crude product was purified on an ODS

column (Rainin 5 × 26 mm), by HPLC (Waters 625 LC system) with a gradient of acetonitrile and water. The acetonitrile concentration was increased from 0% to 40% over 40 min. All solvents contained 10 mM trifluoroacetic acid. The separated CRP solution was concentrated in vacuo and lyophilized. ^{1}H-NMR (in D_2O, 500 MHz) δ = 1.226 (m, 2H), 1.953 (m, 2H), 2.711 (q, 1H), 2.818 (m, 4H), 3.081 (s, 15H), 3.124−3.734 (m, majority), 4.140 (m, 4H), 4.303 (t, 1H), 4.989 (s, 1H), 5.263 (q, 2H), 6.525 (s, 2H), 6.711 (d, 2H), 7.133 (d, 2H), 7.486 (d, 1H), 7.965 (d, 1H), 8.163 (s, 1H). FABMS: 1028 $(M + H)^+$.

5-Carboxytetramethylrhodamine Labeled Gentamycin C1 (CRG). CRG was synthesized according to the same procedure as described for CRP. ^{1}H-NMR (in D_2O, 500 MHz) δ = 1.250 (m, 9H), 1.510 (m, 1H), 1.923 (m, 8H), 2.389 (m, 2H), 2.678 (s, 2H), 2.755 (s, 3H), 3.153 (s, 15H), 3.267−4.345 (m, majority), 5.172 (d, 2H), 5.745 (m, 2H), 6.740 (s, 2H), 6.862 (d, 2H), 7.126 (q, 2H), 7.497 (d, 1H), 8.026 (d, 1H), 8.303 (s, 1H). FABMS: 890 $(M + H)^+$.

5-Carboxytetramethylrhodamine Labeled Kanamycin B (CRK). CRK was synthesized according to the same procedure as described for CRP. ^{1}H-NMR (in D_2O, 500 MHz) δ = 1.2895 (q, 2H), 2.499 (m, 2H), 3.142 (s, 15H), 3.363−4.949 (m, majority), 4.985 (d, 1H), 5.784 (s, 1H), 6.714 (s, 2H), 6.868 (t, 2H), 7.133 (s, 2H), 7.514 (d, 1H), 8.031 (d, 1H), 8.275 (s, 1H). FABMS: 896 $(M + H)^+$.

Fluorescence Measurements. Fluorescence intensity and anisotropy measurements were performed on a Perkin Elmer LS-50B luminescence spectrometer equipped with a thermostat accurate to ±0.1 °C. In the study of RRE IIB, samples were excited at 490 and 340 nm and monitored at 525 and 400 nm for Fl-Rev$_{34-50}$ and PYT, respectively. In the study of the decoding region, samples were excited at 550 nm and monitored at 580 nm. The integration time was 4 s. For every single point, 5 measurements were made, and their average values were used for calculation. Measurements were performed in buffer solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM HEPES (pH 7.40). Tracer concentrations were determined spectroscopically at 492 nm using a molar extinction coefficient of 7.80×10^4 M^{-1} cm^{-1} for FL-Rev$_{34-50}$, at 340 nm using a molar extinction coefficient of 5.40×10^4 M^{-1} cm^{-1} for C1-PYT and C4-PYT, and at 550 nm using a molar extinction coefficient of 6.00×10^4 M^{-1} cm^{-1} for CRP.

Determination of Binding Parameters. The affinities of RNAs for the dye labeled Rev$_{34-50}$ and dye labeled aminoglycosides were determined by fluorescence anisotropy or fluorescence intensity measurements. The dissociation constant between tracer and RNA is defined by the following equations:

$$\text{RNA·tracer} \overset{K_d}{\rightleftharpoons} \text{RNA} + \text{tracer} \quad (1)$$

$$K_d = [\text{RNA}][\text{t}]/[\text{RNA·t}] \quad (2)$$

where [t] is the tracer (Fl-Rev$_{34-50}$, or CRP) concentration.

The molar fraction of bound species is obtained by the following equation:

$$f = (i - i_0)/(i_\infty - i_0) \quad (3)$$

where i, i_∞, and i_0 are the fluorescence intensities or the fluorescence anisotropy values of sample, totally bound tracer, and totally free tracer, respectively. The fluorescence intensity or anisotropy values of totally bound species are estimated by measuring samples in the presence of excess RNA or ligand. The value can also be calculated using previously published eq 1 (Wang & Rando, 1995).

The concentrations of each species are denoted by the following equations:

$$[\text{RNA·t}] = [\text{t}]_0 \cdot f \quad (4)$$

$$[\text{t}] = [\text{t}]_0(1 - f) \quad (5)$$

$$[\text{RNA}]_0 = [\text{RNA·t}] + [\text{RNA}] \quad (6)$$

where $[\text{t}]_0$ and $[\text{RNA}]_0$ are the initial concentrations of the tracer and RNA, respectively.

The following equation is obtained from eqs 1−6:

$$[\text{RNA}]_0 = K_d(i - i_0)/(i_\infty - i) + [\text{t}]_0(i - i_0)/(i_\infty - i_0) \quad (7)$$

For the direct (tracer) binding assay, eq 7 is used for the calculation of K_D (dissociation constant) between the tracer and the RNA.

The affinity of the analog RNA for Rev$_{34-50}$ or for the aminoglycosides is determined by competition between either Rev$_{34-50}$ or the aminoglycosides, and tracer (Fl-Rev$_{34-50}$, or CRP).

The dissociation constants (K_D) between RNA and the ligand (Rev$_{34-50}$ or the aminoglycosides) are defined by following equations:

$$\text{RNA·ligand} \overset{K_D}{\rightleftharpoons} \text{RNA} + \text{ligand} \quad (8)$$

$$K_D = [\text{RNA}][\text{L}]/[\text{RNA·L}] \quad (9)$$

where [L] is ligand (Rev$_{34-50}$ or the aminoglycosides) concentration.

The concentrations of each species are given by eqs 4 and 5 and the following equations:

$$[\text{L}] = K_D\,[\text{RNA·L}]/[\text{RNA}] \quad (10)$$

$$[\text{L}]_0 = [\text{L}] + [\text{RNA·L}] \quad (11)$$

$$[\text{RNA}]_0 = [\text{RNA·L}] + [\text{RNA·t}] + [\text{RNA}] \quad (12)$$

where $[\text{L}]_0$ and $[\text{RNA}]_0$ are the initial concentrations of ligand (Rev$_{34-50}$ or the aminoglycosides) and RNA, respectively.

The following equation is readily derived from the equations shown above:

$$[\text{L}]_0 = (K_D(i_\infty - i)/K_d(i - i_0) + 1)([\text{RNA}]_0 - K_d(i - i_0)/(i_\infty - i) - [\text{t}]_0(i - i_0)/(i_\infty - i_0)) \quad (13)$$

In the competitive binding assay, eq 13 is used for the calculation of K_D, the dissociation constant for the RNA and the (Rev$_{34-50}$ or the aminoglycosides) interactions.

The van't Hoff equation, $\ln K_D = \Delta H°/(RT) - \Delta S°/R$, is used to calculate $\Delta H°$ and $\Delta S°$ for the binding reaction between Rev$_{34-50}$ and the RRE constant.

RESULTS

Binding Characteristics of Dye-Labeled Rev$_{34-50}$ Peptide with RREIIB and the M3 and M4 Mutants. Initial studies were performed monitoring the interactions of the fluorescent

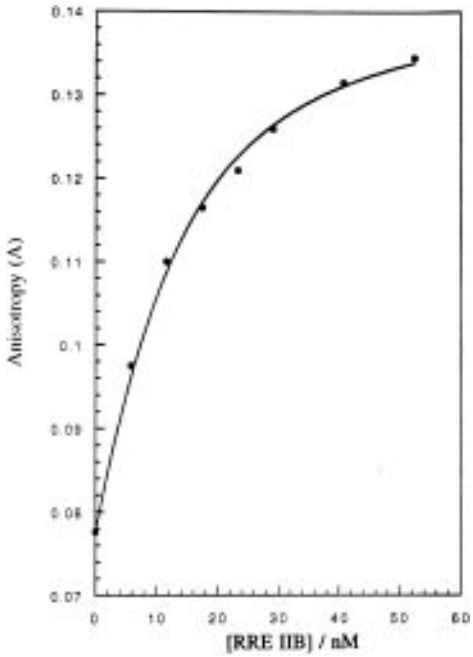


FIGURE 1: Fluorescence titration of Fl-Rev$_{34-50}$ (10 nM) as a function of RRE IIBRNA concentrations. Curve fitting (solid line) using eq 7 gave 7.62 nM as the K_D.

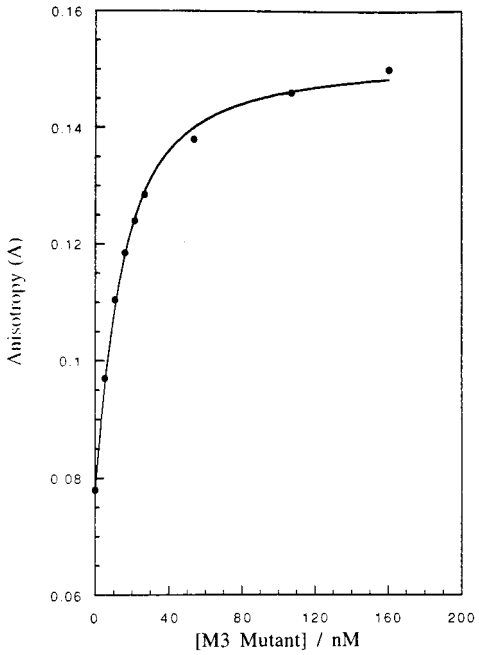


FIGURE 2: Fluorescence titration of Fl-Rev$_{34-50}$ (10 nM) as a function of M3 mutant RNA concentrations. Curve fitting (solid line) using eq 7 gave 9.21 nM as the K_D.

peptide Fl-Rev$_{34-50}$ with RRE IIB RNA (Chart 1). Binding studies were carried out by titrating a solution of Fl-Rev$_{34-50}$ (10.0 nM) with aliquots of the RNA stock solution. Figure 1 shows the change in fluorescence anisotropy (A) as a function of RRE IIB RNA. A remarkably large change in anisotropy was observed; this can be attributed to the large difference in molecular size between the free Fl-Rev$_{34-50}$ (2.1 kDa) and its RNA complex (ca. 17.6 kDa). An anisotropy change of this magnitude is certainly large enough for quantitative calculation. Curve fitting using eq 7 gave a dissociation constant of 7.62 nM for the complex with stoichiometric binding. Additional evidence that the binding interactions are specific was provided by further studies carried out on the two RRE IIB mutants M3 and M4 (Kjems et al., 1992). The M3 mutant (G48 to U) can still bind Rev with about the same affinity as RRE IIB RNA (Kjems et al., 1992). As shown in Figure 2, a dissociation constant of 9.21 nM was measured for the interaction of Fl-Rev$_{34-50}$ with the M3 mutant of RRE IIB. Again, stoichiometric binding is observed. Finally, similar experiments were performed on the M4 mutant (UG46 to AC) (Kjems et al., 1992). This mutation disrupts the binding of Rev (Kjems et al., 1992). Fluorescence anisotropy of Fl-Rev$_{34-50}$, measured as a function of the increasing concentration of M4 mutant, showed no significant change in anisotropy (Figure 3A), suggesting M4 does not bind Fl-Rev$_{34-50}$. However, it is also possible that Fl-Rev$_{34-50}$ forms a complex with the M4 mutant, but that this binding does not produce a change in fluorescence anisotropy. To address this possibility, Fl-Rev$_{34-50}$ was titrated by the M4 mutant in the presence of 17 nM RRE IIB RNA, which does show an anisotropy change upon binding. As shown in Figure 3B, Fl-Rev$_{34-50}$ forms a complex with RRE IIB RNA, as indicated by the observed induced fluorescence anisotropy increase. If the M4 mutant binds to Fl-Rev$_{34-50}$, it would be expected to reduce the anisotropy increase caused by RRE IIB, since the M4 mutant would compete with RRE IIB RNA for Fl-Rev$_{34-50}$. However, no fluorescence anisotropy change was

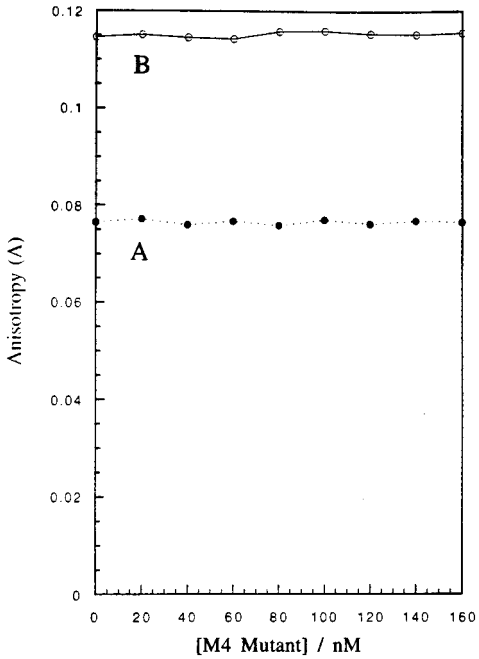


FIGURE 3: Fluorescence titration of Fl-Rev$_{34-50}$ (10 nM) as a function of M4 mutant RNA concentration, in the absence (A) and in the presence (B) of 15 nM RRE IIB. No fluorescence isotropy changes were observed in either case.

observed upon the addition of the M4 mutant RNA (Figure 3B), and hence the M4 mutant does not bind to Fl-Rev$_{34-50}$.

Competitive Binding of Fl-Rev$_{34-50}$ with Rev$_{34-50}$. Since the assay described above provides reliable quantitative data on the binding of the fluorescent probe to RRE IIB RNA, the assay was adapted as a competitive one for general use. In particular, the binding affinity of Rev$_{34-50}$ for RRE IIB RNA was determined by direct competition with Fl-Rev$_{34-50}$. A mixture of Fl-Rev$_{34-50}$ (10.0 nM) and RRE IIB RNA (17 nM) was first prepared. The initial anisotropy value was measured to be 0.122 $\pm$ 0.002. Competitive binding experiments were carried out by adding aliquots of Rev$_{34-50}$

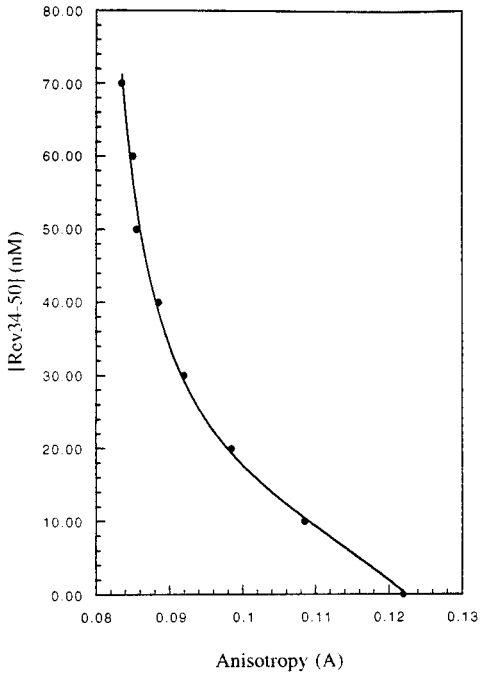


FIGURE 4: Fluorescence titration of Fl-Rev$_{34-50}$ (10 nM) in the presence of RRE IIB (17 nM) as a function of to Rev$_{34-50}$ concentrations. Curve fitting (solid line) using eq 13 gave 1.41 nM as the K_d.

Table 1: Dissociation Constants (K_D's, nM) of Fl-Rev$_{34-50}$ and Rev$_{34-50}$ with RRE IIB and Its Mutants Measured by Fluorescence Anisotropy Assay at 4 °C

	RRE IIB	M3 mutant	M4 mutant
Fl-Rev$_{34-50}$	7.62 ± 0.65	9.21 ± 0.61	no binding
Rev$_{34-50}$	1.41± 0.63	2.08 ± 0.32	

Table 2: K_D's of Fl-Rev$_{34-50}$ and RRE IIB RNA Complex at Different Temperatures

	temp (°C)			
	4	15	27	40
K_D (nM)	7.62 ± 0.65	15.4 ± 4.5	45.5 ± 3.6	216 ± 21

to the mixture. Figure 4 shows that gradually as the Rev$_{34-50}$ concentration was increased, the anisotropy decreased, approaching that of free Fl-Rev$_{34-50}$. Using the data from Figure 4 and a K_d = 7.62 nM (for Fl-Rev$_{34-50}$), the dissociation constant for the interaction of Rev$_{34-50}$ with the RNA can be calculated. Nonlinear curve fitting according to eq 13 gave a K_D = 1.41 nM. This value is in agreement with those described in the literature, estimated by gel shift (Kjems et al., 1992; Powell et al., 1995) and filter binding assays (Daly et al., 1993). Table 1 provides a compilation of the dissociation constants measured.

Entropy and Enthalpy of Binding Reaction. The new fluorescence anisotropy binding assay allows one to measure the thermodynamic values associated with Rev binding accurately. The temperature dependence of the dissociation constants of Fl-Rev$_{34-50}$ with RRE IIB RNA were measured (4, 15, 27, and 37 °C) (Table 2). The results were used to calculate the entropy and the enthalpy change of the binding reaction using the van't Hoff equation (Figure 5). The determined values are $\Delta H° = -66.7 \pm 1.0$ kJ mol^{-1} and $\Delta S° = -83.7 \pm 1.3$ J K^{-1} mol^{-1}. The binding reaction is clearly driven by the large negative enthalpy change, which

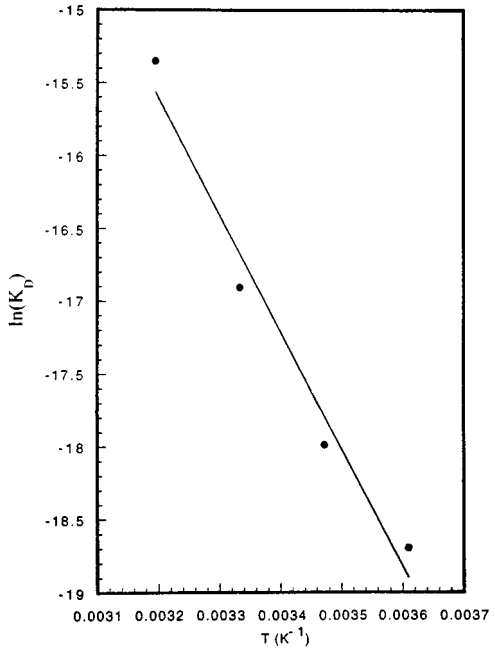
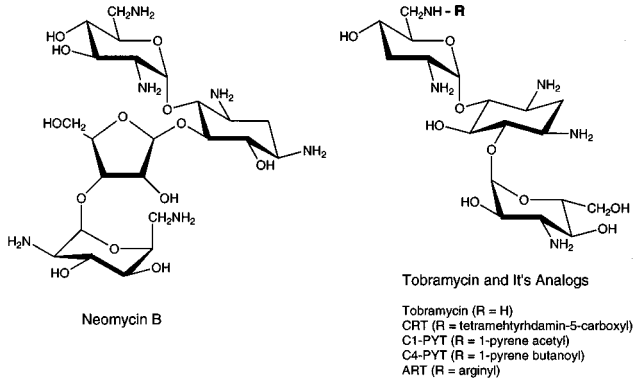


FIGURE 5: The van't Hoff plot of the Fl-Rev$_{34-50}$ and RRE IIB binding reaction.

Chart 3: Aminoglycosides Which Interact with RRE IIB RNA



compensates for the negative entropy change associated with the loss of degrees of freedom as a consequence of Rev binding to RRE.

Competitive Binding of Aminoglycoside Antibiotics with Fl-Rev$_{34-50}$. It has been reported that certain aminoglycoside antibiotics can selectively bind to RRE RNA and selectively block HIV-1 virus production (Zapp et al., 1993). It was of interest to determine if aminoglycosides could directly compete with Fl-Rev$_{34-50}$ and, further, to determine dissociation constants and stoichiometry of binding accurately for the representative aminoglycosides shown in Chart 3. Neomycin B and tobramycin have been reported to bind to the RRE IIB region (Zapp et al., 1993). C1-PYT and C4-PYT are pyrene containing tobramycin analogs previously used to study the interactions of tobramycin analogs with selected RNA structures (Wang & Rando, 1995). ART, an arginyl substituted tobramycin analog, was prepared for study because arginyl moieties have proved to interact strongly with RNA molecules through electrostatic interactions (Calnan et al., 1991). Regiospecific synthesis of tobramycin analogs could be readily achieved, because the 6′-primary amino group is selectively acylated as a consequence of being the sole unhindered primary amino group (Tangy et al., 1983; LeGoffic et al., 1979). Competitive binding experiments

Table 3: Dissociation Constants (K_D's, μM) Determined by Competitive Binding Experiments with Fl-Rev$_{34-50}$ at 4 °C

aminoglycosides	K_D's (μM)	aminoglycosides	K_D's (μM)
neomycin B	1.18 ± 0.24	C1-PYT	0.12 ± 0.04
tobramycin	2.29 ± 0.12	C4-PYT	0.23 ± 0.03
		ART	0.94 ± 0.03

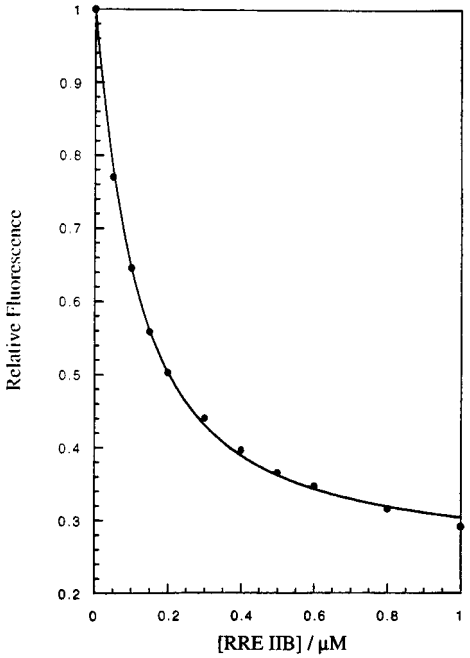


FIGURE 6: Fluorescence titration of 50 nM C1-PYT as a function of RRE IIB concentrations. The solid line is a calculated curve obtained by curve fitting using eq 7.

were carried out under the same conditions as described above for the competition of Rev$_{34-50}$ with Fl-Rev$_{34-50}$. The K_D values, which were determined by competitive binding experiments, are summarized in Table 3. It is clear that the chemically modified tobramycins—C1-PYT and C4-PYT—and ART are more potent inhibitors for Rev$_{34-50}$ binding than the unmodified forms.

Measuring the K_d and Stoichiometry of Complex Formation between C1-PYT and RRE IIB RNA by Directly Monitoring C1-PYT Fluorescence. The competitive assays described above do not allow for a determination of the stoichiometry of aminoglycoside binding, since the measurement records only the displacement of Fl-Rev$_{34-50}$ from the RRE construct. In order to access the specificity of aminoglycoside binding, it is important to determine how many molecules of the drug actually bind to the RNA target. Since C1-PYT is fluorescent, it is possible to study C1-PYT binding by directly measuring C1-PYT fluorescence intensities (Wang & Rando, 1995). The fluorescence intensity of C1-PYT is quenched upon binding to RNA molecules (Wang & Rando, 1995). However, because the fluorescence of C1-PYT suffers significant photobleaching and quenching by oxygen, it is difficult and inconvenient to perform experiments by titrating RRE IIB with C1-PYT directly. In an alternate approach, C1-PYT was titrated with RRE IIB under conditions where the fluorescence intensity was stable before the addition of RRE IIB. To determine the binding stoichiometry of C1-PYT to RRE IIB, various concentrations of C1-PYT (50, 100, 200, and 500 nM) were separately titrated with RRE IIB. Figures 6−9 show the results of these titrations. The experimental data were precisely fitted using

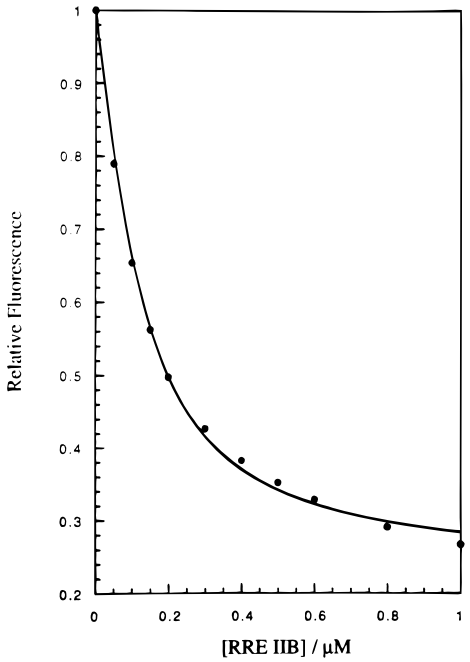


FIGURE 7: Fluorescence titration of 100 nM C1-PYT as a function of RRE IIB concentrations. The solid line is a calculated curve obtained by curve fitting using eq 7.

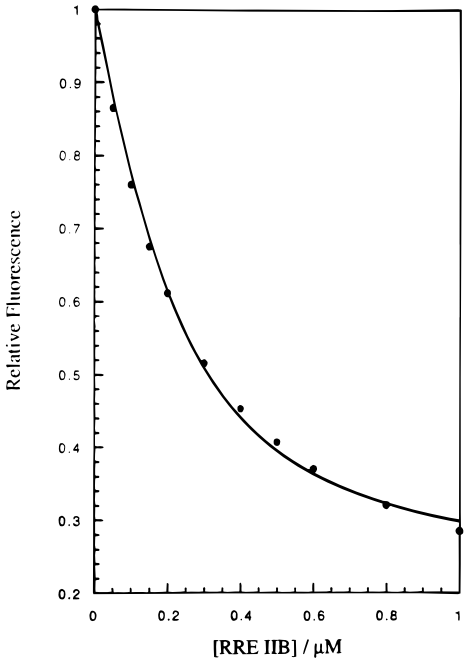


FIGURE 8: Fluorescence titration of 200 nM C1-PYT as a function of RRE IIB concentrations. The solid line is a calculated curve obtained by curve fitting using eq 7.

eq 7 from the Materials and Methods. The results are summarized in Table 4. At all of the examined C1-PYT concentrations, the data can be analyzed by 1:1 complex formation. The K_d values are in agreement with each other and are close to the values determined by competition with Fl-Rev$_{34-50}$. Moreover, the fluorescence quenching factor Q ($Q = (i_0 - i)/i_0$, where i and i_0 are the fluorescence intensities of sample and totally free tracer, respectively) is the same in all cases, showing that the identical binding isotherms are followed. Most importantly, these experiments demonstrate that aminoglycoside binding to RRE IIB RNA is stoichiometric.

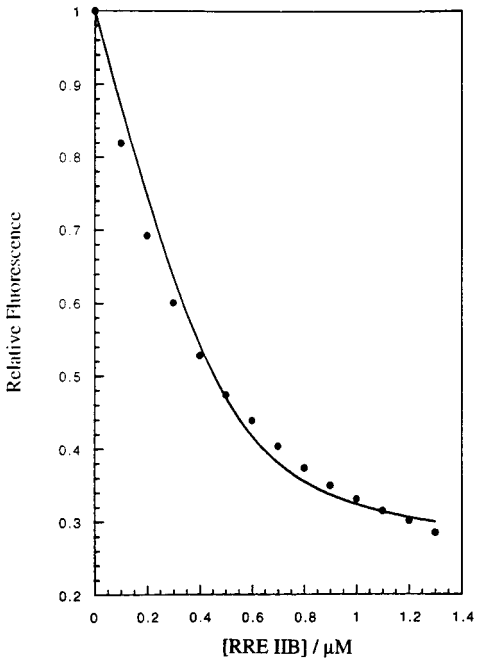


FIGURE 9: Fluorescence titration of 500 nM C1-PYT as a function of RRE IIB concentration. The solid line is a calculated curve obtained by curve fitting using eq 7.

Table 4: K_d's and Fluorescence Quenching Factor (Q) Measured by Titration of C1-PYT with RRE IIB at Different C1-PYT Concentrations (4 °C)

	C1-PYT (nM)			
	50	100	200	500
K_D (nM)	88 ± 7	73 ± 8	89 ± 5	67 ± 9
Q	0.76 ± 0.01	0.77 ± 0.01	0.79 ± 0.02	0.75 ± 0.03

Binding of Fluorescent Dye Labeled Paromomycin to a Prokaryotic 16S Decoding Region Construct. The experiments described above demonstrate that aminoglycosides bind to the RRE IIB region with affinities in the micromolar range. Very little specificity of binding was observed among the aminoglycosides studied. To further investigate more fully the specificity of aminoglycoside binding to biologically relevant RNA constructs, a portion of the prokaryotic 16S rRNA region 1 shown in Chart 2 was prepared and studied with respect to its ability to discriminate among various aminoglycosides. In these experiments, a fluorescent paromomycin (CRP, Chart 4) derivative was used in the binding studies. We had previously shown that fluorescent aminoglycosides could bind to RNA constructs, and that fluorescence anisotropy increases were observed as a consequence of this binding, due to the increased size of the probe−RNA heterodimer compared to the fluorescent probe itself (Wang et al, 1996). The structures of the probes used in the current studies are shown in Chart 4. Paromomycin is expected to bind to the construct used here, since it has been found to interact with the rRNA decoding region (Moazed & Noller, 1987) and with a 49 nt analog derived from it (Purohit & Stern, 1994). Paromomycin also has the advantage that it does not possess a readily modifiable unhindered primary amino group in the A-ring. It would be expected that analogs substituted in the A-ring would not bind to the decoding region because one mechanism of bacterial resistance to aminoglycosides is through acetylation or adenylation of an A-ring amino group in an aminoglycoside (Chambers & Sande, 1996). Presumably, this modification blocks the interaction of the aminoglycoside with the decoding region.

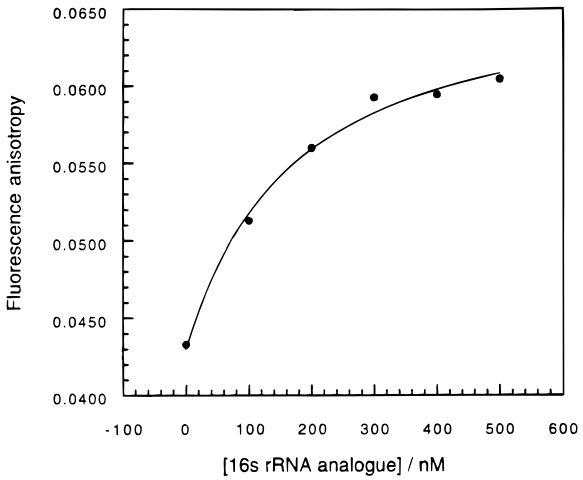


FIGURE 10: Fluorescence anisotropy of CRP (10 nM) as a function of 16S rRNA analog concentrations.

In the studies carried out here, the binding assays were performed by titrating a CRP solution (10 nM) with aliquots of the RNA solution. As expected, the fluorescence anisotropy of CRP increased upon the addition of RNA. Figure 10 shows the titration curve of CRP with the RNA solution. Each data point fits the theoretical curve obtained from eq 7. These results indicate that the binding stoichiometry of the RNA decoding construct with CRP is 1:1. A dissociation constant of 165 ± 31 nM was calculated by nonlinear curve fitting using eq 7 from the Materials and Methods. Thus, CRP is a useful probe for studying aminoglycoside interactions with the decoding region.

Competitive Binding of Aminoglycosides to the Decoding Region Construct. Competition experiments were performed with other aminoglycosides to determine binding affinities and the specificity of binding. In these experiments a solution of CRP (10 nM) and RNA (300 nM) was used as the assay reagents. Competitive binding was determined by titrating this solution with aliquots of aminoglycoside stock solutions. Fluorescence anisotropy values were decreased upon the addition of competing aminoglycosides, as CRP was displaced from the RNA binding site by the aminoglycoside. Figure 11 shows a titration curve of competitive binding, using neomycin B to displace CRP. The data points fit the theoretical curve, which assumes a 1:1 binding mode. Using a series of aminoglycosides in these competition experiments, dissociation constants for each aminoglycoside were determined by nonlinear curve fitting using eq 13 from the Materials and Methods. The results from these competition experiments are presented in Table 5. There are several noteworthy points to be made from this table. First, paromomycin, gentamycin C, kanamycin B, and tobramycin all bound with virtually identical dissociation constants, even though their structures are significantly different (Chart 5). Neomycin B bound significantly tighter than these aminoglycosides. Hygromycin B bound weakly to the construct, but the binding affinity was too weak to measure the dissociation constant accurately. The guanidinyl aminoglycoside, streptomycin, did not measurably bind to the construct. Streptomycin would not be expected to bind to the decoding region (Moazed & Noller, 1987), so its failure

Chart 4: Tracers Used in the Binding Assay of the Decoding Region

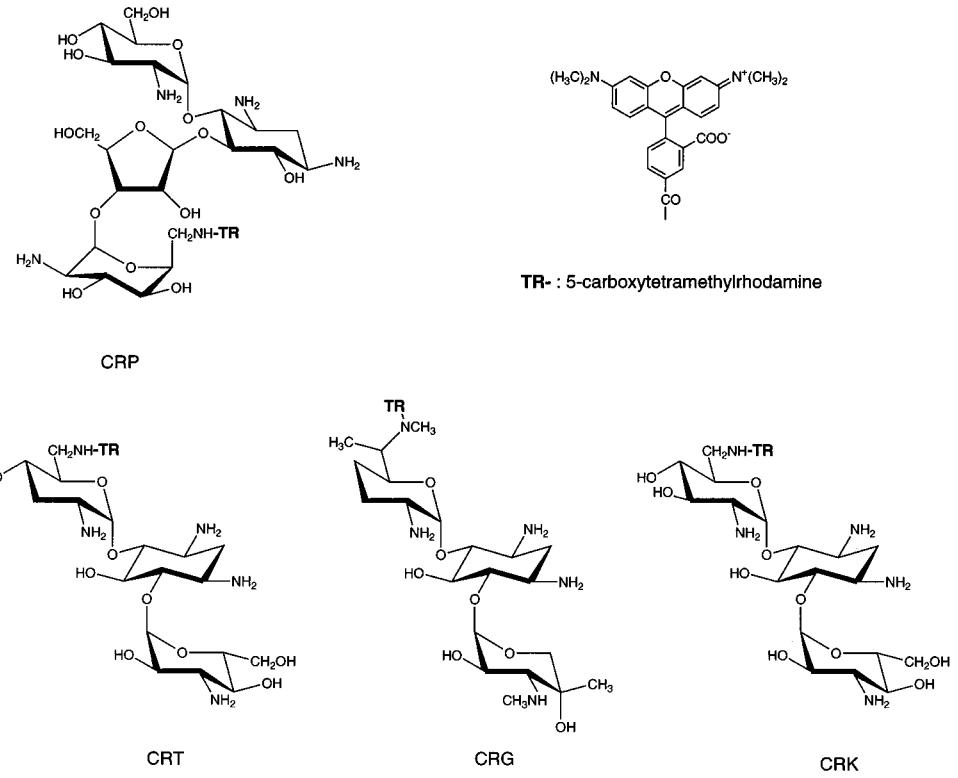


Table 5: K_D's of Aminoglycosides Binding to the 16S rRNA Decoding Region Analog[a]

aminoglycoside	K_D (μM)	aminoglycoside	K_D (μM)
CRP	0.165 ± 0.031	kanamycin B	2.14 ± 0.29
paromomycin	1.85 ± 0.32	tobramycin	1.69 ± 0.16
neomycin B	0.132 ± 0.023	hygromycin B	wb
gentamycin C	1.84 ± 0.39	streptomycin	nb

[a] CRP: 5-carboxytetramethyl rhodamine labeled paromomycin (tracer). wb: very weak binding (anisotropy change was too small to determine accurate dissociation constant). nb: no binding.

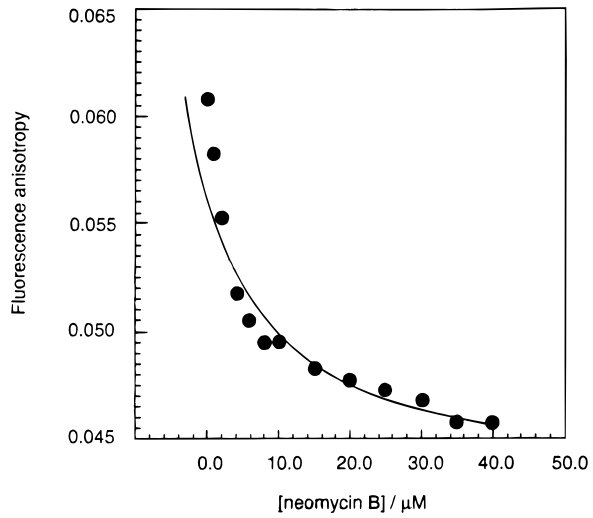


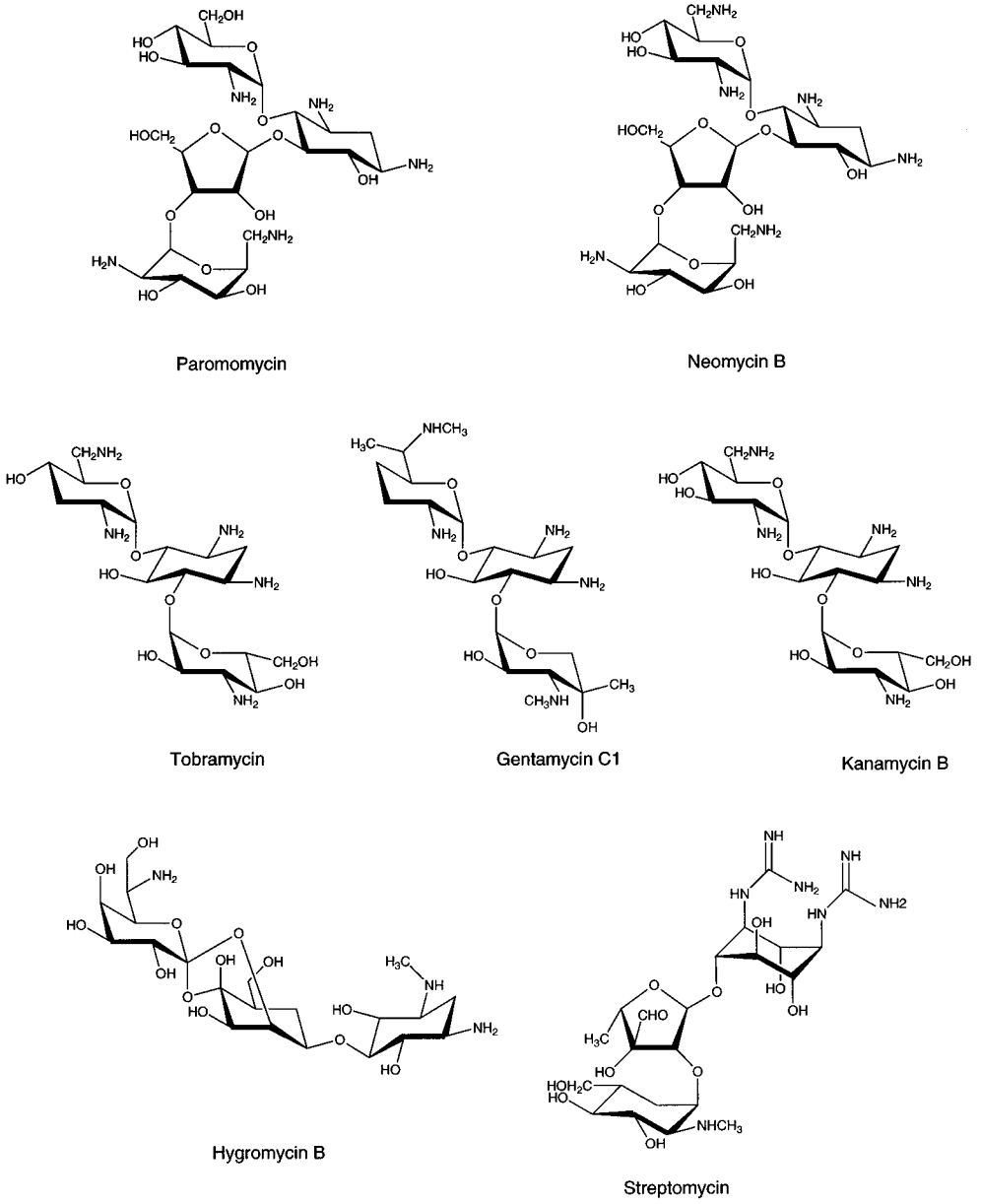
FIGURE 11: Fluorescence anisotropy of CRP (10 nM) containing the 16S rRNA analogue as a function of neomycin B concentrations.

to bind to the construct serves as an indicator of the relevance of our decoding construct to ribosomal−aminoglycoside interactions. Along these lines, it is interesting to note that 5-carboxytetramethylrhodamine labeled derivatives of tobramycin, kanamycin B, and gentamycin C1 did not bind to the decoding region construct. This is also an expected result because the amino groups reacted in these cases appear to be essential for aminoglycoside binding (Chambers & Sande, 1996).

DISCUSSION

The studies described here are aimed at quantitatively determining the nature of the binding interactions between aminoglycoside antibiotics and two RNA constructs of biological relevance: the decoding region derived from the prokaryotic 16S ribosome, and the HIV-1 RRE IIB transcriptional activator region. We initially sought to develop an accurate, quantitative assay system for determining stoichiometries and affinities of antagonist binding to a particular RNA molecule. Typically used methodologies, such as gel shift assays, are nonquantitative and cumbersome to use, and can be misleading, since only kinetically stable complexes are observable and scored. Equilibrium dialysis methods are possible, but they require the very difficult syntheses of very high specific activity aminoglycosides. To our knowledge, molecules of this type have not been reported in the literature. Fluorescence methods appear ideal for the kinds of measurements required because they are extremely sensitive, convenient to use, and readily adaptable for the screening of libraries. We had previously developed a fluorescence method to study the interactions of aminoglycosides with RNA molecules; this method has the advantage of being quantitative and rapid (Wang & Rando, 1995). We describe here a highly specific and quantitative fluorescence depolarization method of studying the interactions of Rev and aminoglycosides with RRE, and the nature of the binding of aminoglycosides with a decoding region construct based on the prokaryotic 16S rRNA. The purpose of the current studies is to determine the affinities of aminoglycoside binding, and to further determine how specific the binding to these biologically relevant constructs is.

Chart 5: Aminoglycosides Used in the Binding Assay on the Decoding Region



Fl-Rev$_{34-50}$ was prepared and shown to interact readily with RRE IIB in a stoichiometric fashion, with a $K_d = 7.6$ nM. To assess the specificity of this binding, the interactions of Fl-Rev$_{34-50}$ with the M3 and M4 mutant constructs of RRE IIB RNA were followed. According to published studies, the M3 mutant should bind the probe, but the M4 mutant should not, as it binds Rev only very weakly (Kjens et al., 1992). Indeed, Fl-Rev$_{34-50}$ interacted with the M3 mutant with a K_d similar to that found with RRE IIB itself, but Fl-Rev$_{34-50}$ did not bind to M4. In competition experiments, it was found that Rev$_{34-50}$ bound to RRE IIB with a $K_D = 1.4$ nM. Thus, the fluorescein moiety of Fl-Rev$_{34-50}$ does not appear to interact strongly with the RNA. The small diminution in binding to Fl-Rev$_{34-50}$ (∼5-fold) compared to Rev$_{34-50}$ could be due to a decrease in the α-helical content in the modified probe, although decreasing the positive charge at the amino terminus would have been expected to stabilize α-helix formation. Published studies lead to the expectation that Rev$_{34-50}$ binding is dependent on α-helical content (Harada et al., 1996; Tan et al., 1993).

Aminoglycosides have been reported to bind to the HIV-1 RRE IIB region (Zapp et al., 1993; Werstuck et al., 1996). The binding of aminoglycosides appeared to be competitive with the Rev protein, but quantitative determination of stoichiometries and binding affinities was not made (Zapp et al., 1993). Using an ultrafiltration binding assay, a K_D of 82 nM was determined for the interactions of neomycin B with an RRE construct different from the one studied in the present article (Werstuck et al., 1996). However, direct competition experiments between neomycin B and Rev suggested a dissociation constant of approximately 5 μM for neomycin B, which is similar to the value reported here (Werstuck et al., 1996). In the present study it is demonstrated by competition with Fl- Rev$_{34-50}$ that the binding affinities for the structurally dissimilar neomycin B and tobramycin are 1.18 μM and 2.29 μM, respectively. ART bound to the RRE IIB construct with a 2−3 fold greater affinity than did tobramycin itself. C1-PYT and C4-PYT bound with somewhat higher affinities (∼10−20-fold) to RRE IIB than did either tobramycin or neomycin B. This

suggests that hydrophobic and/or stacking interactions occur between the pyrene moieties and the RNA molecule. Overall, however, the specificity of aminoglycoside binding is clearly limited with the RRE IIB construct studied here.

The stoichiometry of aminoglycoside binding could not be determined from the kind of fluorescence measurement described here, because only the displacement of Fl-Rev_{34-50} from the RRE region is being measured. To determine the stoichiometry of aminoglycoside binding, a complementary fluorescence assay involving PYT was utilized. The quenching of the fluorescence of the pyrene moiety by RNA when PYT is bound to the RRE regions allows one to determine directly both the affinity and stoichiometry of aminoglycoside binding. The study of C1-PYT binding to RRE IIB showed that the affinity of binding is approximately 90 nM and the stoichiometry of binding is one to one. This stoichiometry was determined by showing that the experimentally determined binding isotherms precisely fit the calculated curve derived for a binding stoichiometry of one to one. It should be noted that while the direct fluorescence quenching assay is useful for the direct measurement of the affinity and stoichiometry of C1-PYT binding to RRE IIB, the assay can be problematic for carrying out competition studies with other aminoglycosides. This is because the pyrene moiety in and of itself can weakly bind to RNA. Displacement of the high affinity binding component by competing ligand can result in incomplete recovery of fluorescence intensity, because of putative low affinity (nonspecific) binding. The relative extents of quenching at the high and low affinity sites are unknown, causing uncertainty in the measurement of binding affinity and stoichiometry for the competing ligand. On the other hand, the direct quenching binding assay is excellent for the measurement of fluorescent probe binding to RNA in the absence of competing ligands.

The specificity of aminoglycoside binding to a decoding region construct derived from the prokaryotic 16S rRNA was also studied. A smaller construct derived from RNA had previously been shown to bind to aminoglycosides by chemical protection experiments (Prohit & Stern, 1994). Of course, neither the binding affinities nor stoichiometries of aminoglycoside binding could be assessed by this method. In the studies reported here, CRP was utilized as the fluorescent probe to determine aminoglycoside binding to the construct directly by fluorescence anisotropy measurements. We had previously reported on use of the same technique to measure affinities of aminoglycoside binding to RNA constructs (Wang et al., 1996). In the presence of the decoding construct, a significant anisotropy increase was measured using CRP. This increase was competitively reversed in the presence of certain other aminoglycosides, allowing for the direct measurement of aminoglycoside binding affinities. Paromomycin, gentamycin C1, kanamycin B, and tobramycin all bound to the construct with approximately the same K_D values (approximately 2 μM). Only neomycin B showed a significantly higher affinity for the construct (132 nM). Thus, little selectivity in the binding of individual aminoglycosides was observed. This is not to say that the binding is nonspecific. For one thing, the binding is stoichiometric. Secondly, aminoglycoside antibiotics known not to bind to the decoding region (Moazed & Noller, 1987), such as streptomycin, did not bind to the decoding region construct. In addition, fluorescent aminoglycoside analogs substituted in ring A did not bind to the construct. This is an interesting and expected result, because mechanisms of aminoglycoside resistance include acylation or adenylation of amino groups in this ring (Chambers & Sande, 1996). Thus, the RNA decoding construct appears to reflect the inherent specificity of the decoding region in prokaryotic 16S rRNA which clearly involves molecular discrimination with respect to binding of the A-ring in aminoglycosides, but perhaps little or no discrimination for the remaining rings of the aminoglycosides. Further support for this view can be gleaned from the pharmacological use of aminoglycoside antibiotics, where it has been found that specificity patterns exist for classes of aminoglycosides rather than for individual aminoglycosides within a class (Chambers & Sande, 1996).

While the specificity of the two "naturally" occurring RNA constructs discussed above in binding aminoglycosides is limited, previously described RNA aptamers selected against the aminoglycosides are much more selective (Wang et al., 1996). We had shown that stringent *in vitro* selection of RNA molecules against the aminoglycoside antibiotic tobramycin could provide RNA molecules which bound to tobramycin with high affinity and great specificity (Wang et al., 1996). For example, the RNA construct J6 binds tobramycin with a 0.77 nM dissociation constant (Wang et al., 1996). The construct strongly discriminates against other aminoglycosides and non-aminoglycoside antibiotics. Neomycin B and gentamycin C1 bind to J6 with K_D values respectively 1337 and 10142 times greater than that measured for tobramycin (Wang et al., 1996). Erythromycin did not measurably bind to J6 (Wang et al., 1996). Thus, J6 and similar constructs bind aminoglycosides with much greater apparent specificities than do the RRE and decoding region constructs described here.

It is interesting to compare the measured affinities of aminoglycosides for the various RNA molecules under discussion here. Measurements of the binding of aminoglycosides to RRE IIB and the decoding construct showed that their K_D values were approximately 1−2 μM. Aminoglycosides can bind to many different RNA structures with similar affinities (Wang & Rando, 1995). In fact, aminoglycosides cannot only bind with moderate specificity to RNA molecules selected under low stringency conditions with micromolar affinities, but they can also bind to hammerhead ribozymes (Stage et al., 1995), group 1 ribozymes (von Ahsen et al., 1991), and tRNA molecules (Y. Wang and R. R. Rando, unpublished experiments) with similar affinities. In fact, the TAR RNA region will also bind to tobramycin with an affinity only approximately 5-fold lower than that measured with RRE IIB RNA (Y. Wang and R. R. Rando, unpublished experiments). In this context, it is of further interest to consider low stringency selections of RNA aptamers directed against tobramycin (Wang et al., 1996). Beginning with approximately 10^{14} RNA sequences, approximately 10^7 of these sequences could bind to tobramycin with low micromolar affinities (Wang et al., 1995). There were no observable consensus sequences for the tobramycin binders, demonstrating that there are a virtually unlimited number of ways for RNA molecules to bind aminoglycosides in the micromolar range of affinities. In this light, it may be somewhat surprising that the aminoglycoside antibiotics are useful drugs, since one might expect an abundance of both host and parasite RNA molecules to bind to these drugs at micromolar levels. In fact, aminoglycosides are quite toxic

to humans (Chambers & Sande, 1996). The major observed toxicities are manifest as kidney and ototoxicities (Chambers & Sande, 1996). It is likely that the aminoglycosides can bind to an extensive number of prokaryotic and eucaryotic RNA molecules in the micromolar range of concentrations. Why the function of prokaryotic 16S rRNA appears to be especially susceptible to the binding of aminoglycosides remains an intriguing question.

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