Specific Binding of Aminoglycosides to a Human rRNA Construct Based on a DNA Polymorphism Which Causes Aminoglycoside-Induced Deafness†

Keita Hamasaki and Robert R. Rando*

Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 250 Longwood Avenue, Boston, Massachusetts 02115

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ABSTRACT: RNA constructs prepared from wild-type and mutant (1555^G) human mitochondrial 12S RNA were studied with respect to their abilities to specifically bind aminoglycoside antibiotics. The 1555^G point mutation had previously been found to be associated with hereditary deafness induced by aminoglycosides. It is shown here that the 1555^G RNA analog stoichiometrically binds aminoglycosides with high affinities, while the wild-type construct does not bind aminoglycosides at all. Analogous mutations in a 16S bacterial rRNA construct show the opposite behavior. Bacterial 16S rRNA constitutes the functional target for aminoglycoside antibiotics. While the wild-type 16S rRNA decoding region construct binds aminoglycosides stoichiometrically with binding affinities in the micromolar range, the mutant is unable to specifically bind aminoglycosides. These results demonstrate the importance of a specific GC base pair in aminoglycoside binding in both the human and the bacterial rRNA constructs. These studies also provide quantitative evidence in support of the hypothesis that the 1555^G point mutation in human mitochondrial 12S RNA causes aminoglycoside induced deafness.

Aminoglycoside antibiotics are bacteriocidal drugs used in the treatment of Gram-negative infections (Chamber & Sande, 1996). These drugs have relatively high affinities for RNA molecules, and are generally thought to operate by binding to bacterial ribosomal (r) RNA, causing mistranslation or premature termination of protein synthesis (Chamber & Sande, 1996). These errors in protein translation result in the observed bacteriocidal effects of the aminoglycoside antibiotics. The use of these drugs is limited by kidney toxicity and ototoxicity (hearing loss) (Chamber & Sande, 1996). A particularly severe form of ototoxicity is of a hereditary nature. A mutation in human mitochondrial DNA is found associated with hereditary deafness induced by aminoglycosides (Hutchin & Cortopassi, 1994). This mutation results in a 1555 A to G transition in mitochondrial 12S ribosomal RNA (rRNA), which has been suggested to create a new C-G base pair, making the secondary structure of this RNA more closely resemble the proposed corresponding region of *E. coli* 16S rRNA. This latter region is referred to as the decoding region of rRNA, and is an important locus of action of the aminoglycoside antibiotics (DeStasio & Dahlberg, 1990; Noller, 1991; Chernoff et al., 1994). Quantitative binding studies on aminoglycoside binding to a 16S rRNA construct using fluorescence anisotropy techniques have demonstrated specific and stoichiometric binding, with dissociation constants in the micromolar range (Wang et al., 1997). The binding of aminoglycosides to the bacterial decoding region *in vivo* results in errors in protein translation with concomitant bacterial death.

The major object of the studies described here is to quantitatively determine whether the 1555 A to G transition in mitochondrial 12S ribosomal RNA (rRNA) leads to increased affinity for aminoglycoside binding over its wild-type counterpart. Using a previously established quantitative fluorescence binding methodology, it is possible to accurately measure both affinities and stoichiometries of aminoglycoside−RNA binding (Wang et al., 1997). It is of substantial interest to determine whether the wild−type 12S rRNA is incapable of binding aminoglycosides and, moreover, whether the 1555^G mutation allows specific binding to occur. It is established here that this is indeed the case. Moreover, a reverse mutation in a bacterial 16S rRNA (1491 G to A) construct abolishes specific aminoglycoside binding. These studies demonstrate that the proposed 1555 A to G transition in a mitochondrial 12S rRNA construct leads to the specific binding of aminoglycoside antibiotics, and provide quantitative evidence in support of the hypothesis that the 1555^G point mutation in human mitochondrial 12S RNA causes aminoglycoside-induced deafness (Hutchin & Cortopassi, 1994). Moreover, these studies also demonstrate the potentially highly specific mode of recognition between RNA molecules and aminoglycosides.

MATERIALS AND METHODS

Materials

Paromomycin, hygromycin B, and kanamycin B were from Sigma. Neomycin, tobramycin, gentamycin, and streptomycin were purchased from Fluka. 5-Carboxytetramethylrhodamine succinimidyl ester was purchased from Molecular Probes. Template DNAs were purchased from Oligo etc. Primer DNAs were purchased from Integrated DNA Technologies, Inc. *Taq* DNA polymerase was from Promega. T7 RNA polymerase was from Ambion or Promega. NICK columns (Sephadex G50) were from Pharmacia, Inc. All other chemicals were purchased from either Aldrich Inc., Fluka Inc., or Sigma Inc. and were of the highest purity available.

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* To whom correspondence should be addressed.

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For the preparation of each double-strand DNA template, the upstream primer, including the T7 promoter (5′AATTTAATACGACTCACTATAGCACAGTACACACCGCCC3′), and the down stream primer (for 12S wild type, 5′GCACAGTCACCCATGTTACGACTTGTCTCCTCTATATAA3′; for 12S mutant, 5′GCACAGTCACCCATGTTACGACTTGCCTCCTCTA TATAA3′; for 16S wild type, 5′GCACAGTCACCCTTGTTACGA CTTCACCCCAGTCATGAA3′; for 16S mutant, 5′GCACAGTCACCC TTGTTACGACTTTACCCCAGTCATGAA3′) were annealed to each template DNA and amplified by PCR. The purity of the products was confirmed by 12% polyacrylamide gel electrophoresis; 10−15 cycles of PCR reactions were employed to successfully generate pure double-stranded DNA template. RNA products of each construct were prepared by transcription from the corresponding double-strand DNA templates using T7 RNA polymerase. The RNAs were purified by gel filtration using NICK columns, and the purity of the RNA constructs was determined by polyacrylamide gel electrophoresis. RNA concentrations were determined spectrophotometrically at 260 nm.

5-Carboxytetramethylrhodamine-labeled paromomycin (CRP) was prepared as previously reported (Wang et al., 1997).

Methods

Fluorescence Measurements. 5-Carboxytetramethylrhodamine-labeled paromomycin (CRP) concentrations were determined spectroscopically at 550 nm using a molar extinction coefficient of 6.00×10^4 M^{-1} cm^{-1}.

Fluorescence anisotropy measurements were performed using a Perkin Elmer LS-50B luminescence spectrometer equipped with a thermostat accurate to ±0.1 °C. Fluorescence anisotropy methods are extremely useful and reliable for quantitative ligand−receptor binding in solution. The measurements provide direct equilibrium binding data (Checovich et al., 1995). Fluorescence anisotropy changes depend on molecular mobility in solution, and anisotropy is enhanced when the tracer molecule (fluorescent dye labeled aminoglycoside in this study) binds to the RNA construct. The increase in the observed fluorescence anisotropy occurs because of the large mass difference between tracer aminoglycoside and the RNA. Thus, binding of the fluorescent aminoglycoside to RNA is directly observable as a fluorescence anisotropy increase. By setting up a competitive binding assay with unlabeled aminoglycosides, binding constants for the unmodified aminoglycosides can also readily be determined. In this case, the interaction of the unlabeled aminoglycoside with the RNA causes a decrease in the measured fluorescence anisotropy because of competition with the probe (fluorescent aminoglycoside).

The tracer solution was excited at 550 nm, and monitored at 580 nm. The integration time was 5s. For every single point, six measurements were made, and their average values were used for calculations. Error limits for measured anisotropy values are lesser than 0.0015. Measurements were performed in buffer solutions containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 20 mM HEPES (pH 7.5).

Determination of Dissociation Constants. The following equation was used for the determination of the dissociation constant for the interactions between RNA and CRP (K_d) (Wang & Rando 1995):

$$A = A_0 + \Delta A\{[\mathrm{RNA}]_0 + [\mathrm{CRP}]_0 + K_d - [([\mathrm{RNA}]_0 + [\mathrm{CRP}]_0 + K_d)^2 - 4[\mathrm{RNA}]_0[\mathrm{CRP}]_0]^{1/2}\}/2 \quad (1)$$

where A and A_0 are the fluorescence anisotropy of CRP in the presence and in the absence of RNA, respectively, and ΔA is the difference between the fluorescence anisotropy of 1 nM CRP in the presence of an infinite concentration of RNA and the fluorescence anisotropy in the absence of RNA. $[RNA]_0$ and $[CRP]_0$ are the initial concentrations of RNA and CRP, respectively.

In the competitive binding assay, eq 2 is used for the calculation of the $K_D$values (Wang et al., 1996):

$$[\mathrm{aminoglycoside}]_0 = (K_D(A_\infty - A)/K_d(A - A_0) + 1] \times [[\mathrm{RNA}]_0 - K_d(A - A_0)/(A_\infty - A) - [\mathrm{CRP}]_0(A - A_0)/(A_\infty - A_0)] \quad (2)$$

Both K_d and K_D were determined by nonlinear curve-fitting using the equations described above (Wang et al., 1996; Wang & Rando 1995).

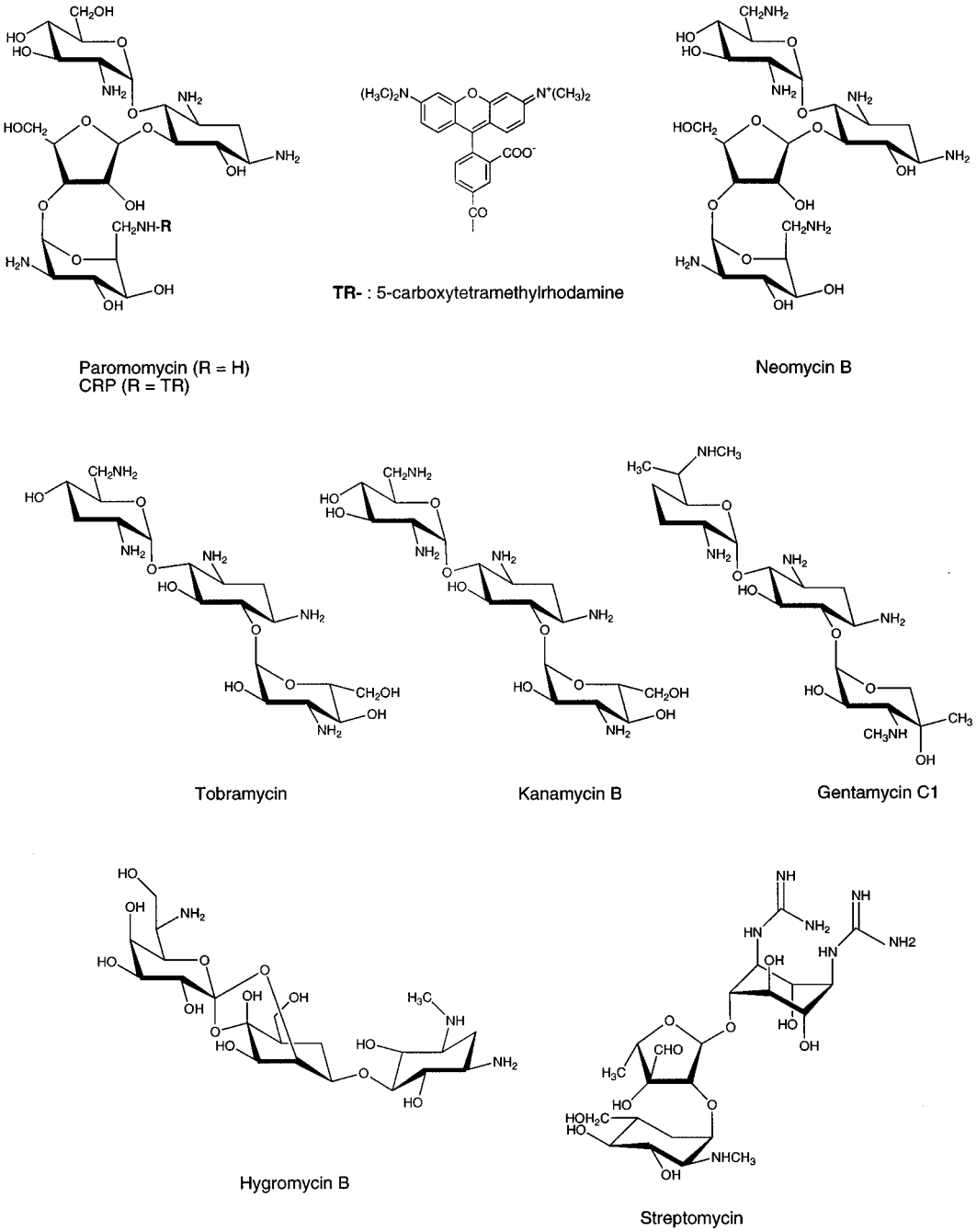
RESULTS

Aminoglycoside−RNA interactions can be quantitatively measured by fluorescence anisotropy measurements using fluorescently labeled aminoglycosides (Wang et al., 1997). While the labeled aminoglycoside freely travels and rotates in solution, its motion becomes relatively frozen when it is bound to the large RNA construct. This leads to polarized fluorescence emission. Fluorescent (5-carboxytetramethylrhodamine labeled) paromomycin (CRP) (Scheme 1) was used as a tracer in the binding assays described here. We have shown that CRP is a extremely useful aminoglycoside probe, which can be used to quantitatively establish the binding of aminoglycosides to various RNA molecules, including a bacterial 16S decoding region rRNA construct (Wang et al., 1997). This latter construct would be predicted to possess a similar secondary structure to the human mitochondrial 12S 1555^G mutant rRNA.

Two constructs of wild-type and mutant (1555^G) 12S rRNAs were prepared and studied here (Scheme 2). Aminoglycoside binding RNA molecules of the size of the construct studied here show substantial anisotropy increases when they bind fluorescent aminoglycosides (Wang et al., 1996, 1997). The fluorescence anisotropy of CRP substantially increases upon the addition of the 12S 1555^G mutant RNA, as shown in Figure 1. Each data point fits the calculated theoretical curve (Wang et al., 1996, 1997) described under Methods Section, exceedingly well, and gives a $K_D = 0.184 \pm 0.028$ μM for binding to CRP. Curve-fitting also shows that the stoichiometry of binding is 1:1, an expected result when specific aminoglycoside binding to RNA molecules occurs. Importantly, the wild-type human mitochondrial 12S rRNA analog does not bind CRP, as revealed by the lack of fluorescent anisotropy enhancement of CRP (10 nM) by 600 nM RNA (Figure 1).

It was of great interest to measure the dissociation constants between the 1555^G mutant and various aminoglycosides. To make these measurements, competitive binding assays with CRP were performed with aminoglycosides and

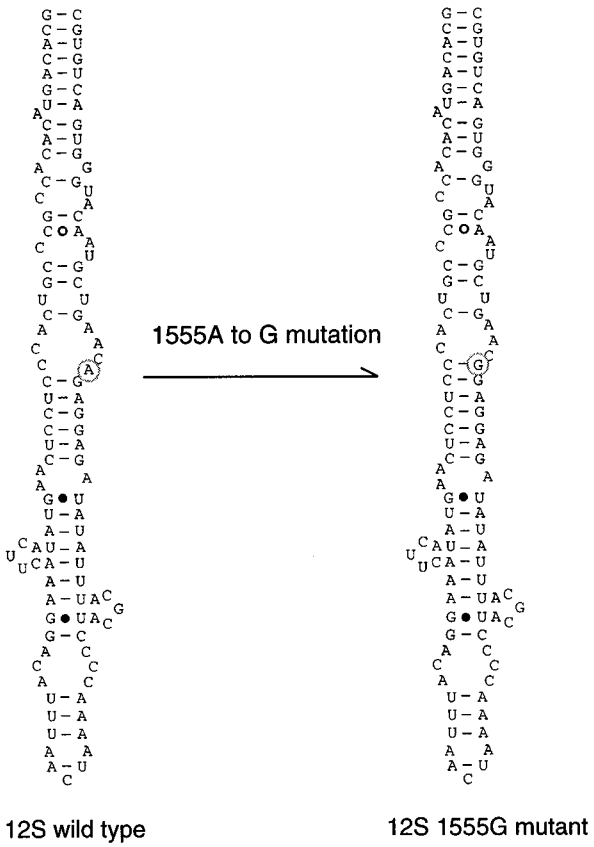
Scheme 1: Aminoglycosides Used in the Binding Assays

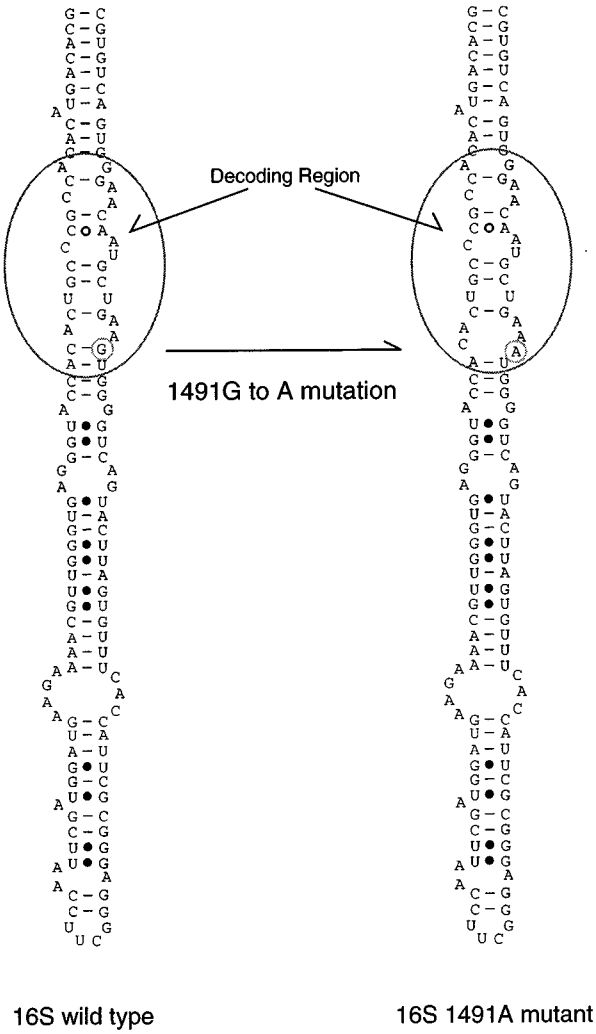


the RNA construct. The initial sample included 10 nM CRP and 250 nM 1555^{G} mutant RNA. Competitive binding experiments were performed by titrating this sample with various aminoglycosides. Figure 2A−C shows the titration curves of the competitive binding of paromomycin, gentamycin, and hygromycin B to the 1555^{G} mutant RNA, respectively. Fluorescence anisotropy values decreased upon addition of the aminoglycosides, and the data points fit well to the theoretical curve calculated using the previously derived equation (Wang et al., 1996, 1997), shown under Methods, which assumes 1:1 binding stoichiometry. The dissociation constants for the various aminoglycosides are summarized in Table 1. Not a great deal of selectivity is found in the aminoglycoside binding properties of the RNA construct. Nevertheless, the 1555^{G} mutant does bind the tetracyclic aminoglycosides paromomycin and neomycin (K_D values ~2 μM) (Scheme 1), more tightly than it does the tricyclic aminoglycosides kanamycin B, tobramycin and gentamycin (K_D values ~40 μM) (Scheme 1). Hygromycin B proved to have the lowest affinity (182 ± 52 μM) among the aminoglycosides studied, while streptomycin showed no binding affinity when tested at a concentration of 4 mM.

The wild-type 16S rRNA decoding region construct shown in Scheme 2 has been previously shown to specifically bind aminoglycosides (Wang et al., 1997). Given the results with the 12S construct, it was of interest to determine whether the 1491^{A} 16S rRNA mutant (Scheme 2) could bind aminoglycosides specifically. As shown in Figure 3, weak, non-saturable, binding of CRP was observed with the mutant. This kind of binding is nonspecific in nature, and is not a function of the general affinity of aminoglycosides for RNA molecules.

Scheme 2: Proposed Secondary Structures of the Human Mitochondrial 12S rRNA Analog, the *E. coli* 16S rRNA Analog, and Their Mutants





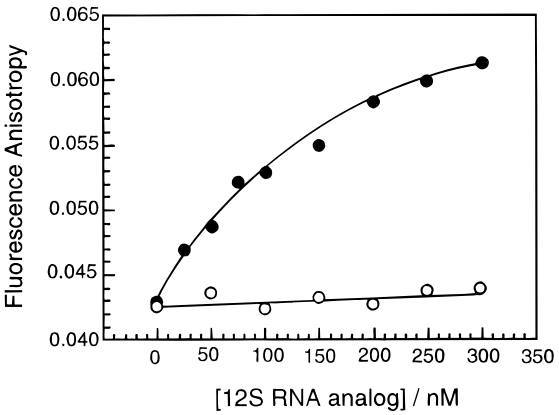


FIGURE 1: Fluorescence anisotropy of CRP (10 nM) solution as a function of 12S 1555^{G} mutant RNA concentrations (solid circles) or 12S 1555^{A} wild-type RNA concentrations (open circles).

DISCUSSION

We previously reported that the small RNA construct derived from the *E. coli* 16S rRNA decoding region (Scheme 2) binds various medically relevant aminoglycosides specifically with 1:1 stoichiometries (Wang et al., 1997). Paromomycin, gentamycin, kanamycin B, and tobramycin all were bound stoichiometrically with dissociation constants of approximately 2 μM (Wang et al., 1997). Only neomycin was bound with a significantly higher affinity (K_D = 0.132 $\pm$ 0.023 μM) (Wang et al., 1997). Neither hygromycin B nor streptomycin bound to the 16S RNA construct, an expected result given that these aminoglycosides are not thought to specifically bind to the decoding region (Wang et al., 1997). These observations on aminoglycoside binding to the wild-type 16S rRNA construct are similar to those observed in the studies reported here on the 12S 1555^{G} mutant construct. Thus, the wild-type 16S construct behaves similarly to the 12S 1555^{G} mutant construct with respect to aminoglycoside binding.

Various mutational and chemical protection experiments in the presence of aminoglycosides point to the importance of the 1409−1491 C-G base pair for functional aminoglycoside binding to bacterial 16S rRNA (DeStasio & Dahlberg, 1990; Noller, 1991; Chernoff et al., 1994). Indeed, the corresponding base pair appears to play a parallel role in the binding of aminoglycosides by the mitochondrial 12S construct described here. Not surprisingly then, the 1491^{A} 16S rRNA mutant (Scheme 2) proved not to bind CRP tightly at concentrations up to 0.5 μM (Figure 3). Weak, nonsaturable, binding was observed that is almost certainly nonspecific in nature.

Further substantial evidence in the literature points to the importance of the 1409−1491 C-G base pair both for ribosomal function (DeStasio & Dahlberg, 1990; Noller, 1991; Chernoff et al., 1994) and for aminoglycoside binding

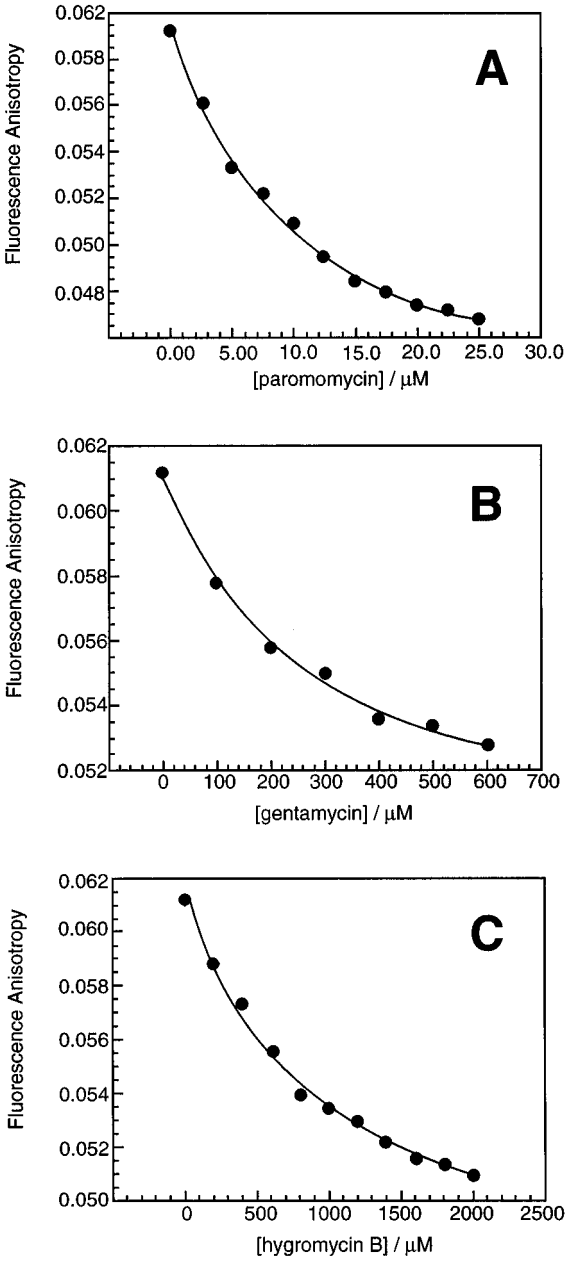


FIGURE 2: Fluorescence anisotropy of CRP (10 nM) solution containing 12S 1555^{G} mutant RNA (250 nM) as a function of added paromomycin (A), gentamycin (B), and hygromycin B (C).

Table 1: Dissociation Constants of 12S 1555G Mutant RNA

aminoglycosides	K_D (μM)	aminoglycosides	K_D (μM)
CRP[a]	0.184 ± 0.028	tobramycin	34.4 ± 13.7
paromomycin	1.39 ± 0.28	gentamycin	46.9 ± 11.1
neomycin	2.16 ± 0.42	hygromycin B	182 ± 52
kanamycin B	47.8 ± 5.6	streptomycin	nb[b]

[a] CRP: 5-carboxytetramethylrhodamine-labeled paromomycin. [b] nb: no binding by 4 mM streptomycin addition.

to 16S rRNA. The 1491^{A} mutant in *E. coli* 16S rRNA proved to be a lethal one (DeStasio & Dahlberg, 1990). G-C base pairs at equivalent sites in yeast mitochondrial 15S rRNA (Li et al., 1982) and *Tetrahymena thermophila* (Spangler & Blackburn, 1985) have also proved to be important for aminoglycoside binding. This G-C pair is found at the predicted base of the penultimate helix of the *E. coli* rRNA (DeStasio & Dahlberg, 1990; Noller, 1991; Chernoff et al., 1994). As such, this base pair is predicted

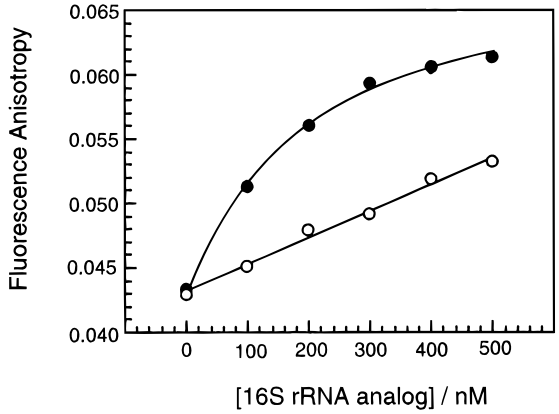


FIGURE 3: Fluorescence anisotropy of a CRP (10 nM) solution as a function of 16S 1491^{G} wild-type RNA concentrations (solid circles; Wang et al., 1997) or 16S 1491^{A} mutant RNA concentrations (open circles).

to have an important role to play in the structure of the rRNA decoding region, the region containing the A and P sites of the translational apparatus (DeStasio & Dahlberg, 1990; Noller, 1991; Chernoff et al., 1994). A recent NMR investigation of paromomycin binding to a 27 nt A site rRNA construct, while not indicating a particular role for the 1409−1491 C-G base pair in antibiotic binding, did suggest a role for this base pair in the overall structure of the aminoglycoside binding site (Fourmy et al., 1996). Moreover, molecular dynamics calculations on the 1555^{G} 12S mutant indirectly suggest that the G-C base pair in question may be important in local RNA folding and aminoglycoside binding (Hutchin, et al., 1993). The quantitative aminoglycoside binding experiments reported here certainly point to an important functional role for the 1409−1491 C-G base pair in aminoglycoside binding.

That being the case, it is not surprising that the equivalent base pair in the 12S mitochondrial construct is essential for aminoglycoside binding. The studies reported here demonstrate that this base pair is also the sine qua non of aminoglycoside binding to the mitochondrial mutant, as was predicted based on studies on DNA polymorphism found in individuals hypersensitive to aminoglycosides. It is important to note that the quantitative studies reported here allow one to demonstrate an all or nothing aspect to the binding of aminoglycosides to the 12S constructs. It is not that the wild-type 12S construct binds aminoglycosides with a lower affinity than the mutant; the 12S construct simply does not bind aminoglycosides at all. While the structural basis of the profound sensitivity of rRNA primary sequence to aminoglycoside is obscure, it is clear from the studies reported here that subtle alterations in rRNA sequence can give rise to qualitative changes in functional effector binding. This could be of substantial interest for the design of RNA-specific antagonists.

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