Neomycin Inhibition of the Hammerhead Ribozyme Involves Ionic Interactions†

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Received March 14, 1995; Revised Manuscript Received June 15, 1995®

ABSTRACT: To investigate the properties of the neomycin−hammerhead interaction, inhibition of hammerhead activity was measured as a function of magnesium concentration and pH. The data are consistent with a simple competition between magnesium and neomycin with about five magnesium ions required to displace neomycin from the hammerhead. The pH dependence of the inhibition of hammerhead cleavage by neomycin and two related aminoglycosides was also determined. The data indicate that at least three of the five positively charged ammonium ions present on neomycin are critical for inhibiting hammerhead function. Taken together, these results suggest that the neomycin−hammerhead interaction is mostly ionic in character.

It has long been known that the antibiotic neomycin binds tightly to ribosomal RNA (Dahlberg et al., 1978). Like other aminoglycoside antibiotics, neomycin is a potent inhibitor of ribosome function, causing extensive misreading of the genetic code (Cundliffe, 1981) by binding to a unique site on the ribosome (Moazed & Noller, 1987). Several recent reports have shown that neomycin can also interfere with the function of other RNAs. Neomycin is an effective inhibitor of group I intron self-splicing, acting primarily by blocking the second step of splicing (von Ahsen et al., 1991, 1992). Footprinting experiments revealed that neomycin interacts with the intron near the guanosine cofactor binding site (von Ahsen & Noller, 1993). Neomycin was also shown to block the binding of the HIV Rev protein to its viral RNA recognition element significantly better than any other antibiotic tested (Zapp et al., 1993). Chemical footprinting experiments revealed that neomycin interacts within the core element of the Rev-binding site (Zapp et al., 1993). More recently, neomycin was found to be the best inhibitor of hammerhead cleavage among a set of antibiotics for inhibiting the hammerhead ribozyme cleavage reaction (Stage et al., 1995). By making use of two kinetically characterized hammerheads, it was shown that neomycin primarily affects the rate of chemical cleavage but also slightly alters the binding properties of the ribozyme−substrate complex (Stage et al., 1995).

The ability of neomycin to inhibit the activities of RNAs having such a wide variety of structures and functions influenced us to further investigate the properties of the neomycin−hammerhead interaction. One clue toward understanding the interaction of neomycin with RNA is its polycationic character. Neomycin has six amino groups, five of which are protonated in the range of pH between 6 and 7.5 (Botto & Coxon, 1983). Since RNA is a polyanion, it seems likely that ionic interactions could contribute significantly to neomycin's ability to bind RNA.

Two different approaches were used to evaluate the contribution of the protonated ammonium ions to neomycin inhibition of hammerhead activity. First, inhibition of hammerhead cleavage by neomycin was measured as a function of Mg^{2+}concentration. If the interaction of neomycin with the hammerhead is primarily ionic, increasing amounts of Mg^{2+} should alleviate neomycin inhibition of hammerhead activity by acting as a competing counterion. In a second approach, the pH dependence of neomycin inhibition of hammerhead cleavage was investigated. Since several of the amino groups of neomycin are titrated in the pH range where the hammerhead is active, it was possible to evaluate the relative contribution of different ammonium ions to binding. This analysis was extended by comparing the pH dependence of hammerhead inhibition by two additional antibiotics that are closely related to neomycin.

MATERIALS AND METHODS

Ribozyme 16 RNA (E16) was synthesized by *in vitro* transcription of a synthetic DNA template with T7 RNA polymerase and subsequently gel-purified (Milligan & Uhlenbeck, 1989). Substrate 16 RNA (S16) was chemically synthesized, gel-purified, and 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P] ATP (Fedor & Uhlenbeck, 1992). Solutions of neomycin, paromomycin, and ribostamycin (from Sigma) were prepared in 50 mM buffer by a serial dilution procedure.

Cleavage reaction conditions were 500 nM E16, trace 5′-^{32}P-labeled substrate (S16*),[1] 0−10 mM antibiotic in 50 mM buffer, and 5−50 mM $MgCl_2$. Sulfonate buffers used were Mes (pH 6), Pipes (pH 6.5), Mops (pH 7−7.8), and Taps (pH 8.2−8.5). A typical reaction was carried out in 20 μL. 1 μM E16 and trace [5′-^{32}P]S16 were heated separately in 50 mM buffer at 95 °C for 2 min and slowly cooled down to 25 °C for 5 min. $MgCl_2$ was added to each solution to a final concentration of 10 mM. One microliter of H_2O or neomycin solution (0.25 μM to 10 mM) was added to 9 μL of ribozyme solution and to 9 μL of substrate solution. Ribozyme and substrate solutions were then incubated 5 min at 25 °C. For the zero time point, 1 μL of the S16* solution was removed and mixed in 10 μL of stop solution (8 M urea, 50 mM EDTA, and 0.05% bromophenol blue and xylene

† This work was supported by NIH Grant AI30242 to O.C.U. and by a long-term postdoctoral fellowship from EMBO to B. C.-O.

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® Abstract published in *Advance ACS Abstracts,* August 15, 1995.

[1] Abbreviations: E, ribozyme; S, substrate; HH, hammerhead; S*, 5′-^{32}P-labeled substrate.

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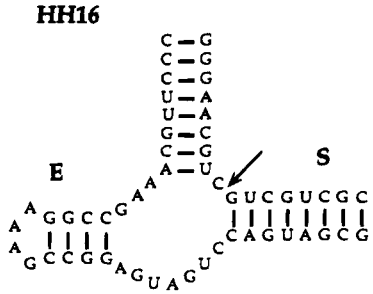

FIGURE 1: Secondary structure of hammerhead 16 (HH16). The arrow indicates the site of cleavage.

cyanol). The cleavage reaction was initiated by addition of 9 μL of R16 to 9 μL of S16*; 2 μL aliquots were removed at specific time intervals and quenched in 10 μL of stop solution. Substrate and product were separated on 20% polyacrylamide/7 M urea gels and quantitated using a Molecular Dynamics phosphorimager. Cleavage rates were obtained as described previously (Fedor & Uhlenbeck, 1992).

The concentration dependence of neomycin, paromomycin, and ribostamycin inhibition of HH16 cleavage was measured by varying the concentration of the antibiotic from 0.25 μM to 10 mM and measuring the rate of cleavage under single-turnover conditions. The observed rate of cleavage is the rate of chemistry, k_2, for all the reactions studied (Stage et al., 1995). A cleavage reaction with no antibiotic added was carried out in parallel. The fraction inhibition (I) at each antibiotic concentration was calculated as $1 - (k_{2\text{antibiotic}}/k_2)$ where k_2 is the rate of chemistry without antibiotic and $k_{2\text{antibiotic}}$ is the rate of chemistry in the presence of antibiotic. The fraction inhibition (I) versus antibiotic concentration was plotted and fit to a hyperbolic binding equation to obtain N_I, the antibiotic concentration needed to inhibit half of the rate of cleavage. The error in N_I measurements was about 20%.

RESULTS

HH16, a kinetically well-characterized hammerhead (Figure 1), was used to evaluate the effects of neomycin on the rate of chemical cleavage (k_2). S16 binds E16 through the formation of 16 base pairs. As a result, the substrate dissociation rate of HH16 is so slow that any substrate that binds the ribozyme is cleaved (Hertel et al., 1994). All kinetics were done under single-turnover conditions at a saturating ribozyme concentration. Therefore, the appearance of products directly reflects the rate of chemistry (Hertel et al., 1994).

To evaluate the effect of pH and magnesium concentration on neomycin inhibition of hammerhead cleavage, a variety of buffer conditions were used. Therefore, it was important to account for the effects of buffer alone on the intrinsic rate of hammerhead cleavage. This was done by defining the fraction of inhibition, I, from the rate of chemical cleavage in the presence of neomycin (k_{2neo}) and the rate in the absence of neomycin (k_2) as $I = 1 - (k_{2neo}/k_2)$. For example, $I = 0.8$ for a reaction with 5 μM neomycin in 10 mM Mg^{2+} at pH 7 (Figure 2A). An inhibition curve was obtained for a given set of buffer conditions (Figure 2B) by determining I at a series of neomycin concentrations. The concentration of neomycin needed to give $I = 0.5$ is defined as N_I and is equal to 1.5 μM under the conditions illustrated in Figure 2B.

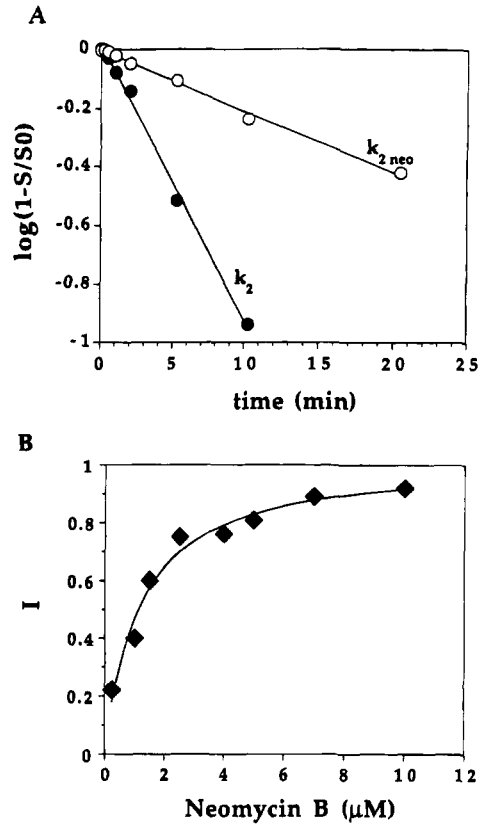

FIGURE 2: (A) Rate of hammerhead cleavage in the absence (●, $k_2 = 0.21$ min) and in the presence (○, $k_{2neo} = 0.04$ min^{-1}) of 5 μM neomycin at pH 7, 10 mM Mg^{2+}, gives a value of $I = 1 - (k_{2neo}/k_2) = 0.8$. (B) Concentration dependence of inhibition of hammerhead cleavage by neomycin at pH 7, 10 mM Mg^{2+}. The line is a simple binding curve with $N_I = 1.5$ μM.

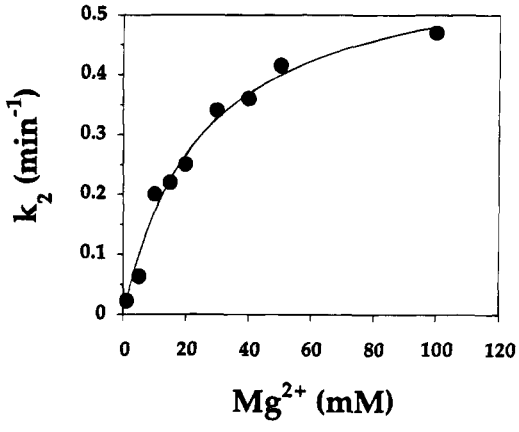

FIGURE 3: Rate of cleavage (k_2) of HH16 versus magnesium concentration at pH 7 fit a hyperbolic binding equation giving $K_s = 22.5 \pm 2.5$ mM.

The rate of HH16 cleavage increases with magnesium concentration (Figure 3). The data between 5 and 100 mM Mg^{2+} fit well to a simple binding isotherm giving an apparent dissociation constant, K_s, for the HH16−Mg^{2+} complex of 22.5 ± 2.5 mM at pH 7 (Figure 3). This indicates that Mg^{2+} ions bind non-cooperatively to HH16 as has been observed with other hammerheads (Dahm & Uhlenbeck, 1991; Perreault et al., 1991; Yang et al., 1992; Dahm et al., 1993). These data do not reveal the number of magnesium ions that are bound.

To evaluate the effect of Mg^{2+} ions on the inhibition of hammerhead cleavage by neomycin, inhibition curves similar

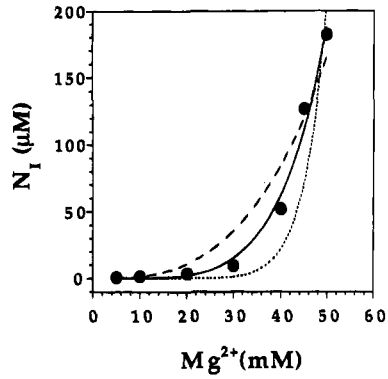

FIGURE 4: N_I versus magnesium concentration. The data fit a curve described by the equation described under Results (solid line) with $\alpha = 5$ and $K_I > 50$ nM. Curves for $\alpha = 3$ (dashed line) and $\alpha = 8$ (dotted line) are also shown.

to Figure 2B were obtained for a series of magnesium concentrations ranging from 5 mM to 50 mM, and N_I values for each Mg^{2+} concentration were determined. As shown in Figure 4, N_I increases from 1.5 μM to 180 μM with increasing magnesium concentrations, suggesting that competition between the two molecules occurs. A minimal model for such a competition is that α magnesium ions compete with a single neomycin for a site on the hammerhead and that cleavage only occurs when magnesium is bound and not when neomycin is bound:

$$E{\cdot}S + \alpha Mg \overset{K_{Mg}}{\rightleftharpoons} E{\cdot}S{\cdot}Mg_\alpha \overset{k}{\rightarrow} E{\cdot}P{\cdot}Mg_\alpha$$

$$E{\cdot}S + neo \overset{K_{neo}}{\rightleftharpoons} E{\cdot}S{\cdot}neo \not\rightarrow \text{no cleavage}$$

where K_{Mg} and K_{neo} are the intrinsic affinities of the relevant magnesium ions and neomycin. This model is reasonable as long as ribozyme is saturating since we have previously shown that neomycin does not significantly alter the affinity of ribozyme to substrate (Stage et al., 1995). From this scheme, the following relation can be derived:

$$N_I = (K_{neo}/K_{Mg})[Mg]^\alpha + K_{neo}$$

If we further assume that K_{Mg} is equal to K_s, the apparent affinity of the bulk magnesium ions determined in Figure 3, the data are most consistent with an intrinsic affinity of neomycin to RNA of 50 nM and a value of α equal to 5 (Figure 4). The data fit less well to α values of 3 and 8 (Figure 4). Thus, in this model about five magnesium ions are required to displace neomycin from the hammerhead. It should be emphasized that this simple competition model may not accurately reflect the true situation. It is possible that more than one neomycin molecule may be required to inhibit the hammerhead or that magnesium competition may not reflect simple binding to well-defined sites, but rather a more general electrostatic effect. Finally, the competition between neomycin and magnesium may not be simple, and neomycin could change its mode of inhibition at different magnesium concentrations. In the absence of additional information, however, this model provides a useful description of the data.

The pK_a values of the six amino groups of neomycin have been determined by nitrogen-15 NMR (Botto & Coxon, 1983, Figure 5A). The 6-aminomethyl groups on the A and

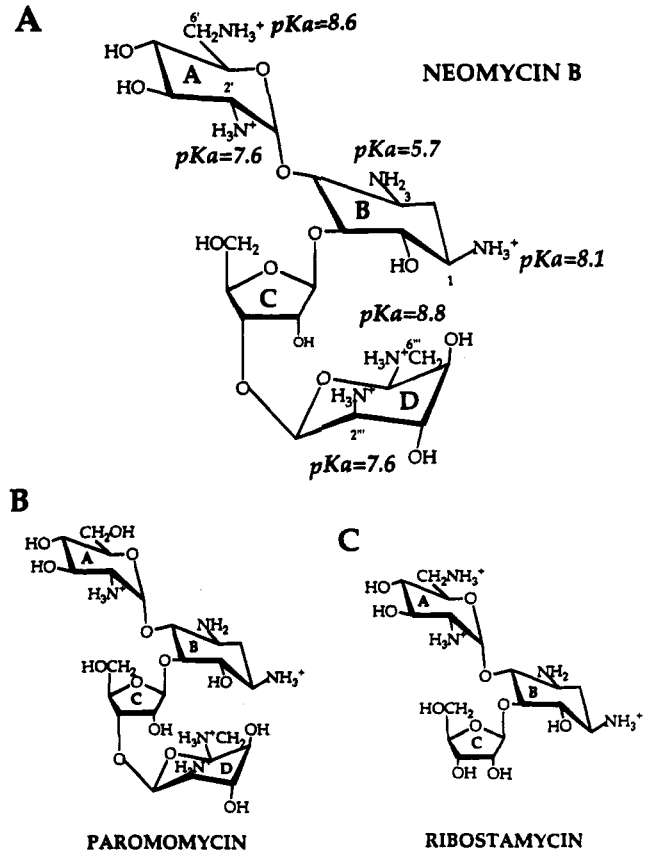

FIGURE 5: (A) Structure of neomycin at pH 7.5 with pK_a values of the six amino groups. (B) Structure of paromomycin. (C) Structure of ribostamycin.

D sugar rings have pK_as near 8.7, the N-1 deoxystreptaminyl group on the B ring has a pK_a of 8.1, the 2-amino groups on the A and D sugar rings have a $pK_a = 7.6$, and the N-3 deoxystreptaminyl group on the B ring has a $pK_a = 5.7$. The possibility of ionic interactions between individual ammonium ions of neomycin and the hammerhead was tested by determining neomycin inhibition as a function of pH. Since the rate of hammerhead cleavage depends on pH (Dahm et al., 1993), it was necessary again to compare the rates of cleavage in the presence and in the absence of varying concentrations of neomycin. The N_I values at 12 different pH values ranging from pH 6 to pH 8.5 in 10 mM Mg^{2+} are shown in Figure 6. In the range between pH 6 and 7, N_I remains constant at about 1.5 μM, indicating that the inhibition is independent of the protonation state of the B3 amino group ($pK_a = 5.7$). If this amino group was important to the interaction with the hammerhead, N_I should have changed significantly between 6 and 6.5. Above pH 7, the value of N_I increases more than seven hundred-fold in a pH range where the remaining five ammonium ions are either partially or fully deprotonated. Between pH 7 and 7.5, N_I increases from 1.5 μM to 24 μM, presumably reflecting the deprotonation of the A2′ and/or D2‴ ammonium ions ($pK_a = 7.6$) and not the three ammonium ions with the higher pK_as. N_I continues, however, to increase rapidly above pH 8 where the A2′ and D2‴ ammonium ions would be mostly deprotonated. This suggests that one or more of the three ammonium ions with higher pK_as (B1, D6‴, and A6′) also contribute to binding. Thus, it appears likely that neomycin uses more than one of its ammonium ions to interact with the hammerhead.

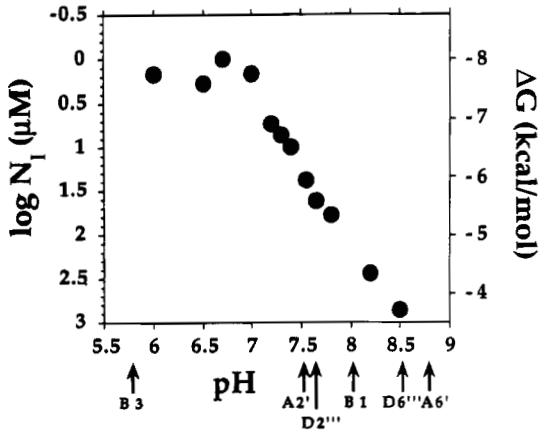

FIGURE 6: N_I and the calculated ΔG values ($\Delta G = -RT \ln N_I$) for neomycin inhibition of hammerhead cleavage as a function of pH in 10 mM Mg^{2+}. The arrows indicate the pK_a values of the six neomycin ammonium ions.

Table 1: N_I and Calculated ΔG Values for Neomycin, Paromomycin, and Ribostamycin at Different pH Values

	neomycin		paromomycin		ribostamycin	
pH	N_I (μM)	ΔG (kcal/mol)	N_I (μM)	ΔG (kcal/mol)	N_I (μM)	ΔG (kcal/mol)
6.5	1	−8.2			140	−5.2
7	1.5	−7.9	140	−5.2		
7.5	25	−6.2	1000	−4.0	880	−4.1

Ribostamycin and paromomycin, derivatives of neomycin, also inhibit the hammerhead cleavage reaction (Stage et al., 1995). Ribostamycin lacks the D ring, resulting in the loss of the 2‴- and 6‴-amino groups (Figure 5A). Paromomycin is identical to neomycin except for the replacement of the 6′-amino group with a hydroxyl (Figure 5B). Although the pK_as of the amino groups for these two antibiotics have not been determined, the resemblance of their structures to neomycin suggests that they should be similar. Previous results indicated that both of these antibiotics were less effective than neomycin at inhibiting hammerhead cleavage (Stage et al., 1995). In order to evaluate their inhibition more quantitatively, the concentration dependence of inhibition for both ribostamycin and paromomycin was determined at two different pH values (Table 1).

At pH 6.5, where all of the amino groups required for maximal neomycin binding are fully protonated, the N_I for ribostamycin is 144 μM as compared to 1.5 μM for neomycin. The apparent ΔG values for the interaction of the hammerhead with the different antibiotics can be calculated from the N_I values ($\Delta G = -RT \ln N_I$). At pH 6.5, $\Delta G = -8.2$ kcal/mol for neomycin and $\Delta G = -5.2$ kcal/mol for ribostamycin. While it is not known whether the two antibiotics bind to the hammerhead in the same way, it is reasonable to propose that ribostamycin can bind at any site accessible to neomycin. Thus, the D ring contributes about −3 kcal/mol to the binding energy of neomycin to the hammerhead. However, at pH 7.5 a similar comparison of ΔG values indicates that the contribution of the D ring has been reduced to −2 kcal/mol (Table 1). This 1 kcal/mol reduction of the contribution of the D ring to neomycin inhibition between pH 6.5 and 7.5 is consistent with the partial deprotonation of the D2‴ ammonium ion that occurs in this range. It is interesting that the absolute affinity of neomycin to the hammerhead changes by 2 kcal/mol in the same pH range. This is too large of a change to be accounted for solely by 1 kcal/mol due to the D2‴ ammonium ion, suggesting that the A2′ and/or the B1 ammonium ion contributes to ΔG as well.

A similar analysis of paromomycin inhibition confirms the previous conclusion (Stage et al., 1995) that the A6′ ammonium ion contributes substantially to the binding of neomycin to the hammerhead. At both pH 7 and pH 7.5, the apparent affinity of paromomycin is substantially weaker than neomycin (Table 1). Not unexpectedly, the ability of paromomycin to inhibit the hammerhead is reduced significantly between pH 7 and 7.5, presumably due to the titration of the A2′ and/or D2″ ammonium ions.

DISCUSSION

Two types of evidence suggest that neomycin's ammonium ions contribute significantly to the inhibition of hammerhead cleavage. First, inhibition of hammerhead cleavage by neomycin is reduced by increasing Mg^{2+} ion concentrations. By assuming a simple competition model, about five Mg^{2+} ions are required to compete with one neomycin molecule under conditions where neomycin has an average of five positive charges. Similar competition between neomycin and magnesium ions has been reported previously. The mechanism for transport of aminoglycosides across the outer membrane of Gram-negative bacteria was observed to involve Mg^{2+} ion competition (Davies et al., 1993). Second, neomycin inhibition of hammerhead cleavage is very pH-dependent. From pH 6 to 7, the B3 ammonium ion is deprotonated; however, no change in affinity is observed, suggesting that this group is not directly involved in hammerhead contact. In contrast, several of the remaining ammonium ions (pK_as ranging from 7.6 to 8.8) appear to be involved in binding to the hammerhead since the apparent ΔG for neomycin binding to the hammerhead is increased from −8 to −4 kcal/mol between pH 7 and 8.5. Since the decrease in apparent affinity occurs over a broad pH range, it is unlikely that the titration of a single amino group is involved. Instead, at least two amino groups with different pK_as contribute to binding. The pH dependence of the inhibition of hammerhead cleavage by two related antibiotics, paromomycin and ribostamycin, supports this view. While it is tempting to combine these data and assign free energy contributions of individual ammonium ions to binding of neomycin to the hammerhead, there are a number of reasons why this may not be worthwhile. First, the pK_a values of individual ammonium ions may be different when bound to the hammerhead than when free in solution. Second, it is not known whether the other antibiotics bind to the hammerhead in the same way as neomycin. Finally, it is not clear whether neomycin interacts with the hammerhead in the same way at every pH. Despite not being able to access the contribution of individual ammonium ions, it appears that three or more of five ammonium ions in neomycin contribute to at least half of its binding energy to the hammerhead.

It is not possible from these experiments to identify the functional groups of RNA that interact with neomycin's ammonium ions. While it is reasonable to expect that the anionic phosphates participate in ion pairs with neomycin, it is also possible that charged hydrogen bonds could form with the N7 of purines, nucleotide carbonyl, and/or ribose hydroxyl groups. In addition, other functional groups of

neomycin may contribute to the overall binding free energy. Examination of inhibition of hammerhead cleavage by additional neomycin analogs having specific functional group replacements may help clarify this point. Physical approaches such as diffusing neomycin into hammerhead crystals (Pley et al., 1994) or analyzing the NMR spectra of the neomycin−hammerhead complex could also help to identify the specific functional groups involved in interaction of neomycin with the hammerhead.

At very low Mg^{2+} concentrations and pH values below 7, neomycin is able to inhibit the hammerhead with an apparent affinity of less than 100 nM. At more physiological Mg^{2+} concentrations, the affinity is in the range of 1 μM. This value can be compared to an apparent inhibition constant of 0.06 μM for inhibition of protein synthesis (Lando et al., 1973), 0.5 μM for inhibition of group I intron (von Ahsen et al., 1992), and less than 1 μM for the disruption of the Rev−RRE interaction (Zapp et al., 1993). While comparing these affinities is complicated by the fact that experiments in each system were done at different pH values and magnesium concentrations, it is clear from these data that neomycin can bind with a similar affinity to RNAs with quite different structures. This moderate specificity of neomycin binding to RNA can, to some extent, be understood from the structural properties of the oligocation. The five critical positive charges are attacted to sugar rings that have only a limited number of conformations. Although free rotation around the four single bonds that connect the sugars will allow neomycin to adapt itself to many different RNA structures, in most cases it will not be possible to have sufficiently close contract between the ammonium ions and functional groups of the RNA. In this sense, neomycin differs from the more flexible polyamines, spermine and spermidine, which show much less specificity for binding RNA. It is interesting that spermine and spermidine do not generally inhibit RNA reactions (at low concentrations), and sometimes stimulate them presumably by acting as counterions (Dahm & Uhlenbeck, 1991). One can therefore expect that other conformationally constrained oligocations will only be able to bind RNA at a limited number of sites and, thus, would be good candidates for specific inhibitors of RNA function.

ACKNOWLEDGMENT

We thank David M. Long and Klemens J. Hertel for helpful discussions.

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BI950568Y