Hairpin ribozyme cleavage catalyzed by aminoglycoside antibiotics and the polyamine spermine in the absence of metal ions

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ABSTRACT

The hairpin ribozyme is a small catalytic RNA that achieves an active configuration by docking of its two helical domains in an antiparallel fashion. Both docking and subsequent cleavage are dependent on the presence of divalent metal ions, such as magnesium, but there is no evidence to date for direct participation of such ions in the chemical cleavage step. We show that aminoglycoside antibiotics inhibit cleavage of the hairpin ribozyme in the presence of metal ions with the most effective being 5-epi-sisomicin and neomycin B. In contrast, in the absence of metal ions, a number of aminoglycoside antibiotics at 10 mM concentration promote hairpin cleavage with rates only 13–20-fold lower than the magnesium-dependent reaction. We show that neomycin B competes with metal ions by ion replacement with the positively charged amino groups of the antibiotic. In addition, we show that the polyamine spermine at 10 mM promotes efficient hairpin cleavage with rates similar to the magnesium-dependent reaction. Low concentrations of either spermine or the shorter polyamine spermidine synergize with 5 mM magnesium ions to boost cleavage rates considerably. In contrast, at 500 µM magnesium ions, 4 mM spermine, but not spermidine, boosts the cleavage rate. The results have significance both in understanding the role of ions in hairpin ribozyme cleavage and in potential therapeutic applications in mammalian cells.

INTRODUCTION

A number of small self-cleaving RNAs undergo trans-esterification reactions which are chemically identical in that the two cleavage products contain a 5'-hydroxyl group and 2',3'-cyclic phosphate, respectively. Yet the structures and mechanistic pathways used to achieve such cleavages appear quite dissimilar (for recent reviews see 1,2). The hairpin ribozyme occurs in the negative strand of the satellite RNA of tobacco ringspot virus (TRSV), as well as in two other closely related nepovirus satellite RNAs, and is the second smallest, naturally occurring, self-cleaving RNA (reviewed in 3–6).

The minimum catalytic motif consists of two helical domains (A and B) each containing an internal loop flanked by two regions of Watson–Crick double helix. The hairpin is commonly studied in trans-cleavage form, with a substrate strand and a ribozyme component consisting of one or two strands (Fig. 1). The two domains are hinged between residues 14 and 15 in the ribozyme strand. The hairpin motif undergoes site-specific cleavage in domain A in the presence of magnesium ions as well as a reverse ligation reaction. The cleavage reaction is favoured when the two helices in domain A are short (usually 6 and 4 bp, respectively), such that dissociation of the cleaved fragments is not rate-limiting in the presence of magnesium ions (7).

Much recent effort has gone into understanding the steps involved in the cleavage activity. The association of substrate strand with the substrate binding strand to form domain A occurs extremely rapidly, if the substrate sequence is modified so as to avoid self-complementarity, with values of $k_{on}$ in the region of $2 \times 10^9$M/min, one of the fastest association rates of any known ribozyme (7–9). The complex is quite stable with a $k_{off}$ of <0.01/min. The kinetics of hairpin cleavage are biphasic showing a fast phase with a $k_{cat}$ of ~0.2/min, some 5-fold lower than efficient hammerhead ribozyme reactions (7–9). The subsequent slow phase has been shown to be due to an inactive conformation where the two domains A and B are stacked in an extended configuration (8,10). The conformers are non-exchangeable and the inactive complex must dissociate in order to rebind to form an active ribozyme. The active conformation is achieved through an initial docking of the two domains A and B in an antiparallel fashion. The docking process has been monitored by attachment of fluorescent dyes to each domain and by measurement of fluorescence resonance energy transfer (FRET) upon folding induced by metal ions such as calcium, magnesium and strontium, all of which support cleavage. Docking was found to be required for both cleavage and ligation but in the case of cleavage, docking is a fast process (~0.5/min) that is not rate-limiting (11).

In the context of the TRSV satellite RNA, the hairpin motif is part of a four-way helical junction, the folding of which in the presence of magnesium or other group IIA metal ions in the micromolar range also favours the interaction of domains A and B (12). Further metal ions are needed for cleavage activity which is achieved in the millimolar range. However, some metal ions which allow correct folding, such as manganese, are less able to support cleavage (13). This dual role of metal ion in the natural...
hairpin context is paralleled by earlier studies of the ionic requirements for cleavage of the hinged two-domain hairpin (14).

In the case of the well-documented hammerhead ribozyme, in addition to playing an important structural role, metal ions have been shown to participate directly in the chemical cleavage step. The precise role is controversial, but proposals for metal ion involvement have included interaction with the attacking 2'-hydroxyl group, direct chelation to the pro-R oxygen atom of the cleaved phosphate in the transition state, and/or interaction with the leaving 5'-oxygen atom (discussed in 15,16). In contrast, recent studies of the metal ion requirements for hairpin ribozyme cleavage have failed to provide any evidence for direct participation of metal ions in the cleavage mechanism, either by 2'-hydroxyl activation or by chelation to a non-bridging oxygen atom in the transition state of the cleaved phosphate. Furthermore, cobalt (II) hexamine, which cannot form inner sphere co-ordination complexes, was found to be particularly efficient at supporting hairpin ribozyme cleavage (17–19). Although participation of metal ion as an outer sphere complex cannot be ruled out, a more likely possibility is that metal ions play a structural role in a step just prior to chemical cleavage, perhaps a metal-dependent conformational change.

The polyamine spermidine was the first non-metallic ion shown to support hairpin cleavage, but at 2 mM the cleavage rate is extremely slow, some 10^3 lower than for 10 mM magnesium ions (14). More interestingly, low concentrations of spermidine were found to be able to stimulate the magnesium-dependent cleavage rate somewhat (14). To gain further insight into the structural and unusual ionic requirements of hairpin ribozyme cleavage, we decided to investigate a wider range of non-metallic polycations, such as aminoglycoside antibiotics and other polyamines. We now report that aminoglycoside antibiotics cause inhibition of hairpin cleavage when added to a magnesium ion-catalyzed reaction. Neomycin B gives moderate inhibition, but only one, 5-epi-sisomicin, inhibits strongly. More significantly, and in contrast to other self-cleaving domains, aminoglycoside antibiotics stimulate hairpin ribozyme cleavage in the absence of metal ions, with the best (neomycin B, apramycin and kanamycin B) being only 13–20-fold lower in rate at 10 mM than for the same concentration of magnesium ions. We have further found that the tetra-amine spermine supports a very efficient hairpin cleavage reaction, almost as fast as magnesium ions at the same concentration. In addition, certain concentration combinations of spermine and magnesium ions lead to substantial stimulation of hairpin ribozyme cleavage and more efficiently than the triamine, spermidine. The results have important implications for potential therapeutic use of the hairpin ribozyme in mammalian cells.

**MATERIALS AND METHODS**

Oligoribonucleotides were synthesized by 1 µmol solid-phase synthesis on controlled pore glass and purified by anion exchange chromatography on a NucleoPac PA-100 column (Dionex, Surrey, UK) as described previously (20,21). 2'-O-methyladenosine phosphorimidate was obtained from Glen Research (via Cambio, Cambridge, UK). Desalting was achieved via extensive dialysis against water. Oligoribonucleotides were 5'-end-labeled with γ-[32P]ATP and T4 polynucleotide kinase (22). Parities were checked by electrophoresis on a 20% denaturing polyacrylamide gel (PAGE) and by MALDI-TOF mass-spectrometry (21). Aminoglycoside antibiotics were purchased from either Sigma or Fluka except for neamine which was obtained from Affiniti (Exeter, UK) and 5-epi-sisomicin which was kindly provided by Julian Davies. Neomycin (Sigma) was a mixture of neomycin B (>90%) and neomycin C, with the difference being the stereochemistry of the carbon at position 1 of the B ring. Kanamycin A (Fluka) contained minor components B and C (<5%). Gentamicin (Fluka) was a mixture of two components as illustrated (Fig. 5). Spermine and spermidine were purchased from Calbiochem (Nottingham, UK). Fresh solutions of antibiotics or polyamines were prepared just prior to each experiment.

**Determination of ribozyme kinetic parameters**

Kinetic parameters of cleavage of metal-, antibiotic- and polyamine-dependent reactions were determined under single turnover conditions (20). Separate solutions of ribozyme (equimolar mixture of RzA and RzB strands, 20–200 nM, 90 µM) in Tris–HCl (pH 7.5) and γ-32P-labeled substrate RNAs (10 nM, 10 µM) in water were each incubated at 70°C for 1 min and then cooled to room temperature over 15 min. The cofactor solution was added to its required concentration in the ribozyme solution and then both were incubated at 37°C for 15 min. Reactions were initiated by mixing the ribozyme and substrate solutions to give a final volume of 100 µl of 40 mM Tris–HCl in the case of metal ion reactions or 100 mM in the case of the other co-factors. Non-metal catalyzed reactions contained 1 mM EDTA and 1 mM EGTA which were added to the ribozyme strand prior to the 70°C incubation. No differences in rates were observed in the concentration range of 0.1–2 mM for these chelators but 10-fold rate reductions were observed at 50 mM EDTA, most likely due to the inhibition by sodium ions (data not shown) (14).

Aliquots (10 µl) were removed at six suitable time intervals and the reactions quenched by addition to 10 µl of urea stop mix (7 M urea, 50 mM EDTA, 0.04% w/v xylene cyanol, 0.04% w/v bromophenol blue). Samples were loaded onto a 20% denaturing polyacrylamide gel and subjected to electrophoresis at 12 W for 80 min. The resultant gels were dried and scanned using a PhosphoImager (Molecular Dynamics, Buckinghamshire, UK), and the data processed using the programme Image Quant (Molecular Dynamics) and quantitated by use of the Geltrak programme as described previously (20). The initial velocities of the reactions (k_{obs}) at different ribozyme concentrations were determined from a plot of product formation against time, usually up to 30% cleavage. Data from at least three independent experiments were obtained and usually fell within 20% of the mean. k_{obs} values were plotted against ribozyme concentration/k_{obs}, using Eadie–Hofstee plots as described previously (20). Multiple turnover kinetic parameters were carried out using substrate concentrations of 50–100 nM and ribozyme concentrations of 1–80 nM as described previously (23,24). In the cases of aminoglycosides, it was necessary to keep the ribozyme concentration constant or within a narrow range and to vary substrate concentration.

**Inhibition of ribozyme cleavage by antibiotics**

The inhibition constants (K_i) of hairpin ribozyme cleavage for the antibiotics were obtained by varying the concentration of antibiotic from 0.1 µM to 100 nM and by measuring the k_{obs} at 10 mM MgCl_2 under conditions described for the single turnover conditions. Ribozyme (RzA and RzB) concentration was 50 nM and 32P-labeled substrate (10 nM) was used to initiate the reaction. Control cleavage reactions with antibiotic alone and
with magnesium ions alone were also carried out in parallel. For neomycin B inhibition, the fraction of inhibition at each concentration of antibiotic \((1 - k_{\text{obs}}/k_{\text{con}})\) was calculated from the \(k_{\text{obs}}\) for the antibiotic reaction in the presence of 10 mM magnesium ions \((k_{\text{neo}})\) and the \(k_{\text{obs}}\) for the control reaction of 10 mM magnesium ions alone \((k_{\text{con}})\) (25). The fraction of inhibition was plotted against neomycin B concentration (Fig. 2), and the \(K_i\), the antibiotic concentration needed to inhibit half of the rate of cleavage, was obtained from a simple hyperbolic bimolecular binding fit. For the other antibiotics, estimates of \(K_i\) were made at 50% inhibition with an error of <20%. Under the conditions of 10 mM MgCl₂, the contribution to the rates from the antibiotics was negligible in the calculation of the values of \(K_i\).

In the delayed inhibition reactions (Fig. 7) the cleavage reaction was carried out in the presence of 10 mM MgCl₂ and after 90 s antibiotic was added to 10 mM.

pH profiles

The pH dependences of the \(k_{\text{obs}}\) of hairpin cleavage reactions (Fig. 6) were carried out under the single turnover conditions described above with ribozyme and substrate concentrations as used for the inhibition reactions. The polyamine and antibiotic experiments contained 1 mM each of EDTA and EGTA, but we found that for neomycin B and spermine in the absence of EDTA and EGTA, rates were essentially unchanged (data not shown). To maintain pH at the extremes of the range tested, it was necessary to reduce the concentration of the antibiotics and polyamines to 5 mM and to increase the buffer strength to 100 mM. Solutions of antibiotics (5 mM) mixed with final buffer concentration (100 mM) were tested for pH by use of a pH meter as well as pH indicator strips (BDH-Merck, Lutterworth, UK). The following buffers were used to maintain pH, pH 4.8–5.5, potassium acetate/acetic acid; pH 5.5–6.7, potassium MES [2-N-morpholinoethanesulfonic acid]; pH 6.1–7.5, potassium PIPES [piperazine-N,N’-bis(2-ethanesulfonic acid)]; pH 7.5–8.8, Tris–HCl, pH 8.6–10, potassium CHES [2-(N-cyclohexylamino)ethanesulfonic acid]. Ribozyme cleavage reactions were carried out over the pH profile in buffer alone at 100 mM as a control. Experiments were carried out in triplicate or until high reproducibility was obtained (5–10% error). For spermine, neomycin B and MgCl₂, the pH profiles were also carried out at 1, 5 and 10 mM concentrations with buffers at concentrations of 40, 100 and 200 mM, respectively, under the same single turnover conditions without significant differences (data not shown). The pH profiles were also carried out under multiple turnover conditions for 5 mM spermine, neomycin B and MgCl₂ at 100 mM buffer again without significant differences (data not shown).

**Competition cleavage assays**

Ribozyme cleavage rates \((k_{\text{obs}})\) for competitive cleavage reactions (Figs 8, 11 and 12) were determined under single turnover conditions as described above with titrations of either neomycin B or polyamine against a fixed concentration of magnesium ions or vice versa. The ribozyme and substrate concentrations were the same as for the inhibition reactions. The data shown in Figure 9 involved a fixed concentration of neomycin B at 1 mM and a range of magnesium ion concentration in the presence of 100 mM Tris–HCl (pH 7.5), MES (pH 6) or CHES (pH 8.5).

**Filter binding**

Filter binding assays utilizing a 96-well 0.45 µM ‘MultiScreen’-HA Millipore mixed cellulose ester filtration plate were carried out essentially as described previously for protein–RNA filter binding (21,26) and similarly to the method reported for measurement of neomycin B binding to RRE RNA (27). To prevent substrate cleavage during the binding assay, an uncleavable 2’-O-methyl analogue at the A₁ position of the substrate strand was used. Hairpin ribozyme (25 nM) with one strand 5’-32P radio-labelled (200 000 c.p.m.) was annealed in 40 mM Tris–HCl (pH 7.5) by incubation of equimolar amounts of the three strands at 70°C for 1 min and cooled slowly to 4°C. Antibiotic was added to give concentrations of 50 nM to 1200 µM and a volume of 250 µL. Parallel experiments were also carried out in the presence of 10 mM MgCl₂. Binding reactions were incubated on ice for 60 min in individual wells of the 96-well plate which was precooled and washed in ice-cold 40 mM buffer. After 60 min the plate was vacuum-filtered, and the wells washed once with ice-cold 40 mM buffer (200 µL), dried and counted by liquid scintillation as described previously (21). Apparent \(K_i\) values were determined by plotting the percentage of labeled ribozyme bound to the filter against the antibiotic concentration and calculation of the concentration at which binding was half maximal.

In control experiments we found that ribozyme B and substrate strands alone did not bind aminoglycosides significantly, but ribozyme A strand alone was retained on the filter in the presence of antibiotics. Therefore, to check that binding of the complete hairpin was measured, three parallel experiments with neomycin B were carried out where one strand in turn was 32P-labelled. Identical filter retention results were found in each case. In the tRNA competition reactions, 250 nM and 2.5 µM concentrations of yeast tRNA (Sigma) were also present.

**RESULTS**

**The effect of aminoglycoside antibiotics on hairpin ribozyme cleavage**

Several years ago, aminoglycoside antibiotics were shown to inhibit the self-splicing activity of the group I intron (28,29). More recently, it was shown that a range of aminoglycosides inhibit the cleavage of the hepatatis delta virus ribozyme (30) and the hammerhead ribozyme (25,31), with neomycin B being particularly effective. We therefore investigated the effect of increasing concentrations of neomycin B on the magnesium ion-dependent hairpin ribozyme cleavage reaction. For these experiments we utilized our well-characterized three-stranded hairpin ribozyme (Fig. 1) which we have used previously both for functional group analysis (23,24) and for interdomain cross-linking (20). Cleavage rates were measured at pH 7.5, 10 mM magnesium ions and 37°C under single turnover conditions at a ribozyme concentration of 50 nM, conditions where we have observed optimal values of \(k_{\text{obs}}\). As anticipated, we found that neomycin B inhibited the hairpin ribozyme cleavage reaction. To obtain the concentration of neomycin that inhibited cleavage by 50% \((K_i)\), the fraction of inhibition \((1 - k_{\text{obs}}/k_{\text{con}})\) was plotted as a function of neomycin B concentration and the data was found to fit a bimolecular binding equation, giving a \(K_i\) of 190 ± 29 µM (Fig. 2). This result shows that neomycin B is a far less potent inhibitor of the hairpin ribozyme than of the hammerhead \((K_i = 13 ± 3 \mu M)\) (25) and of the hepatitis delta virus ribozyme \((K_i = 28 ± 10 \mu M)\) (30).
Figure 1. Secondary structure of a three-stranded model hairpin ribozyme showing the two domains that are required to dock in order to effect cleavage. The cleavage site in domain A is shown by an arrow. The residue A–1 adjacent to the cleavage site is highlighted in green. The cleavage site in domain A is shown by an arrow. The residue A–1 adjacent to the cleavage site is highlighted in green. The residue A–1 adjacent to the cleavage site is highlighted in green.

Figure 2. Concentration dependence of neomycin B inhibition of the magnesium-induced cleavage of the hairpin ribozyme. The data was fitted to a hyperbolic, bimolecular binding equation to give $K_i = 190 \pm 29 \mu M$. $k_{cat}$ is $k_{obs}$ of the 10 mM magnesium-induced reaction at the particular neomycin concentration. $k_{cat} = k_{obs}$. of the 10 mM magnesium-alone catalyzed reaction.

We screened a range of other antibiotics in the inhibition of magnesium-dependent hairpin ribozyme cleavage. These included other members of the neomycin family (Fig. 3), the tobramycin class (Fig. 4) and a number of other aminoglycoside antibiotics (Fig. 5). Of these, only 5-epi-sisomicin was found to be a potent inhibitor ($K_i = 0.6 \mu M$), and all others were found to be much poorer inhibitors than neomycin B.

The unusual ionic dependence of the hairpin ribozyme prompted us to test whether neomycin B could carry out the hairpin ribozyme cleavage reaction in the absence of metal ions. We found that at 37°C, pH 7.5 and in the presence of chelators EDTA and EGTA, 10 mM neomycin B catalyzed specific cleavage of the hairpin with products which migrated identically to those seen in the magnesium-dependent reaction. No other cleavage products were seen (data not shown). The $k'_{cat}$ was only 18-fold slower than for the same concentration of magnesium ions alone (Fig. 3) and was unaltered if the EDTA and EGTA were omitted (data not shown). Under multiple turnover conditions at the same pH, temperature and buffer conditions, the $k_{cat}$ value (0.0080 ± 0.0003/min) was similar to that under single turnover, but the $K_M$ was somewhat higher (137 ± 16 nM). The cleavage rate was not significantly altered when substrate was added last to ribozyme strands plus neomycin B, to initiate the reaction, rather than initiation by addition of neomycin B to a preformed ribozyme–substrate complex (data not shown). This suggests that the rate of association of ribozyme and substrate strands was not limiting for the neomycin B-induced cleavage reaction.

All other members of the neomycin family showed significantly poorer $k'_{cat}$ values, although butirosin and neamine showed $K_M$ values which were 10-fold lower (Fig. 3). Tobramycin was a relatively poor stimulator of hairpin cleavage, as were all others in this family tested except for kanamycin B (Fig. 4), which had a slightly lower $k'_{cat}$ value than neomycin B. Another effective
aminoglycoside antibiotic at hairpin ribozyme cleavage at pH 7.5 was apramycin, which showed a $k'_{cat}$ value for cleavage only 13-fold slower than for magnesium ions (Fig. 5). In contrast, gentamicin, gentecin G418, sisomicin and 5-epi-sisomicin were all much poorer. Streptomycin and several non-aminoglycoside antibiotics did not induce cleavage of the hairpin ribozyme (data not shown).

In order to obtain an estimate of the relative affinities of the various antibiotics for the hairpin ribozyme, we measured their apparent dissociation constants by determination of the concentrations required for 50% retention of the ribozyme–drug complex on an ice-cold mixed cellulose ester filter (Figs 3–5) (27). For these experiments we used an inactive ribozyme where the nucleoside carrying the hydroxyl group at the cleavage site (A–1) is replaced by the corresponding 2′-O-methyl adenosine analogue, a modification that does not significantly impair docking of the two hairpin domains (11). Almost all the antibiotics had a relatively low binding affinity to the hairpin RNA (apparent $K_d$ 150–4000 µM). However neomycin B was significantly better (40 µM). 5-Epi-sisomicin was by far the strongest binder with a $K_d$ of 1 µM. In all cases tested, in the presence of 10 mM magnesium ions (i.e. under conditions where the antibiotic would be expected to inhibit the cleavage reaction), the binding was significantly poorer. Although the apparent $K_d$s of the antibiotics measured in this way may not reflect the true equilibrium dissociation constants, a comparison of their relative values shows a good correlation with the corresponding inhibition constants for the magnesium-dependent reactions, but a poorer correlation with the rates of the antibiotic-induced hairpin cleavage reactions. Binding was specific for the hairpin since competition experiments in the presence of 10- and 100-fold excesses of tRNA resulted in $K_d$ reductions for neomycin B only to 100 and 250 µM, respectively.

We next compared the pH profiles for antibiotic-induced and magnesium-dependent cleavages (Fig. 6). At 5 mM and under single turnover conditions, the magnesium-dependent reaction showed an approximately linear pH dependence through the range 5–9 with the greatest activity at the highest pH tested (inset). The slope (4-fold change between pH 5.5 and 9) is only slightly greater than that reported by others (18). In contrast, the pH dependence of neomycin B-induced cleavage was reversed compared with the magnesium-dependent reaction, with the greatest value at lower pH. The dependence was approximately linear between pH 6 and 8.5 but dropped rapidly to a very low cleavage rate at pH 9, identical to the cleavage rate induced by buffer alone. Similar pH profiles for neomycin B were obtained at 1 and 10 mM antibiotic concentrations, and under multiple turnover conditions (data not shown). The pH dependence of the kanamycin B-induced cleavage reaction followed a similar linear
dependence between pH 5 and 8, but activity dropped rapidly above this. When a relatively poor inducer of cleavage at pH 7.5, ribostamycin, was studied, it also showed substantially better cleavage at lower pH, with a similar slope between pH 5.5 and 7 to that of both the kanamycin B and neomycin B reactions. These results suggest that activity is correlated with a need for a number of protonated amino groups in the antibiotics. The almost complete loss of cleavage activity above pH 8.5 in all cases is in line with the general values of pKₐ for the amino groups, most of which are in the 7.4 to 8.8 region. In contrast, apramycin was the only antibiotic to show a completely different pH dependence with a maximum rate at pH 8 (Fig. 6).

A number of recent studies have suggested that, in the cases of the hammerhead and hepatitis delta virus ribozymes and the self-splicing group I intron, aminoglycoside antibiotics achieve inhibition of ribozyme cleavage by replacement of structurally important magnesium ions by protonated amino groups (25,30,32,33). To address whether this is also the case for hairpin ribozyme cleavage, we first carried out a delayed addition reaction, where 10 mM magnesium ions was used to initiate ribozyme cleavage and then 10 mM of the antibiotic was added after 90 s. For both neomycin B and tobramycin addition, a reduction in cleavage rate occurred very rapidly as compared with the reaction with magnesium ions alone (Fig. 7). The rate of reduction was greater for neomycin B than for tobramycin. For comparison, the rates of the neomycin B- and tobramycin B-induced reactions are also shown. Since tobramycin alone catalyzes only a very slow rate of cleavage, the kinetics of the delayed tobramycin inhibition reaction can be assumed to be due just to the proportion of magnesium-activated ribozyme remaining. This proportion can be estimated (12%) by comparing the initial rate of delayed inhibition (k_{obs} 0.025/min) with the initial rate of a reaction having 10 mM magnesium ions (k_{obs} 0.216/min). In contrast, the kinetics of the delayed neomycin B addition reaction are complex, since in principle there are both magnesium- and neomycin B-dependent rate components. The kinetics are consistent with an extremely rapid replacement of most of the magnesium ions by the antibiotic, since the new rate after addition of neomycin B (k_{obs} 0.0068/min) is very similar to the rate of cleavage with 10 mM neomycin B at the start (k_{obs} 0.0051/min).

Secondly, the concentration dependence of the neomycin B alone cleavage reaction was studied and this showed a biphasic character with a hyperbolic curve up to 25 mM neomycin B followed by an approximately linear dependence thereafter and no evidence of saturation (Fig. 8). In the presence of 1 mM magnesium ions, the neomycin B concentration dependence at very low concentrations of neomycin B showed a drastic reduction in the rate to reach a minimum value at around 500 µM neomycin B (Fig. 8, inset). Above 1 mM, the curve follows a similar shape to the neomycin B alone reaction where the slight rate enhancement in this region in the presence of 1 mM magnesium reflects a small population of magnesium alone promoted hairpin cleavage. The two curves become the same within experimental error by 10–20 mM neomycin B. The experiment shows that low concentrations of neomycin B readily displace magnesium ions, which results in inhibition, but that as neomycin B is added, the rate profile becomes very similar to that of neomycin B alone. No synergistic effect was found for a variety of magnesium ions and neomycin B concentration combinations tested (data not shown).

Finally, we determined the effect of 1 mM neomycin B on the magnesium dependence of hairpin ribozyme cleavage at three pH values (Fig. 9). In each case the presence of 1 mM neomycin B reduced the cleavage rate, but the magnitude of the effect varied from ~2-fold at pH 8.5 to 5-fold at pH 7.5 and 20-fold at pH 6.0. This indicates that the amount of inhibition is dependent on the
Figure 8. Rate-dependence of cleavage on neomycin B concentration. Graph showing $k_{obs}$ of cleavage as a function of neomycin B concentration for the antibiotic alone reaction and also antibiotic in the presence of 1 mM Mg$^{2+}$. The $k_{obs}$ of the 1 mM Mg$^{2+}$ alone cleavage reaction is marked (0.0143/min). Inset, the effect of low concentrations of neomycin B (<1 mM) on cleavage for the antibiotic in the presence of 1 mM Mg$^{2+}$.

Figure 9. pH dependence of neomycin B inhibition of the magnesium-dependent cleavage reaction. Graph showing $k_{obs}$ of cleavage as a function of Mg$^{2+}$ concentration for three pH conditions (pH 6, 7.5 and 8.5) in the presence or absence of 1 mM neomycin B.

acquisition on the aminoglycoside of increasing positive charge. This experiment, together with the two previous experiments, are all consistent with the replacement of magnesium ions in the hairpin structure by protonated amino groups in the antibiotic.

Figure 10. (a) Structures of the tetramine, spermine, and the triamine, spermidine, and their pK$_a$ values (44). (b) Table showing single and multiple turnover kinetic parameters for the polyamine co-factors and in combination with Mg$^{2+}$. Also for spermine and spermidine the apparent dissociation constants (K$_d$) and percentage filter retention are shown.

### Cleavage of the hairpin ribozyme by polyamines

It has been reported previously that a very slow, but specific cleavage of the hairpin ribozyme could be effected by the triamine spermidine in the presence of EDTA and EGTA ($k_{obs}$ 0.008/min at 50 mM spermidine) (14). We found that under single turnover conditions 10 mM spermidine specifically cleaved the hairpin ribozyme with $k'_{cat}$ of 0.003/min, ∼45-fold slower than the $k'_{cat}$ for magnesium ions (Fig. 10). To our knowledge, no other polyamine had been investigated previously for hairpin ribozyme cleavage. We therefore investigated the longer polyamine spermine, which has four amino groups compared with three for spermidine (Fig. 10). We found that in the presence of EDTA and EGTA, spermine catalyzed an extremely efficient cleavage reaction, with a $k'_{cat}$ (0.14/min) very close to that of the magnesium-dependent reaction (0.16/min). Under multiple turnover conditions, the rate was increased 10-fold. From filter binding experiments, the apparent K$_d$s of binding of spermine and spermidine to the 2'-O-methyl A–U hairpin ribozyme were found to be 200 and 2000 µM, respectively.

The shorter polyamine diethylene triamine catalyzed only an extremely slow cleavage reaction, some 3000-fold poorer than spermine (data not shown). Similarly, diaminoalkanes of C$_2$–C$_9$ chain length at 50 mM concentrations showed very low rates of hairpin cleavage, the best, 1,6-diaminohexane, still having a 1000-fold lower rate than spermine under single turnover conditions. 1,12-Diaminododecane, which is the same length as spermine but has only two amino groups, was poorly water soluble but in the presence of 2.5% DMSO, which does not affect the magnesium reaction significantly, it showed a rate still some 500-fold poorer than spermine (data not shown).
Figure 11. Graph showing the rate ($k_{\text{obs}}$) of hairpin cleavage as a function of magnesium concentration in the presence of 500µM spermine or spermidine. The magnesium concentration dependence for the Mg$^{2+}$ alone reaction is also shown. A concentration of 500µM spermine or spermidine was found to be optimal for the enhancement of the magnesium-induced cleavage, since both 250 and 750µM concentrations gave rise to less stimulation (data not shown).

The pH dependence of the spermine-induced cleavage showed very little change in rate between pH 5 and 8 (data not shown). There was a slight maximum at pH 7 just 2-fold above the rates at pH 5 and 8, which were almost identical. The rate dropped to a very low value at pH 9. More significantly, there was a substantial stimulation of the initial rate of the magnesium-dependent reaction by addition of spermine. Under single turnover conditions, maximum stimulation (3.5-fold) was obtained for spermine at 500µM when the magnesium concentration was ∼5mM to give a $k_{\text{obs}}$ of 0.35/min (Fig. 11), whilst spermidine enhanced the rate by almost as much (3-fold), and again the maximum stimulation occurred at 500µM. The stimulatory effect of spermidine on the magnesium-dependent reaction has been reported previously (14, 19). A much greater effect of spermine compared with spermidine was seen when the magnesium concentration was low. Thus, a maximum 4-fold stimulation in rate was seen at 500µM magnesium ions when ∼4mM spermine was present, whereas the stimulatory effect of spermidine at 500µM magnesium ions was marginal (Fig. 12). These results show that spermine can efficiently replace both types of magnesium ions required for cleavage activity, those involved in folding and those involved in attainment of the catalytically active structure.

DISCUSSION

Cleavage and inhibition of the hairpin ribozyme by aminoglycoside antibiotics

We have shown that specific cleavage of the hairpin ribozyme can be effected by a range of aminoglycoside antibiotics in the absence of metal ions. The rates of cleavage at pH 7.5 under single turnover conditions are at best 13–18-fold less than that for magnesium ions, but at pH 5.5 the rates of cleavage for neomycin B and kanamycin B are more comparable with that for magnesium ions. The antibiotic-induced cleavage reaction is identical in specificity to the magnesium-dependent reaction and occurs much faster than internucleotide hydrolysis. For example, the pseudo-first order rate constant for hydrolysis of ApA by 300mM neomycin B is 1.6 × 10$^{-5}$/min at 50°C and pH 8 (34). Furthermore, ribozyme cleavage is not a general property of aminoglycoside antibiotics, since we found no cleavage of the hammerhead ribozyme up to 100mM neomycin B (data not shown).

A detailed aminoglycoside structure–activity relationship is beyond the scope of this discussion, but we can draw a number of general conclusions. First, the most important feature of aminoglycoside antibiotics for hairpin ribozyme cleavage seems to be the presence of at least four protonated amino groups. For example, at pH 7.5 in the neomycin family, those antibiotics lacking rings III and IV are less active, but at lower pH, ribostamycin, which lacks ring IV, becomes almost as active as neomycin B. All of the antibiotics tested in the neomycin and tobramycin families are more active at lower pH, suggesting that protonation of the amino group at position 3 of ring II, which has a nominally low pK$_a$ value, is important for activity. A second determinant of activity is the presence and orientation of certain hydroxyl groups; for example, as seen in the tobramycin family, where the removal of 3′- and 4′-hydroxyl groups results in loss of activity and in the substantial difference in activity obtained with sisomicin and its synthetic derivative, 5-epi-sisomicin. Structurally important hydroxyl contacts have been observed, for example, in interaction of aminoglycoside antibiotics with the A site of 16S ribosomal RNA (35).

Apramycin is unusual in having a pH optimum at pH 8. This was unexpected because at this pH several of the amino groups...
would be expected to be unprotonated or less protonated. The different ring structure, arrangement of amino groups and the narrower spread of pK_a values (6.6–8.2) suggest that apramycin does not follow the same mode of action throughout the pH range tested. For example, at lower pH there may perhaps be a different conformation or binding mode that stabilizes an inactive form of the hairpin.

Under conditions when magnesium ions are present, aminoglycoside antibiotics inhibit the hairpin ribozyme cleavage. The degree of inhibition is correlated with the apparent binding constant of the antibiotic; in general the tighter the binding, the greater the degree of inhibition. Thus, 5-epi-sisomicin is by far the most potent inhibitor, but neomycin B also has significant inhibition properties. All others tested were much poorer inhibitors. Neomycin B is the only antibiotic tested which is both a good inhibitor of magnesium-dependent cleavage and is also reasonably active in the metal-free cleavage reaction. Competition experiments were therefore particularly revealing. For example, we have provided several lines of evidence that the inhibition by neomycin B occurs by replacement of metal ions by the amino groups of the antibiotic, similarly to the hammerhead, HDV and group I intron ribozymes (25,30,32,33). From curve fitting of the neomycin B inhibition and from preliminary isothermal calorimetry experiments (D.J. Earnshaw and M.J. Gait, unpublished results), the stoichiometry of neomycin B binding to the hairpin appears to be 1:1. In the competition experiment (Fig. 8), maximal inhibition occurs at a molar ratio of one neomycin B to two magnesium ions, and at >1 mM neomycin B, the cleavage reaction quickly takes on the characteristics of the neomycin-alone reaction. This suggests that at equimolar ratio of ligands, most hairpin molecules select neomycin B as the catalytic ligand rather than magnesium ions. It would seem very likely, therefore, that most or all of the structurally important metal ion binding sites are filled simultaneously by protonated amino groups when the antibiotic binds. However, the ability of the antibiotic-bound hairpin to adopt a catalytically active structure must be a reflection in addition of the structural features of the antibiotic itself (i.e. its flexibility and other steric factors) and not just its mere metal ion displacement ability. Thus, structural and mapping studies of the binding sites on the hairpin of a good inhibitor (such as 5-epi-sisomicin) as well as a good cleaver (such as neomycin B) might prove valuable in the future in order to throw light on how the nature of the ligand influences the ability of the hairpin to be cleaved.

The mechanism of hairpin ribozyme cleavage

Early studies of the ionic requirements for hairpin ribozyme cleavage showed that at least two types of ion were needed for efficient hairpin ribozyme cleavage (14). One of these types seems to play a role in the folding and docking of the two domains and can be satisfied by a range of metal ions (magnesium, calcium, strontium, manganese) (11,13). In contrast, it has been shown by FRET studies of the hinged hairpin that 10 mM spermidine in the absence of metal ion does not promote efficient docking, but instead is able to boost the docking rate of 2 mM magnesium or manganese ions (11). It was suggested that spermidine stabilizes the metal-dependent docking. In the case of the natural hairpin junction, spermidine does promote docking, albeit at much higher concentrations than metal ions (13).

Our kinetic data show that the longer polyamine spermine must be able to fulfill the docking requirement very efficiently in the hinged hairpin. It may be noted that spermine has been shown to promote folding of the hammerhead ribozyme and to allow ions such as cadmium to catalyze efficient ribozyme cleavage, which are unable to do so in the absence of spermine (36). We have no information at this stage concerning the effect of aminoglycoside antibiotics on hairpin docking, and therefore as to whether the lower rates of the antibiotic-induced cleavage reaction compared with magnesium ions are due to impaired docking or to a suboptimal ability to replace catalytically important magnesium ions in the transition state, or to both.

Once docking has taken place, a second type of ion is required for attainment of a catalytically competent hairpin configuration. Magnesium, calcium and strontium ions are all effective in this role (14). Use of manganese ions has been reported to result in 10-fold (14) or 2.6-fold (18) lower cleavage rates for the hinged hairpin and to be catalytically inactive in the case of the natural junction hairpin (13). Cobalt hexamine is quantitatively the most effective ion in both docking (11,13) and in hairpin cleavage (17–19). We have also found that under single turnover conditions at pH 7.5 at 37°C, 0.25 mM Co^{2+}(NH_3)_6 or Ru^{3+}(NH_3)_6 promote hairpin ribozyme cleavage with k_cat values 2.9- and 2.3-fold higher, respectively, than are achieved with 10 mM concentrations of Mg^{2+}, but Pb^{2+}(NH_3)_4 was completely inactive (D.J. Earnshaw and M.J. Gait, unpublished results). In the current study we have shown that the polyamine spermine can carry out this second ionic role very efficiently, but the effectiveness of spermidine in this role is less clear. Clearly, both polyamines can synergize with 5 mM magnesium ions to boost cleavage rates. But at low magnesium concentration, where docking is effective but cleavage is slow, we found that spermidine is much less effective than spermine in enhancing the cleavage rate. Under such conditions, the polyamine may be required to play predominantly the role of attainment of the catalytically active structure, and for this spermidine appears sub-optimal.

It is interesting to note that addition of a low concentration of either spermine or spermidine dramatically enhanced the rate of the magnesium-dependent cis cleavage of the Neospora VS ribozyme and higher concentrations facilitated the trans cleavage reaction (37). Furthermore, spermidine was shown to be essential to allow self-cleavage of the coconut cadang cadang viroid but magnesium ions could not promote such cleavage (38). Thus, it seems likely that there is an important general effect of polyamines in facilitating interstrand interactions in ribozymes, which may be due partly to the reduction in charge repulsion between nucleic acid strands expected upon polyamine binding, as observed for tRNA (39). However, the particular success of spermine in promoting hairpin ribozyme cleavage suggests that there is in addition an important structural component both to hairpin interdomain interaction and subsequent configuration for cleavage that is satisfied by the particular length and arrangement of the four positive charges on the spermine backbone. If fully extended, spermine would contain regularly spaced amino groups spanning a distance of 15 Å, which is very similar to the spacing and distance spanning the amino groups in rings I and II of aminoglycoside antibiotics (32).

Curve fitting of preliminary isothermal calorimetry experiments at concentrations of <120 µM suggest that the molecule of spermine may bind to the hinged hairpin (D.J. Earnshaw and M.J. Gait, unpublished results). This is in line with FRET data showing a 1:1 stoichiometry of spermidine binding to the natural hairpin junction (13). Our data support the hypothesis that the
positively charged amino groups on spermine replace structurally important metal ions, one or more of which may be required to be located between the two domains to ligand functional groups or to form ionic interactions, as part of a network of interdomain contacts that may include, for example, the recently proposed ‘ribose zipper’ between pairs of neighbouring hydroxyl groups on the two domains (Fig. 1) (20).

Spermine, and to a lesser extent aminoglycoside antibiotics, must also be able to mimic the second role of metal ion in attaining a catalytically competent configuration. This role also appears to be structural, since no direct catalytic role for metal ion has yet been shown and indeed as we have shown, metal ion can be dispensed with completely. Furthermore, whilst this manuscript was in preparation we have learnt that 4 M ammonium ions can also induce efficient hairpin ribozyme cleavage (40).

Our pH titration data confirm previous studies showing the lack of a log linear pH dependence of the magnesium-dependent reaction, such that the cleavage rate cannot be dependent on the concentration of MgOFP (18). The pH dependence is approximately linear in the range 5–8 (Fig. 6, inset). Since the magnesium ions seem to play merely a structural role, then the pH dependence of cleavage may reflect instead the pK_a values of one or more functional groups within the hairpin. One functional group may provide a general base for proton abstraction from the 2′-hydroxyl group and another a general acid (for proton donation to the 5′-leaving oxygen atom). Based on the crystal structure of a cleaved form of the hepatitis delta virus RNA, another ribozyme where no catalytic role for metal ion seems likely, it has been proposed that the exocyclic amino group of C75 may have an abnormally high pK_a value and may thus act as a general base for proton abstraction from the 2′-hydroxyl group at the cleavage site (41). Candidates for a proton acceptor in the case of the hairpin ribozyme are G+1 in the substrate strand, since removal of the by the pK_a values of the various amino groups on the ligand. For spermine, the pK_a values of all four amino groups are high (8.6–11.2) (44). The flat pH profile between pH 5 and 8 and the very low activity at pH 9 indicates that there is a requirement for all four positive charges, but it seems very unlikely that any of the amino groups can directly participate either as a general base or a general acid. The pH profiles for the aminoglycoside- and polyamine-dependent cleavages would be expected to be dominated by the pK_a values of the various amino groups on the ligand. For spermine, the pK_a values of all four amino groups are high (8.6–11.2) (44). The flat pH profile between pH 5 and 8 and the very low activity at pH 9 indicates that there is a requirement for all four positive charges, but it seems very unlikely that any of the amino groups can directly participate either as a general base or a general acid. The pH profiles for the aminoglycoside- and polyamine-dependent cleavages (Fig. 6) reflect the wider spread of pK_a values and the generally better cleavage rates at pH 5 are also consistent with the need for four positive charges. Presumably the poorer rates of cleavage are due to the lower flexibility, compared with spermine, for the amino groups to reach the optimal locations normally occupied by metal ion in the final, active hairpin configuration.

Overall we favour a structural role for the ligand in hairpin ribozyme cleavage where the metal ions, or positively charged amino groups, are involved in a rate-determining structural rearrangement that follows docking and precedes chemical cleavage. Our previous functional group studies of the requirements for hairpin cleavage in loop B (23,24) indicated that there may be a secondary structure in the transition state different from that proposed in the ground state by chemical probing, molecular modelling and other structural data (20,45,46). Another precedent comes from the finding that neomycin B, spermine and cobalt hexammine, but not platinum tetrammine, share common structural motifs which are important in another structural rearrangement, the conversion of B- into A-DNA (47).

**Implications for therapeutic use of hairpin ribozymes**

There have been a number of studies where the hairpin ribozyme has been engineered for vector delivery into mammalian cells (reviewed in 4,5,48). Further, intracellular cleavage has been demonstrated by a hairpin ribozyme construct (49). It has been estimated that the concentration of free magnesium within cells is in the 1 mM range (49 and references therein). Whereas at 5 mM magnesium ions, both spermidine and spermine boost cleavage rates, at lower magnesium ion concentration, we have found that spermine is much more effective. Polyamines are found in all cells and are essential for eukaryotic proliferation. Of the well known polyamines, spermine is the most abundant polyamine in eukaryotes, for example in hepatocytes (50) and in human colon tumour cells (51), and occurs in amounts up to twice as much as spermidine. Although the quantities of free intracellular polyamines are unknown, it seems likely that hairpin ribozyme cleavage inside cells could be enhanced significantly by spermine. This may help to explain why such promising preliminary results have already been obtained in cleavage by the hairpin ribozyme inside mammalian cells and may lead to improved prospects for the use of the hairpin ribozyme as a therapeutic agent. Furthermore, the ability of aminoglycosides and polyamines to promote hairpin ribozyme cleavage adds strength to suggestions that low molecular weight compounds may have played roles as modulators of RNA-catalyzed reactions during evolution (52).

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