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Inhibition of Klenow DNA polymerase and poly(A)-specific ribonuclease by aminoglycosides

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ABSTRACT

Aminoglycosides are known to bind and perturb the function of catalytic RNA. Here we show that they also are potent inhibitors of protein-based catalysis using *Escherichia coli* Klenow polymerase (pol) and mammalian poly(A)-specific ribonuclease (PARN) as model enzymes. The inhibition was pH dependent and released in a competitive manner by Mg^{2+}. Kinetic analysis showed that neomycin B behaved as a mixed noncompetitive inhibitor. Iron-mediated hydroxyl radical cleavage was used to show that neomycin B interfered with metal-ion binding in the active sites of both enzymes. Our analysis suggests a mechanism of inhibition where the aminoglycoside binds in the active site of the enzyme and thereby displaces catalytically important divalent metal ions. The potential causes of aminoglycoside toxicity and the usage of aminoglycosides to probe, characterize, and perturb metalloenzymes are discussed.

Keywords: antibiotics; DNA polymerase; metalloenzymes; mRNA deadenylation

INTRODUCTION

Aminoglycosides are potent naturally occurring bactericidal antibiotics, and, as such, they have been widely used in clinical practice (reviewed in Davies, 1994; Davies & Wright, 1997; Zembower et al., 1998). Aminoglycosides are known to perturb protein synthesis (Moazed & Noller, 1987; Woodcock et al., 1991) by binding the A-site of the ribosome (Fourmy et al., 1996, 1998; Yoshizawa et al., 1998; Brodersen et al., 2000; Carter et al., 2000). Besides, the ribosome aminoglycosides also bind and inhibit various ribozymes (see, e.g., von Ahsen et al., 1991, 1992; Stage et al., 1995; Rogers et al., 1996; Mikkelsen et al., 1999). Several studies have revealed that aminoglycosides frequently interact with asymmetric internal loops of the RNA (Fourmy et al., 1996; Hermann & Westhof, 1998; Yoshizawa et al., 1998; Vicens & Westhof, 2001) and as a general model, it has been proposed that aminoglycosides bind RNA in negatively charged binding pockets (Hermann & Westhof, 1998). It is believed that aminoglycoside binding interferes with RNA function by distorting the structure and/or by displacing functionally important divalent metal ions (Rogers et al., 1996; Hermann & Westhof, 1998; Hoch et al., 1998; Mikkelsen et al., 1999, 2001; Walter et al., 1999, 2002). Displacement of divalent metal ions complexed with RNA by aminoglycosides has recently been established by structural evidence demonstrating that a neomycin B-binding site on yeast $tRNA^{Phe}$ overlaps with a known divalent metal-ion binding site (Mikkelsen et al., 2001).

Divalent metal ions are frequently used by nucleic acid metabolizing enzymes to catalyze phosphodiester bond breakage or formation (reviewed by Steitz & Steitz, 1993; Joyce & Steitz, 1995). The crystal structures of some of these enzymes have been determined and several active sites have been defined both structurally and functionally. In general, these sites are characterized by the presence of carboxylates as exemplified in *Escherichia coli* DNA polymerase I (DNA pol I), where two aspartates are involved in coordinating two catalytically important divalent metal ions in the active site of polymerization. The structure of the 3′ exonuclease domain of *E. coli* DNA pol I has the same general organization, and here three aspartates and one glutamate coordinate the catalytically important divalent metal ions. This site is the paradigm for nucleases belonging to the RNase D family of nucleases (Mian, 1997) of which poly(A)-specific ribonuclease (PARN) is a member (Korner et al., 1998). PARN is a recently discovered eukaryotic poly(A)-specific 3′ exonuclease that efficiently degrades mRNA poly(A) tails (Åström et al., 1991, 1992; Korner & Wahle, 1997; Korner et al., 1998;

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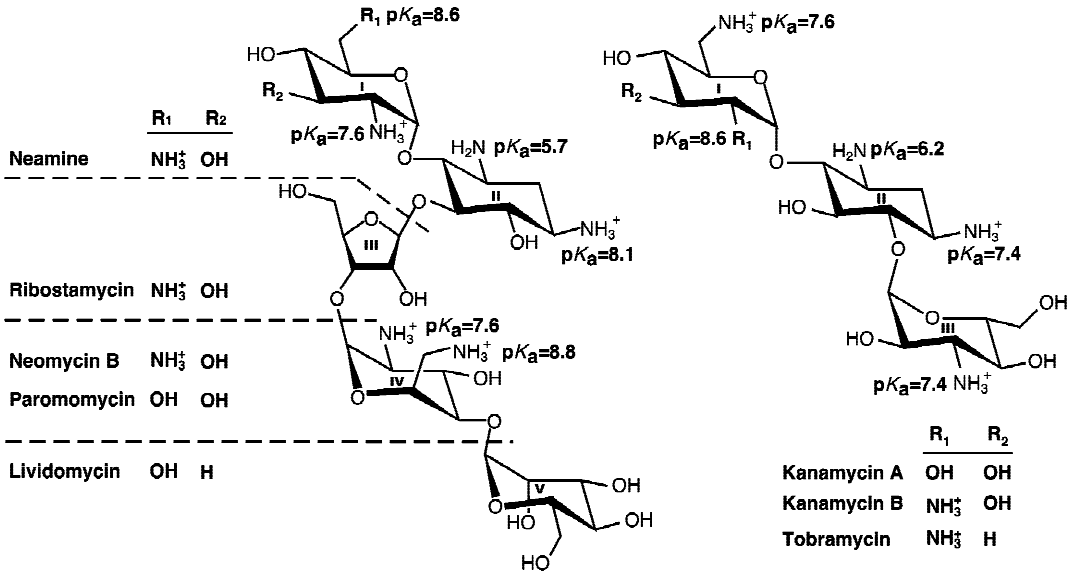

FIGURE 1. Structures of aminoglycosides belonging to the neomycin B and kanamycin B families are shown. Positions where the aminoglycosides differ are indicated with R_1 and R_2. The pK_a values for the different ammonium groups are given in the figure.

Martinez et al., 2000; Copeland & Wormington, 2001) and its active site resembles the 3′ exonuclease active site of *E. coli* DNA pol I (Ren et al., 2002).

Hermann and Westhof (1998) noted that several active sites of nucleic acid metabolizing enzymes resemble the negatively charged binding pockets critical for aminoglycoside binding to RNA. Furthermore, they suggested that the similar arrangements of divalent metal ions in these pockets would form possible electrostatic complementary binding sites for polycationic aminoglycosides. Accordingly, they proposed that aminoglycosides should have the capacity to bind and inhibit such enzymes as well as they bind and inhibit ribozymes (Hermann & Westhof, 1998). We have addressed this issue and investigated if aminoglycosides inhibit the enzymatic activities of *E. coli* Klenow DNA pol and mammalian PARN.

Here we demonstrate that both Klenow pol and PARN activities can be inhibited by several aminoglycosides. We propose that inhibition is caused by displacement of catalytically important divalent metal ions in analogy with inhibition of ribozyme activity by aminoglycosides. We suggest that aminoglycosides are potent inhibitors of enzymes depending on metal ions for their catalytic activity, and, as such, they can be used to probe metal-ion binding sites on enzymes.

RESULTS

Inhibition of Klenow pol and PARN activities by aminoglycosides

Several aminoglycosides (Fig. 1) known to inhibit a variety of ribozymes (see, e.g., von Ahsen et al., 1991, 1992; Stage et al., 1995; Rogers et al., 1996; Mikkelsen et al., 1999) were tested for their ability to inhibit Klenow pol polymerization and PARN exonuclease activities. For the studies of Klenow pol, we used a variant polypeptide in which the 3′ exonuclease active site was inactivated to avoid any interference from this activity (see Materials and Methods). Figure 2A shows typical inhibition curves of Klenow pol and PARN, using neomycin B as the inhibitor. The apparent inhibition constants ($_{app}K_i$) were determined to be in the low millimolar range in the case of Klenow pol and in the high nanomolar range for PARN (Table 1). The $_{app}K_i$ was defined as the concentration of aminoglycoside resulting in 50% inhibition of the enzymatic activity. Similar inhibition

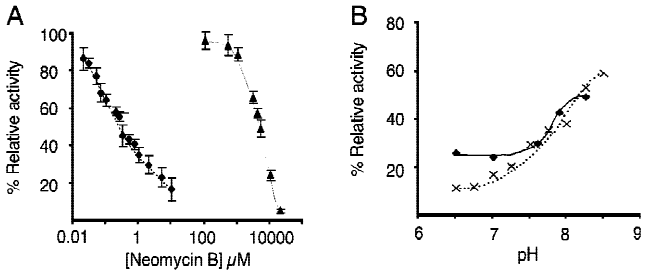

FIGURE 2. Inhibition of Klenow pol and PARN activities by the addition of neomycin B. **A:** Relative activity in percent is plotted as a function of increasing concentrations of neomycin B. Reactions in the presence of Klenow pol (▲) or PARN (♦) were performed as outlined in the text and Materials and Methods. The average activities ± experimental errors are shown for each concentration of neomycin B. **B:** Increasing the pH releases inhibition of Klenow pol and PARN activities by neomycin B. Relative activities for Klenow pol (♦) and PARN (×) in percent are plotted as a function of pH. The neomycin B concentrations were 1 mM (♦) or 10 μM (×). The data points are averages of at least three independent experiments.

TABLE 1. Inhibition of PARN and Klenow pol by aminoglycosides[a]

Aminoglycoside	PARN $_{app}K_i$ (μM)	Klenow pol I $_{app}K_i$ (mM)
Neomycin B	0.4 ± 0.1	4.8 ± 0.3
Paromomycin	17.3 ± 3.5	10.3 ± 0.8
Lividomycin	18.7 ± 2.8	11.6 ± 2.0
Kanamycin B	7.3 ± 0.4	9.2 ± 0.3
Kanamycin A	64.7 ± 7.8	14.3 ± 1.7
Tobramycin	7.1 ± 0.2	9.3 ± 0.7

[a]The listed $_{app}K_i$ values were determined as described in Materials and Methods. The given values are average ± experimental error of several independent experiments.

curves were generated for five additional aminoglycosides and the $_{app}K_i$ values are listed in Table 1. Our data shows that aminoglycosides inhibit both Klenow pol polymerization and PARN exonuclease activities and reveal the importance of the number of amino groups, most obviously by comparing neomycin B with paromomycin and lividomycin or kanamycin A with kanamycin B and tobramycin (Fig. 1; Table 1). In comparison with the aminoglycoside K_i values for ribozymes, the $_{app}K_i$ value for PARN is in the same range, whereas the $_{app}K_i$ value for Klenow pol is significantly higher, approximately one order of magnitude higher than the K_i values for the hairpin ribozyme and eukaryotic RNase P (Tables 1 and 2), although it is in the same range as has previously been reported for inhibition of *E. coli* DNase I and DNA pol I (Tables 1 and 2).

Inhibition requires electrostatic interactions

It has been established that electrostatic interactions are important for inhibition of RNA function by aminoglycosides (von Ahsen et al., 1991, 1992; Zapp et al., 1993; Stage et al., 1995; Werstuck et al., 1996; Wang & Tor, 1997; Tor et al., 1998). To investigate if electrostatic interactions were important for the inhibition of Klenow pol and PARN, we investigated the pH dependence for inhibition because the pK_a values of the ammonium groups (Botto & Coxon, 1983; Szilagyi et al., 1993; Fig. 1) indicate that they are deprotonated at pH values above 7. Figure 2B shows that an increase in pH released the neomycin B inhibitory potential on both enzymes. Similar pH dependence was observed for paromomycin, kanamycin B, and kanamycin A (data not shown). Thus, our data suggests that electrostatic interactions are important for inhibition and that at least some of the ammonium groups have to be protonated.

Neomycin B is a mixed noncompetitive inhibitor

To investigate the mechanism of inhibition more carefully, we performed several sets of kinetic analyses. For Klenow pol we titrated the amount of DNA template or the dCTP nucleotide substrate at various concentrations of neomycin B, and, in the case of PARN, we titrated the polyadenylated RNA substrate at various concentrations of neomycin B. The results were plotted

TABLE 2. Inhibition effects of neomycin B

Inhibited system	Neomycin B (μM)	Reference
Ribozymes[a]		
Group I intron	0.5	von Ahsen et al. (1991)
Sun Y splicing	1.3	von Ahsen et al. (1992)
Hammerhead	13.5	Stage et al. (1995)
HDV self-cleavage RNA	28	Rogers et al. (1996)
Hairpin ribozyme	190	Earnshaw and Gait (1998)
RNase P (M1) RNA	35	Mikkelsen et al. (1999)
Eukaryotic RNase P	143	Tekos et al. (2000)
RNA-protein complexes[b]		
RRE-rev	1	Zapp et al. (1993)
TAR-tat	1	Mei et al. (1997)
Enzymes[c]		
Phospholipase C	3–10	McDonald and Mamrack (1995)
DNase I	$\sim 1 \times 10^3$	Woegerbauer et al. (2000)
DNA pol I	$\sim 5 \times 10^2$	Lazarus and Kitron (1973)
Phenylalanylation of tRNA[d]	300	Mikkelsen et al. (2001)

[a]The given neomycin B values correspond to determined K_i values for the listed ribozymes.
[b]The given neomycin B values correspond to the concentrations of neomycin B that abolish RNA–protein complex formation.
[c]The given neomycin B values correspond to the concentrations of neomycin B that inhibit the enzymatic activity.
[d]Inhibition of phenylalanyl-tRNA-synthetase is caused by neomycin B binding the tRNA substrate (Mikkelsen et al., 2001). It has been shown that tobramycin inhibits tRNA aminoacylation by binding and inducing a conformational change in tRNA (Walter et al., 2002).

using Lineweaver-Burk formalism (Fig. 3A–C). In all cases, the results were compatible with a mixed noncompetitive mechanism of inhibition, indicating that the neomycin B-binding sites do not completely overlap with the binding sites for the substrates. We also investigated if single- or double-stranded DNA of sizes similar to those of the DNA template could release the inhibitory effect of neomycin B on Klenow pol and found no evidence for this (data not shown). These results are not compatible with a model of inhibition based on neomycin B binding to any of the substrates, making them inappropriate as substrates for the enzymes. To determine the K_i values kinetically we replotted the slopes of the lines in Figure 3 as a function of neomycin B concentration (see insets, Fig. 3). From this analysis, the K_i values for neomycin B were determined to be 0.4 μM for PARN and 2.8 mM titrating the DNA template or 3.1 mM titrating the dCTP substrate for Klenow pol, in keeping with the $_{app}K_i$ values listed in Table 1.

Inhibition by aminoglycosides is released by addition of Mg(II) ions

The data presented so far is in agreement with a model of inhibition where aminoglycosides and divalent metal ions compete for overlapping binding sites. This model predicts that an increase in divalent metal ions should release the aminoglycoside inhibition in a competitive manner. To investigate this, we performed deadenylation reactions at various concentrations of neomycin B (0.36, 0.8, 1, or 2 μM). To these reactions, increasing amounts of Mg(II) ions (from 0.1 to 0.4 mM) were added. Figure 3D shows that addition of Mg(II) ions released the aminoglycoside inhibition in a competitive manner, that is, only the K_M parameter and not the V_{max} value was affected. From these plots we also determined the K_i value to 0.4 μM, similar to the estimated $_{app}K_i$ (Table 1) and kinetically determined K_i (Fig. 3A–C) values, suggesting that the K_i value of neomycin B for PARN is independent of the concentration of Mg(II) ions. We also found, in analogy with PARN, that the inhibition of Klenow pol was released by increasing the concentration of either Mg(II) or Mn(II) ions (data not shown). In conclusion, these data suggest that neomycin B and divalent metal ions compete for the same or at least overlapping binding sites.

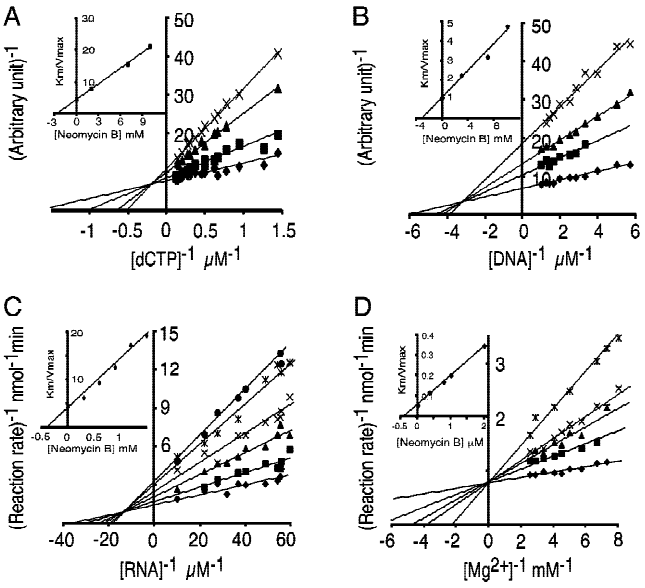

FIGURE 3. Kinetic analysis of inhibition. **A, B, C:** Neomycin B is a mixed noncompetitive inhibitor. Double reciprocal plots 1/v versus 1/[substrate] of indicated substrate for Klenow pol (**A** and **B**) and PARN (**C**) activities in the presence of neomycin B are shown. The neomycin B concentrations were: **A:** (♦) 0 mM, (■) 2 mM, (▲) 7 mM, and (×) 10 mM; **B:** (♦) 0 mM, (■) 3 mM, (▲) 7 mM, and (×) 10 mM; C: (♦) 0 μM, (■) 0.3 μM, (▲) 0.6 μM, (×) 0.9 μM, (∗) 1.2 μM, and (●) 1.5 μM. Insets: The slopes (K_m/V_{max}) of the double reciprocal lines versus the neomycin B concentrations. **D:** Inhibition of PARN activity by neomycin B is released in a competitive manner by Mg(II) ions. Double reciprocal plots, 1/v versus 1/[Mg^{2+}] for PARN activity in the presence of neomycin B (♦) 0 μM, (■) 0.36 μM, (▲) 0.8 μM, (×) 1 μM, and (∗) 2 μM, are shown. Inset: The slopes (K_m/V_{max}) of the double reciprocal lines versus the neomycin B concentrations.

Aminoglycosides perturb iron-mediated cleavage in the active sites of Klenow pol and PARN

The active site for polymerization of Klenow pol has been defined functionally by mutagenesis and structurally by crystallographic studies (reviewed in Joyce & Steitz, 1995; Brautigam & Steitz, 1998; Steitz, 1999) whereas the active site of PARN has been identified by site-directed mutagenesis (Ren et al., 2002). A two-metal-ion mechanism for polymerization has been suggested for Klenow pol and the crystal structure reveals acidic amino acid residues involved in coordinating essential divalent metal ions at the polymerase active site (Brautigam & Steitz, 1998). The active site of PARN resembles the 3′ exonuclease active site of Klenow pol, and the key acidic amino acid residues involved in the coordination of divalent metal ions are structurally conserved between the two sites (Korner et al., 1998; Ren et al., 2002).

To visualize the divalent ions located in the active sites of Klenow pol and PARN experimentally, we performed iron-mediated hydroxyl radical cleavages of the corresponding polypeptides (Fig. 4). Prominent cleavage products, being approximately 50 kDa for Klenow pol and 35 and 48 kDa in sizes for PARN, accumulated over time (Fig. 4B, lanes 10–12). It has been established that the accumulation of the iron-mediated cleavage products of PARN requires the conserved acidic residues in the active site, suggesting that the iron(II) binding sites are located in the vicinity of the PARN active site (Ren et al., 2002). To identify amino acids important for the iron-mediated cleavage of the Klenow pol fragment, we mutated individually each acidic amino acid, involved in coordinating the divalent metal ions of

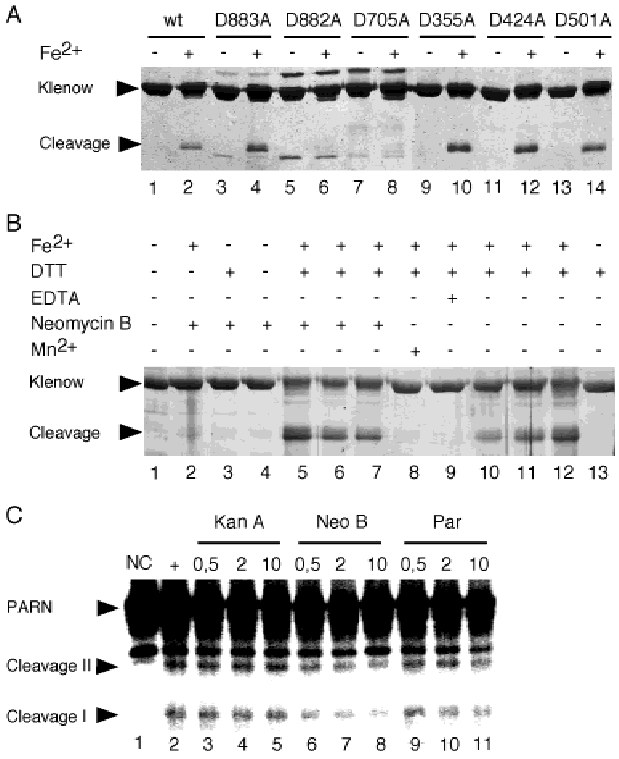

FIGURE 4. Aminoglycosides perturb Fe (II)-mediated cleavages of Klenow pol and PARN. **A:** The acidic amino acid residues D705 and D882 in the active site of the polymerase domain of Klenow pol are required for Fe (II) mediated cleavage. Indicated Klenow pol polypeptide was subjected to Fe(II)-mediated cleavage as outlined in Materials and Methods. The resulting fluorogram is shown. Presence (+) or absence (−) of 20 μM Fe (II) in the cleavage reactions are indicated. The location of Klenow pol and cleavage product is indicated. The polypeptide band migrating slightly faster than the cleavage product is a contaminating polypeptide already present in some of our preparations before Fe(II)-mediated cleavage. **B:** Fe (II)-mediated cleavage reactions of Klenow pol were performed as described in Materials and Methods in the absence (−) or presence (+) of indicated reagent, EDTA (lane 9, 50 μM), Mn^{2+} (lane 8, 10 mM), or Neomycin B (lanes 2–4, 1,000 μM; lane 5, 10 μM, lane 6, 100 μM, and lane 7, 1,000 μM). The incubation times were 5, 10, and 20 min in lanes 10, 11, and 12, respectively. **C:** Fe (II)-mediated cleavage reactions of [^{32}P]-labeled recombinant PARN polypeptides were performed in the presence of neomycin B (Neo B), paromomycin (Par), or kanamycin A (Kan A) as outlined in Materials and Methods. The resulting fluorogram is shown. The concentrations of the aminoglycosides were 0.5 μM (lanes 3, 6, and 9), 2 μM (lanes 4, 7, and 10) and 10 μM (lanes 5, 8, and 10). In lane 1 (NC) PARN was incubated in the absence of aminoglycoside, Fe^{2+}, and DTT. In lane 2 (+) PARN was incubated in the absence of added aminoglycoside but in the presence of Fe^{2+} and DTT.

the polymerase and exonuclease active sites, to alanine residues. Subsequently, each mutated Klenow pol fragment was subjected to iron-mediated hydroxyl radical cleavage. Figure 4A shows that amino acids Asp(705) and Glu(882) located in the active site of polymerization were crucial for generating the 50-kDa cleavage product, suggesting that the iron ions that generate the 50-kDa cleavage product are coordinated at this site of Klenow pol. Finally, we found that the iron-mediated cleavage of Klenow pol fragment was dependent on the presence of Fe(II) ions (Fig. 4B, lanes 10–13) and was abolished by the addition of Mn(II) (Fig. 4B) or Mg(II) (data not shown) ions, suggesting that the Fe(II) ions' binding sites overlapped with the Mn(II) or Mg(II) ions' binding sites.

An important prediction from our aminoglycoside inhibition results (Table 1; Figs. 2 and 3) is that the addition of aminoglycosides should bind and displace the iron ions in the active sites and thereby attenuate the iron-mediated cleavages. To test this, we added increasing amount of aminoglycosides and investigated if this perturbed iron-mediated cleavage. Figure 4B, lanes 5–7, shows that the accumulation of the Klenow pol 50-kDa cleavage product was inhibited by the addition of an increasing amount of neomycin B. In the case of PARN, an increase in the concentration of neomycin B reduced iron-mediated cleavage at both cleavage sites (Fig. 4C, lanes 6–8). In contrast, kanamycin A, a poor inhibitor of PARN, did not significantly affect the iron-mediated cleavage whereas paromomycin, which is an intermediary inhibitor of PARN, reduced iron-mediated cleavage moderately (Fig. 4C, lanes 3–5 and 9–11). In conclusion, our data show that aminoglycosides attenuate iron-mediated cleavage in the active sites of both enzymes. These results are in agreement with a model of inhibition in which aminoglycosides displace functionally important divalent metal ions in the active sites of both enzymes.

DISCUSSION

Mechanism of inhibition

In this report, we demonstrate that aminoglycosides inhibit the catalytic activity of two metalloenzymes, Klenow pol and PARN. We propose that the aminoglycoside binds the enzymes in the vicinity of their active sites and thereby inhibits their catalytic activities. In analogy with the inhibition of ribozyme activity by aminoglycosides (see Rogers et al., 1996; Hermann & Westhof, 1998; Hoch et al., 1998; Walter et al., 1999; Mikkelsen et al., 1999, 2001, and references therein) we suggest that the inhibition is caused by the aminoglycoside distorting the active site and/or displacing functionally important divalent metal ions. The kinetic data showing that neomycin B is a mixed noncompetitive inhibitor of both enzymes (Fig. 3) is compatible with this model of inhibition. Further support is provided by the observation that neomycin B perturbed the iron-mediated hydroxyl radical cleavage reactions in the vicinity of the active sites of both enzymes (Fig. 4). Finally, the kinetic evidence that the neomycin B inhibition of PARN was released in a competitive manner by Mg(II) ions (Fig. 3) is in direct line with this proposal.

It has previously been suggested that neomycin B inhibits DNA polymerase I (Lazarus & Kitron, 1973) and DNase I of *E. coli* (Woegerbauer et al., 2000) by binding to the DNA substrates. However, our results provide no

evidence for this mechanism of inhibition, neither for Klenow pol nor PARN. Most importantly, our kinetic analysis (Fig. 3) revealed that neomycin B was a mixed noncompetitive inhibitor of both Klenow pol and PARN. In these analyses, neomycin B should not have affected the V_{max} parameters if inhibition was due to neomycin B being complexed with any of the substrates. We also found, in the case of Klenow pol, that addition of large amounts of nonsubstrate nucleic acids (i.e., single-stranded DNA or double-stranded DNA with blunt or 5′ protruding ends) did not release the inhibition. Finally, we found that the K_i values for both enzymes were independent of the substrate concentrations (Fig. 3). The large excess of Mg(II) ions compared to the amount of aminoglycoside required to inhibit PARN (e.g., the Mg(II) ion concentration was more than 1,000-fold higher than the K_i value for neomycin B) argues that the chelating properties of aminoglycosides were not causing the inhibition.

Taken together we favor a model of inhibition where the aminoglycoside binds the active site of the enzyme and thereby interferes with its function. Furthermore, it is plausible that the aminoglycoside binding site overlaps with the binding sites for essential divalent metal ions and that aminoglycoside binding displaces functionally important metal ions. This model of inhibition resembles models for inhibition of ribozyme activities by aminoglycosides (see Hermann & Westhof, 1998; Hoch et al., 1998; Mikkelsen et al., 1999, 2001, and references therein) and is analogous to how the amino group of 2′-*O*-aminopropyl substituted RNA directly interferes with divalent metal ion binding at the exonuclease active site of Klenow pol (Teplova et al., 1999).

Aminoglycosides and toxicity

Aminoglycosides are among the most potent and best known antibiotics. However, they are not used against bacterial infections as frequently as they used to be due to a variety of severe side effects (reviewed in Zembower et al., 1998). The mechanisms behind toxicity are not understood and several potential causes have been discussed in the literature, for example, inhibition of phospholipase C (McDonald & Mamrack, 1995), increased formation of free radicals (Schacht, 1999), and inhibition of protein synthesis or other essential ribozyme activities (see Walter et al., 1999, and references therein). The observations that aminoglycosides inhibit Klenow pol and PARN suggest that inhibition of nucleic acid metabolizing enzymes may be a common mechanism by which aminoglycosides cause unwanted side effects. In support of this, we have preliminary evidence that several other nucleic acid metabolizing enzymes, among them *Bam*HI restriction enzyme, HIV reverse transcriptase, bacterial RNase H, Taq DNA polymerase, and T7 RNA polymerase, can be inhibited in vitro by neomycin B (our unpubl. observations). It is interesting to note that phospholipase C is, as are Klenow pol and PARN, a metalloenzyme that breaks phosphodiester bonds (Hansen et al., 1993). This implies that binding to and inhibition of metalloenzymes involved in breaking and forming phosphodiester bonds may be a general feature of aminoglycosides that lies behind many of the different toxic side effects caused by aminoglycosides.

Aminoglycosides as “metal mimics”

We have recently suggested that aminoglycosides can be considered as “metal mimics” (Mikkelsen et al., 2001), because they bind to metal-ion binding sites of RNA molecules and interfere with the function of RNA by displacing functionally/structurally important Me(II) ions. In this study, we show that aminoglycosides also interfere with metal-ion binding sites of protein enzymes, showing that the “metal mimics” property of aminoglycosides is not only restricted to the interaction between aminoglycosides and RNA. Thus, aminoglycosides as “metal mimics” have the potential to be used as functional probes to perturb the catalytic activity of both ribozymes and metalloenzymes, and, as functional/structural probes, to map and characterize the active sites of such catalytic activities. The experiments shown in Figure 4 combined with experiments designed to identify amino acids in the active site by site-directed mutagenesis and map divalent metal-ion binding sites by iron-induced hydroxl radical cleavage (as exemplified in Ren et al., 2002) provide a working platform for how aminoglycosides can be used to functionally/structurally probe the active site of such catalytic activities. Finally we note that aminoglycosides as “metal mimics” can serve as starting compounds for development of novel drugs (e.g., antibiotics) and that aminoglycosides or derivatives thereof can be used as ligands to identify metal-binding proteins in systematic screening efforts using the protein microarray technique recently developed by MacBeath and Schreiber (2000).

MATERIALS AND METHODS

Molecular cloning and expression

A DNA fragment encoding Klenow DNA polymerase was obtained by PCR amplification using *E. coli* genomic DNA (strain MG1655) as template and oligonucleotides 5′-TACATATGA TTTCTTATGACAACTACGTC-3′ and 5′-TGCGGATCCTAG TGCGCCTGATCCCA-3′ as the primers. The obtained DNA fragment was cloned into the pCR 2.1TOPO vector (Invitrogen) and then subcloned into the pET-19 vector (Novagen Inc.) between the *Nde*I and *Bam*HI sites. The obtained plasmid was named pKl. Plasmid pKl was subsequently subjected to site-specific mutagenesis using the QuickChange Site-Directed Mutagenesis Kit (Stratagene) following the instructions from the supplier. The sequences of the resulting mutants [i.e., pKl(D355A, E357A), pKl(D355A, E357A, D424A), pKl(D355A, E357A, D424A), pKl(D355A, E357A, D501A),

pKl(D355A, E357A, D705A), pKl(D355A, E357A, E883A), and pKl(D355A, E357A, D882A)] were verified by DNA sequence analysis. The following primer pairs were used for generation of site-directed mutants:

Kl(D355A, E357A): 5′-CGGTATTTGCATTTGCCACCGCAA CCGACAGCCTTG-3′ and 5′-CAAGGCTGTCGGTTGCG GTGGCAAATGCAAATACCG-3′;
Kl(D424A): 5′-CCAGAATACCGCGTGCGTATTTCAGGTTT TG-3′ and 5′-CAAAACCTGAAATACGCACGCGGTATTC GG-3′;
Kl(D501A): 5′-GTTACGCCGCCGAAGCAGCAGATGTCAC CTTG-3′ and 5′-GGTGACATCTGCTGCTTCGGCGGC GT-3′;
Kl(D705A): 5′-GTGATTGTCTCAGCGGCCTACTCGCAG ATTG-3′ and 5′-GGTGACATCTGCTGCTTCGGCGGC GT-3′;
Kl(E883A): 5′-GATCATGCAGGTACACGATGCACTGGTAT TTGAAGTTC-3′ and GAACTTCAAATACCAGTGCATCG TGTACCTGCATGATC-3′;
Kl(D882A): 5′-GATCATGCAGGTACACGCCGAACTGGTAT TTGAAGTTC-3′ and 5′-GAACTTCAAATACCAGTTCGG CGTGTACCTGCATGATC-3′.

His-tagged Klenow fragment and mutant polypeptides were expressed in *E. coli* strain BL21(DE3)pLysS. The extracted recombinant polypeptides were purified by metal affinity chromatography using the TALON (Clontech Inc.) or Ni- (Novagen Inc.) matrices as outlined by the manufacturers. The amount of protein was measured with a BioRad protein assay kit and the purity was determined by SDS-polyacrylamide gel electrophoresis followed by silver staining.

Assay and quantification of Klenow and PARN activities

To test for Klenow polymerase activity, we used the Klenow(D355A,E357A) recombinant polypeptide, in which the 3′-5′ exonuclease active site was inactivated by site-directed mutagenesis, as the enzyme and a double-stranded DNA fragment, generated by hybridization of oligonucleotides 5′-TCGCAGCCGTGAG-3′ and 5′-ATCCAAGCTCACGGCTG CGA-3′, as the template. The DNA template was prepared as described (Eger & Benkovic, 1992). The reactions were performed in 10 μL in the presence of 100 mM HEPES-KOH, pH 7.0, 1 mM DTT, 0.5 μg/μL BSA, 150 mM KCl, 4 mM $MgCl_2$, 10 mM spermidine, 1 μM DNA template, 0.3 μM (α-^{32}P)dCTP (3,000 Ci/mmol; Amersham Pharmacia Biotech) and contained 0.2–1 nM of indicated Klenow pol fragment. Reactions were incubated at 30 °C for 5–20 min and then stopped by the addition of 10 μL loading buffer (80% formamide (v/v), 0.1% xylene cyanol, 0.1% bromophenol blue, 50 mM Tris-HCl, pH 7.9, 50 mM EDTA). The reacted DNA was fractionated by 10% polyacrylamide (19:1 acrylamide/bisacrylamide 30:0.8)-7 M urea gel electrophoresis. The resulting gel was scanned using a 400 S PhosphorImager (Molecular Dynamics). When dCTP was titrated, the DNA template concentration was 1 μM, whereas the dCTP concentration was 50 μM when the DNA template was titrated. The pH of the reactions was 7.0 if not stated otherwise. The consumption of substrate (dCTP or DNA) was controlled so that not more than 20% of its total amount was consumed.

Homogeneously purified calf thymus PARN 54-kDa active fragment was used as the source of PARN (Martinez et al., 2000). Recombinant full-length human PARN was expressed and purified as described (Martinez et al., 2000). As substrate, we used L3(A_{30}) RNA radioactively labeled with ^{32}P in its poly(A) tail (Åström et al., 1991). Assay conditions and 1-D TLC quantitation procedures were as previously described (Åström et al., 1992). The pH of the deadenylation reactions were 7.0, unless stated otherwise. The consumption of L3(A_{30}) RNA substrate was controlled so that no more than 20% of it was consumed.

Aminoglycosides were purchased from Sigma Chemical Co. and added amount was as indicated. The polymerization and deadenylation efficiencies were plotted as a function of the concentration of the aminoglycoside under study. The $_{app}K_i$ was defined as the concentration of added aminoglycoside resulting in 50% inhibition of Klenow pol or PARN activities. K_i values were obtained by plotting the determined $-(K_M/V_{max})$ values against the concentration of aminoglycoside under study.

Fe(II)-mediated hydroxyl radical cleavage

Two to four micrograms of purified recombinant Klenow pol fragment or mutant thereof, dialyzed against buffer F (20 mM HEPES-KOH, pH 7.0, 5 mM NaCl) on a Milipore "V" Series Membrane (Millipore) for 30 min at 4 °C, were incubated for 20 min at 37 °C in 50 mM HEPES, pH 7.0, 5 mM NaCl, 20 μM $Fe(NH_4)_2SO_4$ and 10 mM DTT in a total volume of 10 μL. The incubations were stopped by the addition of 10 μL of 2× SDS-loading buffer (100 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 200 mM DTT, 16% (v/v) glycerol, 0.05% (w/v) bromophenol blue, and 50 mM EDTA). The amount of added Mg(II)-ions, Mn(II)-ions, or aminoglycoside was as indicated. Reacted polypeptides were fractionated by 10% SDS-polyacrylamide gel electrophoresis and subsequently visualized by silver staining.

Recombinant PARN polypeptides expressed in *E. coli* were purified and radioactively labeled with ^{32}P in their N termini as described (Ren et al., 2002). Fe(II)-mediated hydroxyl radical cleavage of PARN was performed as previously described (Ren et al., 2002). Reacted samples were subjected to 10% SDS-polyacrylamide gel electrophoresis and the resulting gel was subsequently visualized and quantified using a 400 S PhosphorImager (Molecular Dynamics). In the Mg(II) and Mn(II) ions competition experiments, $MgCl_2$ or $MnCl_2$ was added at indicated concentration in the presence of 20 μM $Fe(NH_4)_2SO_4$.

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