Enzyme−Substrate Interactions with an Antibiotic Resistance Enzyme: Aminoglycoside Nucleotidyltransferase(2″)-Ia Characterized by Kinetic and Thermodynamic Methods†

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ABSTRACT: Aminoglycoside nucleotidyltransferase(2″)-Ia is one of the most often detected enzymes in aminoglycoside-resistant bacteria. Despite its prevalence, little biochemical and biophysical work has been reported for this enzyme. In the current study, substrate specificity and temperature dependence of k_{cat} are determined by kinetic assays. Dissociation constants and thermodynamic properties of enzyme−substrate complexes are determined by isothermal titration calorimetry, electron paramagnetic resonance, and fluorescence spectroscopy. Kinetic studies show that aminoglycosides with 2′-NH_2 are better substrates (higher k_{cat}/K_m) than ones with 2′-OH when magnesium(II) is used as the catalytically required divalent cation. The activity is reduced 10-fold for substrates with 2′-NH_2 when manganese(II) replaces magnesium as the required metal. However, kanamycin A, which has a 2′-OH, shows a much smaller decrease in activity when manganese substitutes for magnesium as the divalent cation. Temperature dependence studies show the activation energy of catalysis to be 19.2 kcal/mol and the temperature optimum between 30 and 32 °C. The binding of the aminoglycoside substrate tobramycin to the enzyme occurs with a favorable enthalpy which compensates for a large entropic penalty to yield a negative ΔG value for the complex formation. Enthalpy of binding is less exothermic in the presence of metal−nucleotide. However, due to the more favorable entropy, a more favorable ΔG is observed for the formation of the enzyme−metal−nucleotide:aminoglycoside complex. Tobramycin binds to ANT(2″) with a dissociation constant of 0.6 μM, which is further reduced by 3-fold when metal−nucleotide is present. Binding of ATP to the enzyme is determined to be very weak in the absence of a divalent cation, and becomes 2 orders of magnitude tighter when magnesium or manganese is present. Binding studies also show that, in addition to binding to the enzyme in the form of metal−nucleotide complex, a second catalytically required metal binds to an additional site on the enzyme.

Bacterial resistance to treatment with aminoglycoside antibiotics can result from several factors including drug efflux pumps and modified target ribosomal RNA (*1*, *2*). However the most prevalent cause of resistance to aminoglycosides is the presence of enzymes which covalently modify aminoglycosides (*3*, *4*). These covalent modifications inhibit the ability of the antibiotic to bind the 16S rRNA and interfere with protein synthesis (*5*). Over fifty enzymes have been identified which cause resistance to aminoglycoside antibiotics (*6*). These enzymes include *N*-acetyltransferases, *O*-phosphotransferases, and *O*-nucleotidyltransferases. Each type of modifying enzyme contains several members each of which is specific for modifying primarily at one position on aminoglycoside molecules. Furthermore, each enzyme is capable of modifying several different aminoglycosides and each aminoglycoside antibiotic can be modified by several different resistance enzymes.

One of the most important aminoglycoside modifying enzymes (AGMEs) is aminoglycoside nucleotidyltransferase(2″) (ANT(2″)).[1] This enzyme was first identified in a clinical isolate of *Klebsiella* pneumoniae in 1971 (*7*). By 1985 ANT(2″) was the most often detected aminoglycoside modifying enzyme in resistant clinical isolates in North America and had been found in other continents in patients who had never traveled outside their native country (*8*, *9*). Despite the medical importance of this enzyme, very little biochemical work has been done with this protein. This protein is difficult to isolate in large quantities with a high level of purity (*10*, *11*). Also this enzyme is relatively unstable and has a low degree of solubility. ANT(2″) catalyzes the adenylation of aminoglycosides which contain

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[1] Abbreviations: ANT(2″), aminoglycoside nucleotidyltransferase(2″); EPR, electron paramagnetic resonance; ITC, isothermal titration calorimetry; TCEP, tris(2-carboxyethyl)phosphine; AGME, aminoglycoside-modifying enzyme; TAPS, *N*-tris[hydroxymethyl]methyl-3-aminopropanesulfonic acid; AMPCPP, α,β-methyleneadenosine 5′-triphosphate.

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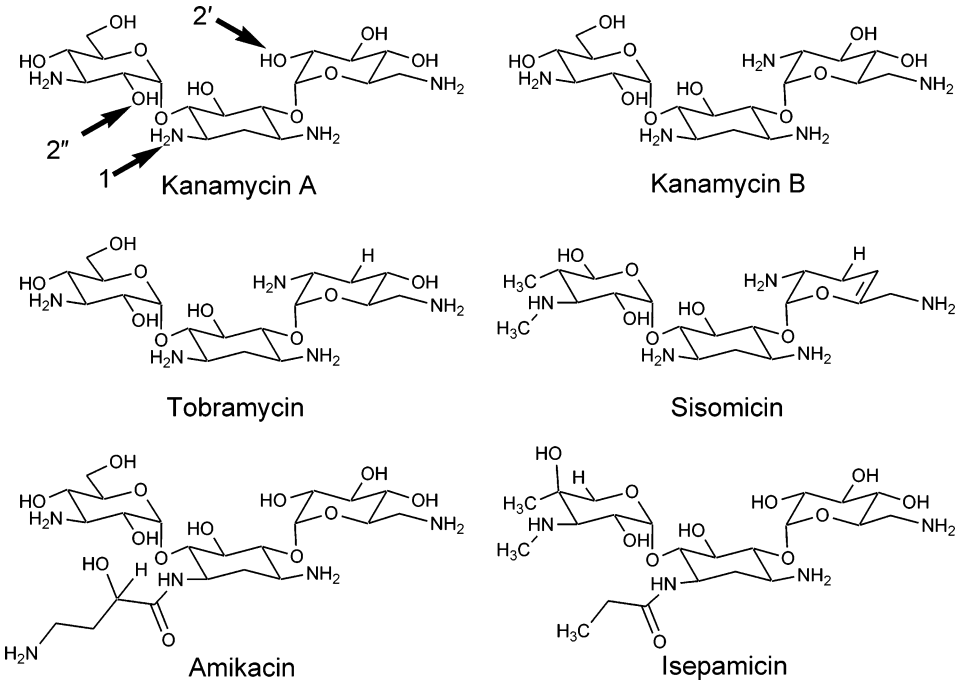


FIGURE 1: The aminoglycosides used in this study. The site of modification (2″) and positions important in enzyme−substrate interactions (1,2′) are indicated on the kanamycin A structure. In all the structures the deoxystreptamine ring (ring B) is in the center with ring C which contains the double-prime (″) positions on the left and ring A which contains the prime (′) positions on the right.

a hydroxyl group at the 2″ position (Figure 1). The reaction is

$$\text{MgATP} + \text{aminoglycoside} \rightleftharpoons \text{AMP-aminoglycoside} + \text{MgPP}_i$$

Early work with ANT(2″) focused on correlation of in vitro substrate specificity determined by kinetic assays with in vivo resistance determined by minimum inhibitory concentration (MIC) testing. Data from these studies were often contradictory, probably due to the use of different assays to monitor activity and the varying levels of purity of enzyme preparations used in the in vitro studies (*10*−*13*). However one observation that was consistent in these studies was substrate inhibition at increasing aminoglycoside concentrations. Later, an extensive kinetic study quantified this substrate inhibition (*14*). This work also showed that the reaction followed an ordered Theorell−Chance mechanism with the metal−nucleotide binding first, followed by the aminoglycoside substrate (*15*). These studies were conducted with a mixture of two enzyme variants, and the amino acid sequence of the enzyme was not known. Even though the primary sequence was not known, an analysis of the amino acid composition was reported (*16*). The enzyme used by Gates and Northrop has a different composition than the one used in the present study. However there is no evidence that the enzyme used in the present study does not follow the mechanism originally proposed by Gates and Northrop.

We have cloned, overexpressed, and purified the aminoglycoside nucleotidyltransferase(2″)-Ia from *Pseudomonas aeruginosa* (*17*). The amino acid sequence of ANT(2″) does not show significant homology to any other aminoglycoside nucleotidyltransferase (less than 5% identity and less than 15% similarity based on BLAST searches and use of CLUSTALW to align the sequences (*18*, *19*)). The acetyl- and phophotransferases are even less similar to ANT(2″). Thus the few well-characterized other AGMEs (*3*, *20*) cannot be reliably used to predict characteristics of this enzyme. Also this enzyme is one of only four of the over fifty known aminoglycoside modifying enzymes to modify primarily at positions on ring C of aminoglycosides (Figure 1) (*6*). Most AGMEs modify positions on the A or B ring.

Because of the prevalence of this unique aminoglycoside-modifying enzyme, an understanding of its interactions with substrates will be a useful tool in the fight against drug resistant bacteria. Here, we describe kinetic and thermodynamic properties of this enzyme. A preliminary account of this work was presented earlier (*21*).

EXPERIMENTAL PROCEDURES

Reagents. All materials were of the highest purity commercially available. All were purchased from Sigma-Aldrich Co. (St. Louis, MO) except for inorganic pyrophosphatase purchased from Roche Diagnostics Corp. (Indianapolis, IN), dithiothreitol (DTT) purchased from Inalco Pharmaceuticals (Milano, Italy), and tris(2-carboxyethyl)phosphine (TCEP) purchased from Fluka (Buchs, Switzerland). The concentrations of adenosine 5′-triphosphate (ATP) and α,β-methyleneadenosine 5′-triphosphate (AMPCPP) were determined by absorbance at 259 nm using an extinction coefficient of 15 400 M^{-1} cm^{-1} (*22*). The concentrations of kanamycin A and amikacin were determined enzymatically using aminoglycoside phosphotransferase(3′)-IIIa (APH(3′)) (*23*). The concentrations of the other aminoglycosides were determined by one-dimensional NMR. The H1′ and H1″ proton peaks of tobramycin, kanamycin B, sisomicin, and isepamicin were integrated relative to the same proton peaks in the kanamycin A and amikacin spectra. Glucose was used as an internal standard to ensure uniform processing of spectra.

Protein Preparation. ANT(2″) was prepared as described previously except that the buffer was exchanged using a Sephadex G-25 column (30 cm × 1.0 cm) (*17*). For all kinetic assays and circular dichroism experiments the final buffer is 50 mM TAPS pH 8.5, 50 mM KCl, 1 mM DTT and 0.1 mM EDTA. For all binding experiments (isothermal titration calorimetry (ITC), electron paramagnetic resonance (EPR), and fluorescence spectroscopy) the final buffer is 50 mM HEPES pH 7.5, 50 mM KCl and 1 mM TCEP.

Kinetic Assays. A discontinuous coupled enzyme assay based on the conversion of one molecule of the reaction product inorganic pyrophosphate to two molecules of inorganic phosphate was utilized to measure the steady-state rate of the reaction catalyzed by ANT(2″) (*17*, *24*). All reaction mixtures contained 50 mM TAPS pH 8.5, 2.0 units of inorganic pyrophosphatase, and a concentration of magnesium acetate to yield 5.0 mM free magnesium at each concentration of ATP used in the assays. These concentrations are based on the dissociation constant of MgATP calculated for the buffer used in the assays (*25*). For the substrate specificity and temperature dependence assays the MgATP concentration was 2.0 mM and each aminoglycoside concentration was varied from 10 to 500 μM. Each assay mixture contained 0.15−0.35 μM ANT(2″). The reaction was started by the addition of aminoglycoside to the reaction mixture. Aliquots were removed at various time points and added to 8% (w/v final) SDS in acetic acid/acetate buffer pH 4.0 to stop the reaction. The amount of inorganic phosphate was determined by previously described methods (*26*, *27*).

For the analysis of the substrate specificity and temperature dependence assays, the data were fit to the following equation (*28*):

$$v = \frac{VA}{K_m + A + A^2/K_i} \quad (1)$$

where v is the measured rate from the kinetic assays, V is the maximal velocity, K_m is the substrate concentration that yields half-maximal velocity, K_i is the inhibition constant, and A is the concentration of the varied substrate. The parameter k_{cat} was calculated by dividing the maximal velocity (V) by the enzyme concentration used in the assays. The assay conditions for the comparison of magnesium and manganese as the divalent cation were identical to the conditions for substrate specificity except for buffer and pH. The magnesium vs manganese assay mixture contained 50 mM HEPES pH 7.5 in place of 50 mM TAPS pH 8.5.

For temperature dependence assays the pH of each stock solution of buffer was adjusted to 8.5 at the temperature of the assay. The reaction mixture except for enzyme and aminoglycoside was incubated at the assay temperature for 10 min and the pH confirmed prior to the start of each assay. For determination of the energy of activation the data were plotted according to the equation

$$\ln k_{cat} = -\frac{E_a}{RT} + \ln A \quad (2)$$

where k_{cat} is the rate constant calculated using eq 1, R is the universal gas constant (1.987 cal mol^{-1} K^{-1}), and T is the temperature in kelvins. The activation enthalpy, activation entropy, and Gibbs free energy of activation were determined according to the equations

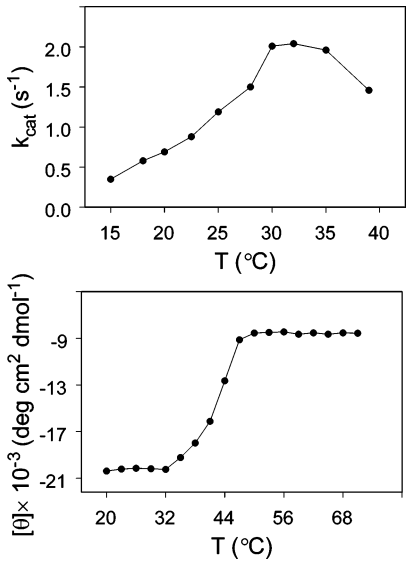


FIGURE 2: The temperature dependence of the reaction catalyzed by ANT(2″) (top panel). The assays were performed at pH 8.5 and contained 2.0 mM MgATP. The free magnesium concentration was 5 mM, and the tobramycin concentration was varied from 10 to 500 μM. The k_{cat} values were calculated using eq 1. The lower panel shows the molar ellipticity as a function of temperature as determined by circular dichroism.

$$\Delta H^{\ddagger} = E_a - RT \quad (3)$$

$$\Delta S^{\ddagger} = R\left(\ln\frac{Ah}{k_B T} - 1\right) \quad (4)$$

$$\Delta G^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger} \quad (5)$$

where k_B is the Boltzmann constant, h is Plank's constant, and A is the preexponential factor determined using eq 2.

Circular Dichroism. Experiments were performed on an Aviv (Lakewood, NJ) model 202 spectrometer with a thermoelectric cell holder. A 2.0 mm path length cuvette containing 5.0 μM enzyme was used for each experiment. The sample chamber was flushed extensively with nitrogen prior to data collection. The CD signal was monitored at 222 nm as the temperature was increased in 3 °C increments. A five minute equilibration time was included at each temperature prior to data collection. The data in Figure 2 represent the average of three separate experiments. Data were collected in the forward direction (increasing temperature) only. A reverse scan could not be performed due to protein precipitation at higher temperatures.

Electron Paramagnetic Resonance. Continuous wave X-band (9.88 GHz) EPR spectra of free Mn^{2+} were recorded using a Bruker (Billerica, MA) EMX spectrometer. All EPR experiments were done at room temperature using a quartz capillary with a volume of 100 μL. Spectra were collected with 20 mW power, 100 kHz modulation frequency, 4.0 G modulation amplitude, 336 s sweep time, and 4 scans. The spectra were baseline corrected and integrated twice using the Win-EPR software from Bruker. A linear relationship between the value for the second integral and the concentration of free manganese was established over the entire range of manganese concentrations used in these experiments. The

dissociation constant for magnesium was determined by competition with manganese as described previously using the equation (*29*)

$$K_d^{Mg} = \frac{[Mg^{2+}]_{1/2}}{1 + \frac{[Mn^{2+}]}{K_d^{Mn}}} \quad (6)$$

where $[Mg^{2+}]_{1/2}$ is the concentration of magnesium required to displace half of the bound manganese.

Isothermal Titration Calorimetry. ITC experiments were performed at 20 °C using a VP_ITC microcalorimeter from Microcal, Inc. (Northampton, MA). Ligand solutions were prepared using the mobile phase from the Sephadex G-25 column used for desalting the enzyme. Both enzyme and ligand solutions were degassed under vacuum for 10 min at 15 °C. Titrations consisted of 29 injections of 10 μL and were separated by 240 s. Cell stirring speed was 300 rpm. Each titration contained 5−10 μM enzyme in the sample cell. For binary enzyme:tobramycin[2] and quaternary enzyme−MgAMPCPP:tobramycin complexes, the tobramycin concentration was 60−120 μM in the injection syringe. For the quaternary titrations the sample and ligand solutions each contained 2.0 mM $MgCl_2$ and 0.8 mM AMPCPP. For the enzyme:MgATP titrations with excess free magnesium, the enzyme and ligand solutions each contained 5.5−6.0 mM $MgCl_2$. The ligand solution contained 0.4−0.8 mM ATP. For the enzyme:MgATP titrations with minimal free magnesium, no magnesium was present in the enzyme solution. The ligand solution contained 0.45 mM $MgCl_2$ and 0.65 mM MgATP. The standard errors represent the deviation including curve fitting errors of the three titrations. Two titrations using AMPCPP instead of ATP in the enzyme:metal−nucleotide titrations were done to ensure that MgAMPCPP binding was similar to MgATP binding to enzyme. Titrations of ATP into the enzyme solution in the absence of Mg^{2+} were performed by using 2−10 mM ATP in the syringe.

All data were fit to the single-site binding model of Origin 5.0 (Microcal, Inc) to determine the binding constant (K_a), enthalpy of binding (ΔH), and stoichiometry (*30*). The free energy (ΔG) and entropy (ΔS) changes associated with binding were determined using the equations

$$\Delta G = -RT \ln K_a \quad (7)$$

$$\Delta G = \Delta H - T\Delta S \quad (8)$$

Fluorescence Spectroscopy. Fluorescence quenching experiments using ATP and MgATP were performed on a Perkin-Elmer (Boston, MA) model LS-5B spectrofluorimeter. For each titration 2−5 μL aliquots from a concentrated stock solution of ATP or MgATP was added to 2.0 mL of 1.0 μM ANT(2″) at room temperature. The excitation wavelength was 295 nm, and the fluorescence emission at 340 nm was measured. Fluorescence intensities were corrected for dilution, scattering, and the inner filter effect. The data were fit to the following equation:

[2] In this manuscript the use of a colon (:) in describing complexes is used exclusively to describe the two components of a titration (sample:titrant).

Table 1: Kinetic Parameters of Selected Aminoglycoside Substrates of ANT(2″)[a]

substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m (M^{-1} s^{-1})	K_i (μM)
tobramycin	2.01 ± 0.14	22.1 ± 3.2	9.10×10^4	74 ± 8.1
kanamycin B	1.77 ± 0.10	20.1 ± 3.4	8.81×10^4	81 ± 14
sisomicin	3.12 ± 0.27	52.6 ± 5.8	5.93×10^4	226 ± 36
kanamycin A	1.96 ± 0.19	49.9 ± 6.3	3.93×10^4	443 ± 81
amikacin	0.17 ± 0.03	84.1 ± 9.2	2.02×10^3	nd[b]
isepamicin	0.19 ± 0.04	163.6 ± 44	1.16×10^3	nd[b]

[a] Data represent average of three separate determinations. [b] Substrate inhibition not detected at substrate concentrations up to 0.5 mM.

$$\left(\frac{\Delta F}{F_0} \times 100\right) = \frac{\left(\frac{\Delta F_{max}}{F_0} \times 100\right)[S]}{K_d + [S]} \quad (9)$$

where $(\Delta F/F_0 \times 100)$ is the percent change in fluorescence resulting from the addition of substrate at a concentration [S].

RESULTS

Substrate Specificity of ANT(2″). Although several studies of the substrate profile of ANT(2″) have been undertaken, many used only partially purified enzyme (*10*). Some used enzyme assays which were linear only at very low substrate concentrations and required distant extrapolation to estimate kinetic values (*13*). In the most comprehensive study of the kinetics of aminoglycoside nucleotidyltransferase (2″) to date, two electrophoretic variants of ANT(2″) were used in the assays (*15*). It is not known whether the two species were due to two different start sites, a covalent modification of one of the species, or some other reason (*16*). In the present study the nature of the ANT(2″) utilized is well-defined. The amino acid sequence is known from DNA sequencing. We used the 226 amino acid version of the enzyme, which has been shown to have higher specific activity than ANT(2″) translated from other start sites (*17*).

Of the aminoglycoside substrates used in this study, tobramycin and kanamycin B had the highest k_{cat}/K_m values (Table 1). The fact that these two substrates had similar kinetic properties is not surprising since these molecules differ only at the 3′ position (Figure 1). Kanamycin A, however, differs from kanamycin B only at the 2′ position, yet the K_m for kanamycin A is 2-fold greater than that observed for kanamycin B. These data illustrate the importance of the 2′ position in aminoglycoside binding to enzyme. Also, amikacin and isepamicin had approximately 2 orders of magnitude lower k_{cat}/K_m when compared to kanamycin B and tobramycin, which may explain why ANT(2″) does not confer resistance to these antibiotics.

Similar to observations made with some of the other AGMEs, substrate inhibition is observed with ANT(2″). The inhibition constant, K_i, shows substrate dependence similar to that observed with K_m values even though these two parameters, K_i and K_m, reflect interactions with different forms of the enzyme. The K_i of kanamycin A is over 4-fold higher than that of kanamycin B. The difference in K_i values between kanamycin A and kanamycin B also confirms the importance of the substituent at the 2′ position in aminoglycoside−enzyme interactions (Figure 1). Tobramycin, which has an amino group at the 2′ position, also follows the same

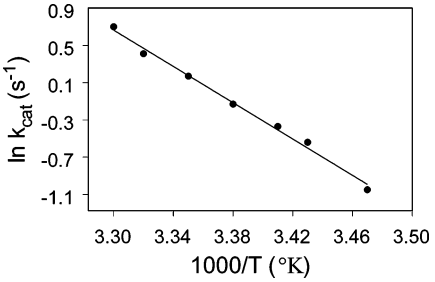


FIGURE 3: An Arrhenius plot constructed from the data for temperatures 15 to 30 °C in Figure 2. The slope of the linear fit of the data was used to calculate the activation energy.

pattern and has a K_i value very similar to that of kanamycin B. K_i values for amikacin and isepamicin could not be determined because no inhibition was observed even at high concentrations (0.5 mM) of substrates. This is consistent with high K_m values observed for these two aminoglycosides.

The substrate with the highest maximal rate per unit enzyme (k_{cat}) is sisomicin. This aminoglycoside contains an unsaturation at position 4′ rather than a hydroxyl. Otherwise the A and B rings are identical to tobramycin (Figure 1). The k_{cat} values for tobramycin, kanamycin A, and kanamycin B were very similar. Both amikacin and isepamicin are very poor substrates for ANT(2″), showing that the alkyl group attached to the 1-NH_2 position of the deoxystreptamine ring in these two aminoglycosides interferes with binding and activity.

Temperature Dependence of Catalysis by ANT(2″). The temperature dependence of the rate of the reaction catalyzed by ANT(2″) was determined using tobramycin as the aminoglycoside substrate. The rate increased steadily between temperatures 15 °C and 30 °C, reached a plateau around 32 °C, and started to decline steeply above 35 °C (Figure 2). The optimum temperature for activity of ANT(2″) is 30−32 °C. This optimum is unusual since this enzyme is present in resistant bacteria within the human body. From the circular dichroism data, shown in Figure 2, it is clear that the enzyme begins to lose secondary structure above 32 °C. This could be because the buffer used in these assays does not adequately mimic conditions inside the cell. The buffer system was chosen because it resulted in the highest enzymatic activity. Increasing salt concentration had no effect on temperature dependence. Only the use of at least 5% glycerol or ethylene glycol increased the temperature optimum to 37−40 °C (data not shown).

Data obtained from the temperature dependence of activity measurements were used to construct an Arrhenius plot, which yielded a straight line for temperatures between 15 °C and 30 °C (Figure 3). An activation energy of 19.2 kcal/mol was determined from the Arrhenius plot of the data. The activation enthalpy ($\Delta H^{\ddagger}$) is 18.6 kcal/mol, the activation entropy ($\Delta S^{\ddagger}$) is 3.9 cal/(mol K), and the Gibbs free energy of activation ($\Delta G^{\ddagger}$) is 17.4 kcal/mol at 25 °C. These results show that the reaction barrier is primarily enthalpic for the reaction catalyzed by ANT(2″).

Manganese Alters Substrate Specificity. Although magnesium is the preferred divalent cation required for ANT(2″) activity, a paramagnetic metal was needed to study metal binding to ANT(2″) by EPR. Therefore the effect of Mn^{2+} on the catalytic activity of the enzyme was studied. Interestingly, the effect of the substitution of MnATP for MgATP was dependent on the aminoglycoside substrate (Figure 4). Catalytic activity of the enzyme was only 7−12% of what was observed with MgATP when tobramycin, kanamycin B, or sisomicin was used as the aminoglycoside substrate with MnATP. In contrast to this, the activity of enzyme was greater than 70% with MnATP as compared to the activity observed with MgATP when kanamycin A was used as substrate (Figure 4A,B). All four aminoglycosides tested showed a similar decrease in K_m resulting in the k_{cat}/K_m profile seen in panel D of Figure 4. Thus, it appears that kanamycin A is a poorer substrate than tobramycin, kanamycin B, or sisomicin when MgATP is the metal−nucleotide substrate but becomes a better substrate than the other

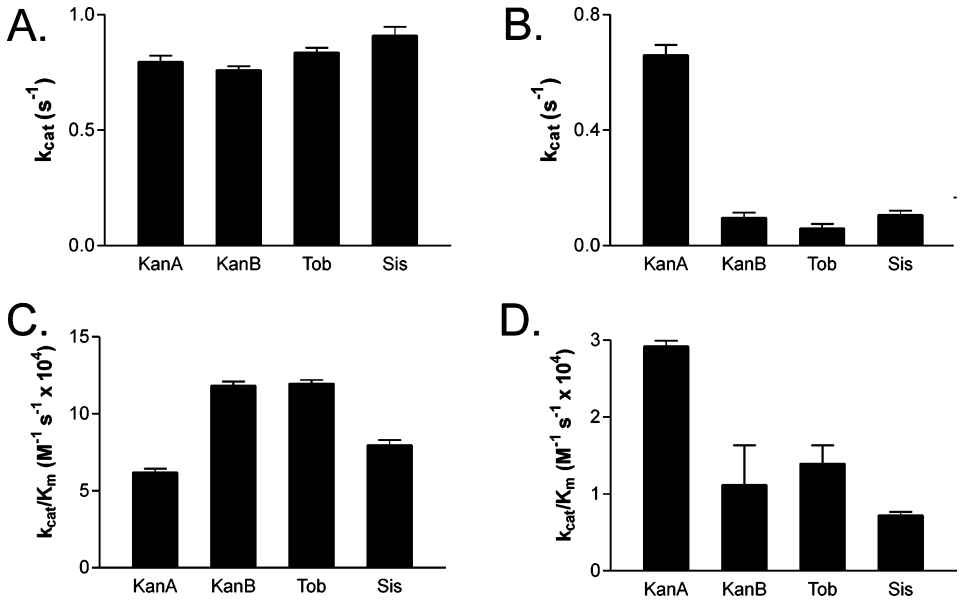


FIGURE 4: The substitution of manganese for magnesium as the required divalent cation alters the substrate specificity of ANT(2″). Panels A and C show the kinetic data for magnesium as the required metal. Panels B and D show the kinetic data for manganese as the required metal. The assays were performed at pH 7.5. Each assay mixture contained 2.0 mM metal−nucleotide and 5.0 mM free divalent cation. The aminoglycoside concentration was varied from 10 to 500 μM and the data fit to eq 1.

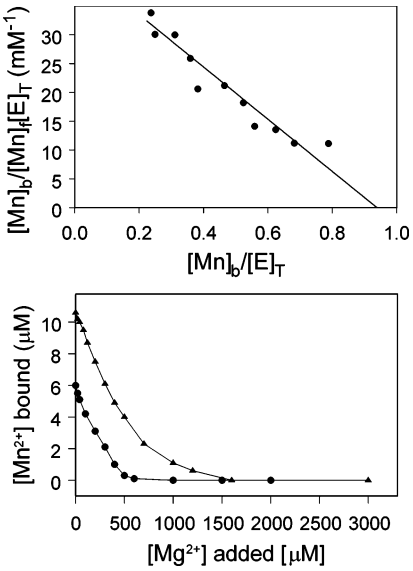


FIGURE 5: Scatchard analysis shows that one manganese ion binds to the enzyme with a dissociation constant of 23 ± 3 μM (top panel). The displacement of manganese by magnesium in solutions containing 20 μM (●) and 50 μM (▲) manganese(II) and 15 μM enzyme (bottom panel). This data is used to determine the binding affinity of magnesium(II) to enzyme.

aminoglycosides when MnATP is the metal−nucleotide substrate. This phenomenon of altered substrate specificity in the presence of manganese versus magnesium has been observed previously in enzymes such as HIV-1 integrase and RNA dependent RNA polymerase (*31*, *32*). For ANT(2″) the reason for this phenomenon is not apparent. It could be due to the metal−nucleotide complex or the free divalent cation that binds to the enzyme (see next section). In either case, one difference between kanamycin A and the three other aminoglycosides studied with manganese is that kanamycin A is the only aminoglycoside with a hydroxyl substituent at the 2′ position while tobramycin, kanamycin B, and sisomicin have an amine at that position.

Metal Binding to ANT(2″). The use of EPR as a tool to determine metal−protein binding affinities is well-established (*33*). The binding of manganese to enzyme broadens the EPR signal to undetectable levels. Thus, the decrease in signal can be quantified to determine the amount of free and bound Mn^{2+} in solutions containing enzyme and Mn^{2+}.

Solutions with ANT(2″) and buffer alone containing matched concentrations of Mn^{2+} were used to determine free and bound Mn^{2+} by EPR. Several different ratios of manganese to enzyme were utilized. Results are shown in Figure 6 in the form of a Scatchard plot. As shown in Figure 5, Mn^{2+} binds to 0.94 ± 0.09 sites on this enzyme with a dissociation constant of 23 ± 3 μM. Addition of Mg^{2+} to a solution containing enzyme−Mn^{2+} complex displaced the enzyme-bound Mn^{2+}, and a complete displacement of Mn^{2+} occurred at higher concentration of Mg^{2+}. A dissociation constant of 111 ± 24 μM was then determined for the enzyme−Mg^{2+} complex by the titration of enzyme−Mn^{2+} complex with Mg^{2+} (Figure 5, lower panel). Although magnesium and manganese are both divalent cations of similar size, manganese binds to the enzyme four to five times more tightly than magnesium. The tighter binding by manganese to enzyme has been observed in several other

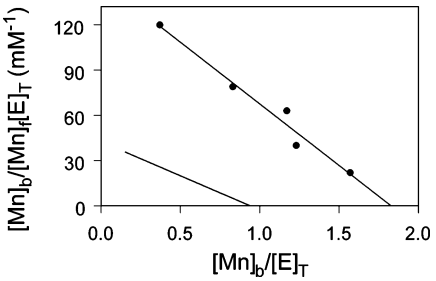


FIGURE 6: ATP changes the stoichiometry from 0.94 ± 0.09 to 1.8 ± 0.3 divalent cations per molecule of enzyme. The linear curve fit of binding in the absence of nucleotide (from Figure 5, top) is shown for comparative purposes.

proteins including glutamine synthetase and T7 RNA polymerase (*34*, *35*).

When Mn^{2+} binding experiments were performed in the presence of ATP, the stoichiometry of bound manganese(II) to the enzyme was increased to 1.8 ± 0.3 (Figure 6). This result suggests that one manganese ion is capable of binding to the enzyme independent of the nucleotide and one binds as MnATP. In these titrations, a further decrease of the free manganese signal was observed after the addition of Mn^{2+} sufficient to completely saturate the ATP. This decrease shows that a divalent cation other than the one present in the metal−nucleotide complex binds to enzyme. Additional EPR experiments confirmed that manganese binds ATP with a one-to-one stoichiometry with a dissociation constant of 8 ± 3 μM. This is similar to values found earlier for the dissociation constant of MnATP (*36*). Scatchard analysis could not distinguish between the two binding events because the dissociation constants for the two binding events are similar to each other (8 μM vs 23 μM). Kinetic data also support these observations; the amount of magnesium required for optimal activity is much greater than the amount required to saturate ATP (*10*). The requirement of two metals for activity is not unusual in ATP utilizing enzymes. The presence of two metal ions in the active site is observed in the aminoglycoside-modifying enzyme APH(3′) (*37*). Also, several other nucleotidyl transferring enzymes such as DNA and RNA polymerases require two metals for activity (*38*−*41*). On the other hand, only one metal per monomeric unit is observed in the crystal structure of the other clinically relevant nucleotidyltransferase, aminoglycoside nucleotidyltransferase(4′,4″) (*42*). Thus, in the absence of structural data, the existence of an additional metal binding site away from the active site, though less likely, cannot be excluded.

Thermodynamic Properties of Enzyme−Substrate Complexes. The binding of substrates to ANT(2″) was studied by ITC. Titrations of MgATP to the enzyme were performed under two different sets of conditions: in the presence and absence of excess Mg^{2+}. These conditions were selected on the basis of the change in stoichiometry of Mn^{2+} binding to the enzyme in the presence of ATP (Figures 5 and 6). In the presence of 5 mM free magnesium the dissociation constant for the enzyme:MgATP was 12 μM (Figure 7). In the absence of excess Mg^{2+}, the affinity of MgATP to the enzyme decreased slightly, indicating that the second metal ion does not play a significant role in metal−nucleotide binding to enzyme (Table 2). In both cases the stoichiometry of binding was 1:1 MgATP:ANT(2″) and enthalpic and entropic contributions to binding were favorable. Interest-

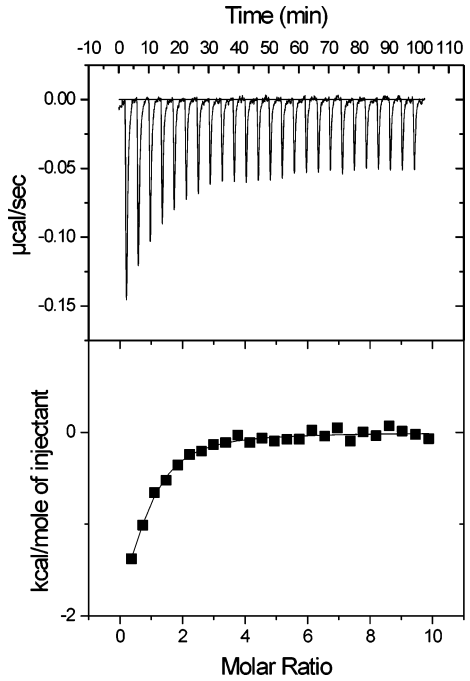


FIGURE 7: The binding isotherm from the titration of MgATP to enzyme in the presence of excess magnesium. Reproducible results were obtained with only 5−10 μM enzyme in the sample cell to obtain thermodynamic parameters for this interaction.

Table 2: Thermodynamic Parameters of Aminoglycoside and Metal−Nucleotide Binding to ANT(2″) Determined by ITC

	K_D (μM)	ΔH (kcal/mol)	$T\Delta S$ (kcal/(mol K))	ΔG (kcal/mol)
E:MgAMPCPP:Tob	0.21 ± 0.09	−12.6 ± 0.3	−3.6	−9.0
E:Tob	0.64 ± 0.17	−18.2 ± 0.4	−10.0	−8.2
E:MgATP[a]	12.1 ± 3.6	−2.4 ± 1.2	4.5	−6.9
E:MgATP[b]	18.4 ± 5.0	−3.8 ± 1.7	2.8	−6.6
E:ATP[c]	nd	nd	nd	nd

[a] Contained 5 mM excess Mg^{2+}. [b] Contained minimal free Mg^{2+} (less than 25 μM). [c] No heat change was observed using 2−10 mM ATP titrated into 10 μM enzyme in the absence of Mg^{2+} at 20 °C.

ingly, titration of enzyme with ATP in the absence of any metal ions did not show an observable heat change signal. The possible explanations are that free ATP binds very weakly or not at all to this enzyme or coincidentally no net heat change is observable at this temperature. Fluorescence experiments were performed to differentiate between these two possibilities. ANT(2″) has eight tryptophan residues. The change in tryptophan fluorescence as a result of adding ATP or MgATP to solutions containing the enzyme showed that ATP binds much more weakly than MgATP (Figure 8). The dissociation constant of enzyme:ATP complex, determined by fluorescence spectroscopy, was 530 μM. In titrations containing Mg^{2+}, the enzyme solution contained matching concentration (5 mM) of free Mg^{2+} to the free Mg^{2+} in MgATP solution. Thus, the change in fluorescence could be attributed to the metal−nucleotide binding and not binding of free magnesium to the enzyme. A dissociation constant of 15 ± 3 μM was determined for MgATP which agrees well with the dissociation constant determined by ITC for the same complex.

Binding of the aminoglycoside substrate to the enzyme was investigated using tobramycin as the substrate. Tobramycin was chosen for these studies since it had the lowest

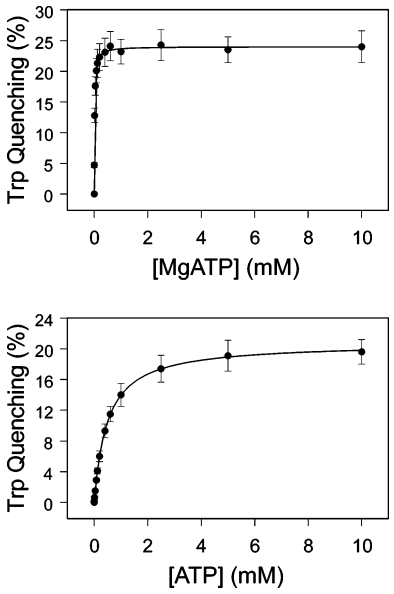


FIGURE 8: The change in intrinsic tryptophan fluorescence of ANT(2″) as a result of nucleotide binding shows that MgATP (top) has a much higher affinity for enzyme than free ATP (bottom).

K_m and K_i values of the aminoglycoside substrates tested kinetically (Table 1). As shown in Figure 9, binding of tobramycin to ANT(2″) is an exothermic reaction. However, unlike metal−ATP, binding of tobramycin to the enzyme is entropically disfavored which was compensated by a large enthalpic contribution yielding a favorable ΔG for the complex formation. Stoichiometry of the binding was 1:1 tobramycin:enzyme, and a dissociation constant of 0.64 μM was determined for the enzyme:tobramycin complex. This value is 2 orders of magnitude lower than the kinetically determined K_i value. The dissociation constant was reduced 3-fold in the presence of saturating metal−nucleotide in the form of the nonhydrolyzable ATP analogue AMPCPP and excess magnesium. Although the enthalpy was less negative in the quaternary complex, the entropic penalty ($T\Delta S$) was much lower compared to the binary enzyme−tobramycin complex (Table 2).

DISCUSSION

This study reports the first kinetic comparison of aminoglycoside substrates that uses a single isoform of the enzyme with a known amino acid sequence. This work confirms the importance of the substituent at the 2′ position in aminoglycoside binding to a 2″-nucleotidyltransferase which was first described by Gates and Northrop (*14*). The importance of this position has also been reported for other aminoglycoside-modifying enzymes (*23*, *43*, *44*). However in these enzymes the K_m and k_{cat} are both decreased for aminoglycosides containing a hydroxyl rather than an amine at the 2′ position with little effect on k_{cat}/K_m. For ANT(2″) K_m is increased while k_{cat} is unaffected by the replacement of a hydroxyl with an amine at this position which results in a much lower k_{cat}/K_m for aminoglycosides such as kanamycin A (2′-OH) compared to tobramycin or kanamycin B (2′-NH_2). The importance of position one of the deoxystreptamine ring in aminoglycoside binding to ANT(2″) is also apparent from these results since isepamicin and amikacin are very poor substrates for this enzyme. These data illustrate why ANT(2″) does not confer resistance against these two aminoglycosides

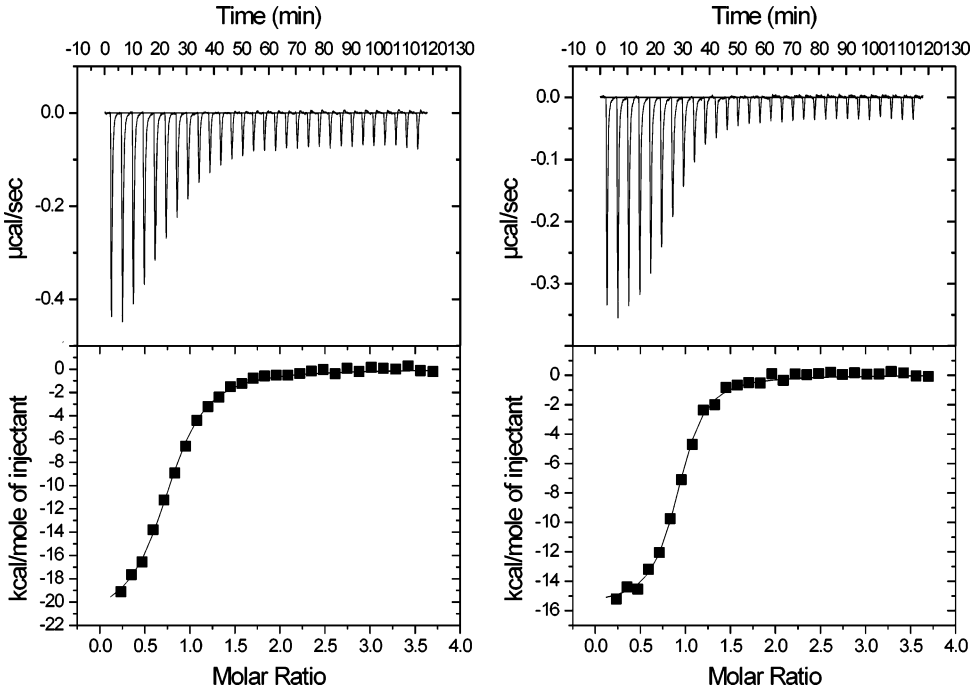
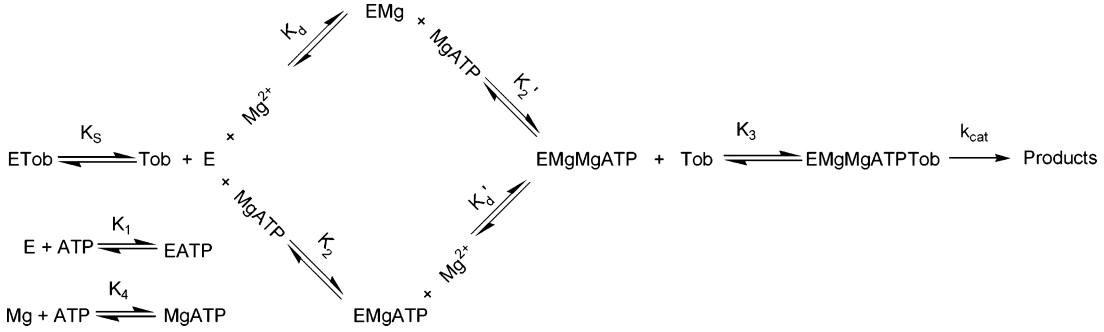


FIGURE 9: The binding of the tobramycin to ANT(2″) in the absence (left) and presence (right) of MgAMPCPP. The presence of metal−nucleotide increases the affinity of aminoglycoside to enzyme.

Scheme 1



even though they each contain a hydroxyl at the 2″ position. These semisynthetic aminoglycosides are also poor substrates for other aminoglycoside-modifying enzymes (*6*, *45*). Even though ANT(2″) is unusual in that it does not share a significant degree of homology with other aminoglycoside-modifying enzymes and modifies on the double-prime ring, it is not surprising that the same groups are important for aminoglycoside binding to the enzyme. The A and B rings of aminoglycoside antibiotics adopt similar conformations when bound to aminoglycoside-modifying enzymes (*46*−*48*). This similarity occurs not only within all three classes of aminoglycoside-modifying enzymes but also in enzymes that modify on each of the three different rings (*49*). These similarities prompted the hypothesis that these two rings form the basic structural unit recognized by many AGMEs (*46*). Although these similarities may seem to provide a rationale for the design of aminoglycoside antibiotics less susceptible to resistance enzymes, these two positions are also important for aminoglycoside binding to RNA (*50*). Therefore altering these positions on the drug will likely reduce its potency, which has been observed with deaminated analogues of kanamycin A and neamine (*51*). Also the increased usage of amikacin has led to the emergence of resistance enzymes which efficiently modify this aminoglycoside (*52*, *53*).

Table 3: Dissociation Constants of Complexes Shown in Scheme 1

param	definition	K_D (μM)
K_d	(E)(Mg^{2+})/(EMg)	111 ± 24^b
K_d'	(EMgATP)(Mg^{2+})/(EMgMgATP)	38 ± 9^c
K_2	(E)(MgATP)/(EMgATP)	18.4 ± 5.0^d
K_2'	(EMg)(MgATP)/(EMgMgATP)	12.6 ± 3.6^d
K_3	(EMgMgAMPCPP)(Tob)/(EMgMgAMPCPPTob)[a]	0.21 ± 0.08^d
K_S	(E)(Tob)/(ETob)	0.64 ± 0.17^d
K_1	(E)(ATP)/(EATP)	530 ± 40^e

[a] The ATP analogue AMPCPP was used to estimate K_3. [b] Determined by EPR. [c] Calculated from the relation $K_2K_d' = K_4K_d$. [d] Determined by ITC. [e] Determined by fluorescence.

This study also measured the binding of substrates and cofactors to ANT(2″). The binding events associated with the formation of the catalytically competent complex are summarized in Scheme 1 and Table 3. Both fluorescence and ITC experiments show that metal−nucleotide binds much more tightly than free nucleotide. This result supports kinetic data which predicted MgATP and not free ATP as the true substrate. The presence of magnesium or manganese is required for ATP to bind to the enzyme with high affinity. Binding studies performed by EPR reveal a separate divalent cation binding site on the enzyme in addition to the metal−

ATP binding site. The affinity for the second metal binding is increased by the presence of metal−ATP. This second binding site is likely to be near the catalytic site because kinetic studies indicated that the full activity of the enzyme requires the presence of additional free divalent cation in addition to metal−ATP. It is possible that the two divalent cations share aspartate ligands on the protein. This feature has been observed in several members of the nucleotidyltransferase superfamily (*38*−*41*, *54*, *55*). Both divalent cations are thought to stabilize the transition state in this mechanism. One metal facilitates the nucleophilic attack while the other helps the exit of the pyrophosphate (*40*, *56*).

The binding of metal−nucleotide also makes the binding of aminoglycoside more favorable. The presence of MgAMP-CPP reduces the enthalpic contribution; however, the entropic contribution becomes more favorable and yields a slightly more favorable ΔG for the enzyme−MgAMPCPP:tobramycin complex compared to the binary enzyme:tobramycin complex. The binding of tobramycin to ANT(2″) in the absence of metal−nucleotide is driven by favorable enthalpic contribution which compensates the large entropic penalty and yields a favorable ΔG for the complex formation. This is similar to what is observed with tobramycin binding to the aminoglycoside phosphotransferase (3′)-IIIa (*57*). The largely exothermic nature of the interaction of aminoglycoside with ANT(2″) explains the large activation enthalpy associated with this reaction since the release of adenylated aminoglycoside is thought to be the rate determining step of this reaction (*58*). Finally, the tight binding of tobramycin to enzyme in the absence of metal−nucleotide may appear to contradict earlier work in which the origin of substrate inhibition was determined to arise from aminoglycoside binding to enzyme complexed with adenylated aminoglycoside and inhibiting product release (*15*). The results of the two studies cannot be directly compared because the earlier kinetic assays were done at pH 9.1 while the binding experiments in the present work were done at pH 7.5.

Even though the aminoglycoside binding is much tighter than MgATP binding, the ordered sequential mechanism is still efficient at modifying aminoglycosides and providing resistance to the bacteria. In the cell the concentration of MgATP and Mg^{2+} is much higher than aminoglycoside concentration. Initially aminoglycoside concentration within the cell is very low. Only after the initial effects on protein translation lead to an increase in permeability of the membrane do large amounts of aminoglycoside enter the cell (*59*, *60*). Therefore, in the presence of modifying enzymes the amount of aminoglycoside remains low. These properties also explain why resistance in vivo for ANT(2″) as well as other aminoglycoside-modifying enzymes correlates better with k_{cat}/K_m than k_{cat} (*61*, *62*).

Detailed structural information from X-ray crystallography or NMR will be difficult to obtain due to the instability of this enzyme and its tendency to form oligomers at concentrations above 40 μM. Also, structural predictions based on amino acid sequence homology are not possible with this enzyme. Therefore the use of biophysical techniques to study ANT(2″) is currently the best strategy to gain insight into this important antibiotic resistance conferring enzyme. Also, comparison of the attributes of this enzyme with the better characterized aminoglycoside-modifying enzymes can provide strategies to design inhibitors of aminoglycoside-modifying enzymes and possibly antibiotics which are not susceptible to resistance enzymes.

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