

# Molecular Determinants of Affinity for Aminoglycoside Binding to the Aminoglycoside Nucleotidyltransferase(2'')-Ia<sup>†</sup>

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Received May 11, 2006; Revised Manuscript Received June 21, 2006

**ABSTRACT:** One of the most commonly occurring aminoglycoside resistance enzymes is aminoglycoside 2''-O-nucleotidyltransferase [ANT(2'')]. In the present study molecular determinants of affinity and specificity for aminoglycoside binding to this enzyme are investigated using isothermal titration calorimetry (ITC). Binding of aminoglycosides is enthalpically driven accompanied by negative entropy changes. The presence of metal–nucleotide increases the affinity for all but one of the aminoglycosides studied but has no effect on specificity. The substituents at positions 1, 2', and 6' are important determinants of substrate specificity. An amino group at these positions leads to greater affinity. No correlation is observed between the change in affinity and enthalpy. At the 2' position greater affinity results from a more negative enthalpy for an aminoglycoside containing an amino rather than a hydroxyl at that position. At the 6' position the greater affinity for an aminoglycoside containing an amino substituent results from a less unfavorable entropic contribution. The thermodynamic basis for the change in affinity at position 1 could not be determined because of the weak binding of one of the aminoglycoside substrates, amikacin. The effect of increasing osmotic stress on affinity was used to determine that a net release of approximately four water molecules occurs when tobramycin binds to ANT(2''). No measurable net change in the number of bound water molecules is observed when neomycin binds the enzyme. Data acquired in this work provide the rationale for the ability of ANT(2'') to confer resistance against kanamycins but not neomycins.

The increasing incidence of bacteria resistant to treatment with antibiotics is a major concern for the medical community. One class of antibiotics particularly susceptible to resistance is the aminoglycoside antibiotics. This group of antibiotics is important because they are often used to treat hospital-acquired infections caused by Gram-negative bacteria. Resistance to aminoglycosides may be caused by a number of mechanisms including altered target RNA, decreased membrane permeability, or drug efflux pumps (1, 2). The most prevalent cause of resistance, however, is covalent modification of aminoglycosides by aminoglycoside-modifying enzymes (AGMEs)<sup>1</sup> (3). Three classes of AGMEs exist. These include *N*-acetyltransferases which catalyze the acetyl-CoA-dependent acetylation of an amino group on an aminoglycoside, *O*-phosphotransferases which catalyze the ATP-dependent phosphorylation of an aminoglycoside, and *O*-nucleotidyltransferases which catalyze the ATP-dependent adenylation of an aminoglycoside. Each type of AGME has several members regiospecific primarily for one position on aminoglycoside compounds. Most of these enzymes are capable of modifying several different aminoglycosides. Also, since several different enzymes exist

which can modify at different positions, each aminoglycoside is capable of being modified by several different enzymes (4).

One of the most prevalent of these enzymes is aminoglycoside nucleotidyltransferase(2'')-Ia [ANT(2'')]. ANT(2'') catalyzes the direct nucleophilic attack on the  $\alpha$ -phosphate of ATP producing AMP-aminoglycoside (5). This enzyme provides resistance against tobramycin, kanamycin, gentamicin, and other aminoglycoside antibiotics. ANT(2'') is one of the most frequently occurring AGMEs. This enzyme has been detected in a large percentage of resistant Gram-negative bacteria in North America as well as the other inhabited continents (6, 7).

Most clinically useful aminoglycoside antibiotics consist of a central 2-deoxystreptamine ring with amino sugars attached by glycosidic bonds at either positions 4 and 6 (4,6-disubstituted) or positions 4 and 5 (4,5-disubstituted). ANT(2'') is unusual in that it is one of only two clinically relevant AGMEs that modify predominantly on ring C of 2-deoxystreptamines (Figure 1). The other enzyme which modifies primarily on this ring is aminoglycoside phosphotransferase (2'') [APH(2'')]. This AGME is usually found as part of the bifunctional enzyme 6'-*N*-acetyltransferase and 2''-*O*-phosphotransferase [AAC(6')-APH(2'')] (8). This enzyme is different than ANT(2''), however, in that it can also modify both 4,6- and 4,5-disubstituted 2-deoxystreptamines (8). ANT(2'') is only capable of modifying 4,6-disubstituted aminoglycosides.

<sup>†</sup> This research was supported by Grant MCB 01110741 from the National Science Foundation (to E.H.S.). E.W. was partially supported by the Center of Excellence for Structural Biology at the University of Tennessee.

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<sup>1</sup> Abbreviations: ANT(2''), aminoglycoside nucleotidyltransferase(2'')-Ia; AGME, aminoglycoside-modifying enzyme.

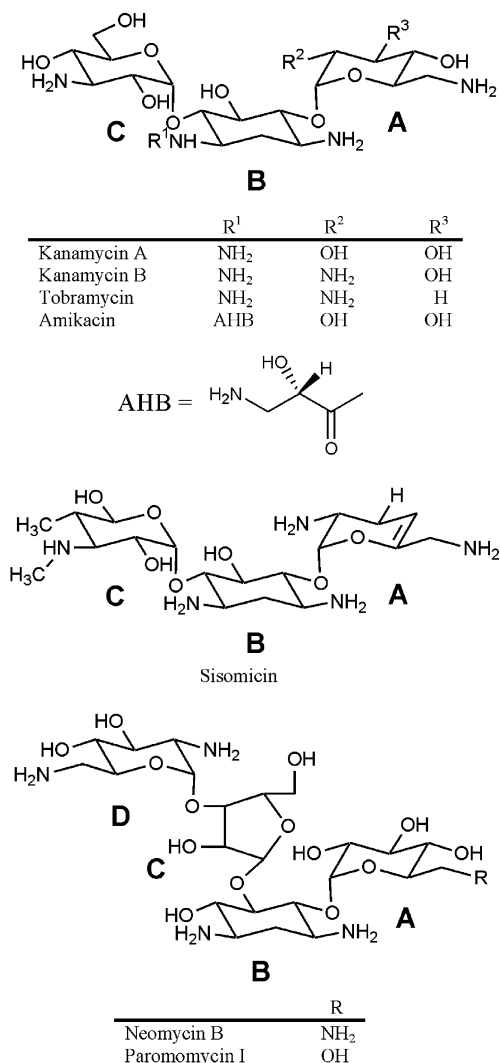


FIGURE 1: Structures of the aminoglycoside antibiotics used in this study. The positions on the A ring are designated as the prime (') positions and the positions on the C ring are designated the double prime (") positions.

Previous studies determining the substrate specificity of ANT(2'') have used kinetic studies to compare the aminoglycoside substrates (9–12). In this work we use isothermal titration calorimetry (ITC) to directly measure the binding affinity of various aminoglycosides for ANT(2''). Thermodynamic parameters for the binding of different aminoglycosides are also determined. ITC has been used previously to characterize aminoglycoside binding to the aminoglycoside acetyltransferase-Iy [AAC(6')-Iy] (13) and the aminoglycoside phosphotransferase APH(3')-IIIa (14). Thermodynamic parameters for MgATP and tobramycin binding have been reported recently for ANT(2'') (15).

## MATERIALS AND METHODS

**Reagents.** All materials were of the highest purity commercially available. All were purchased from Sigma-Aldrich Co. (St. Louis, MO) except for tris(2-carboxyethyl)phosphine (TCEP) purchased from Fluka (Buchs, Switzerland).

**Protein Preparation and Kinetic Assays.** ANT(2'')-Ia was prepared as described previously (16). Immediately prior to use in ITC experiments, the protein was desalted using a Sephadex G-25 column. Kinetic assays of the enzyme were

also performed as described previously (15). All assays were done at pH 7.5. All assay mixtures contained 10 mM MgCl<sub>2</sub> and 1.0 mM ATP. The concentration of tobramycin was varied from 10 to 500 μM. The concentrations of inhibitor were 0, 0.375, 0.75, and 1.5 mM. Kinetic data were fit to eq 1 to determine the kinetic parameters and type of inhibition. Data were fit to eq 2 to determine the inhibition constants for neomycin B and paromomycin:

$$v = \frac{VA}{K_m + A + A^2/K_{i,\text{sub}}} \quad (1)$$

$$K_{m,\text{app}} = K_m(1 + [I]/K_{i,\text{comp}}) \quad (2)$$

where  $v$  is the measured rate of the reaction,  $V$  is the maximal velocity,  $A$  is the concentration of tobramycin,  $K_m$  is the substrate concentration that yields half-maximal velocity, and  $K_{i,\text{sub}}$  is the inhibition constant for substrate inhibition.  $K_{m,\text{app}}$  is the apparent  $K_m$  in the presence of competitive inhibitor, and  $K_{i,\text{comp}}$  is the inhibition constant for the competitive inhibitor. The competitive inhibition constants determined at each concentration of inhibitor were averaged to obtain the values and standard errors for the inhibition by neomycin B and paromomycin I described in the Results and Discussion section.

**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. The final buffer for ITC experiments was 50 mM HEPES or PIPES, pH 7.5, 50 mM KCl, and 2 mM TCEP. The same stock solution of aminoglycoside was used for both the binary and quaternary titrations. The concentration of aminoglycoside was determined by NMR and activity assays as described previously (15). 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 5–20 μM enzyme in the sample cell. For binary enzyme–aminoglycoside and quaternary enzyme–MgAMPCPP–aminoglycoside complexes, the aminoglycoside concentration was 60–150 μM in the injection syringe. For the quaternary titrations the sample and ligand solutions each contained 2.0 mM MgCl<sub>2</sub> and 0.8 mM AMPCPP. The standard errors represent the deviation including curve fitting errors of the three titrations. The  $c$  values ( $c = K_a M_t$ , where  $M_t$  is the concentration of macromolecule binding sites) (17) for all experiments except the amikacin titrations were in the range 5–20. This range is ideal for accurately determining binding constants by ITC. For titrations using amikacin the  $c$  values ranged from 0.2 to 0.5. For all complexes observed in this study the thermodynamic parameters determined were independent of protein concentration.

The heat of dilution for each aminoglycoside was determined by titrating aminoglycoside into buffer in the absence of enzyme and subtracted prior to curve fitting. For all titrations including the osmotic stress experiments the pH was confirmed immediately prior to the start of the experiment. 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 ( $\Delta H$ ), and stoichiometry ( $n$ ) (17).

The free energy ( $\Delta G$ ) and entropy ( $\Delta S$ ) changes associated with binding were determined using the equations:

$$\Delta G = -RT \ln K_a \quad (3)$$

$$\Delta G = \Delta H - T\Delta S \quad (4)$$

Determination of the intrinsic enthalpy of binding was determined by the simultaneous solutions of the equations:

$$\Delta H_{\text{obs}1} = \Delta H_{\text{int}} + \Delta H_{\text{ion}1} \Delta n \quad (5a)$$

$$\Delta H_{\text{obs}2} = \Delta H_{\text{int}} + \Delta H_{\text{ion}2} \Delta n \quad (5b)$$

where the subscripts 1 and 2 refer to different buffers,  $\Delta H_{\text{obs}}$  is the observed enthalpy change upon binding,  $\Delta H_{\text{ion}}$  is the heat of ionization of the buffer, and  $\Delta n$  is the net uptake of protons by the buffer upon complex formation. The heat of ionization for HEPES is +4.87 kcal/mol and for PIPES is +2.67 kcal/mol (18).

The net change in the number of solute-excluding water molecules was determined using the equation (19):

$$d \log K_a / d[\text{solute}]_{\text{osmolal}} = -2.303 \Delta n_w / 55.56 \quad (6)$$

where  $\Delta n_w$  is the number of water molecules coupled to the binding process and 55.56 is the number of moles of water in 1 kg.

## RESULTS AND DISCUSSION

**Thermodynamics of Aminoglycoside Binding.** The binding interactions between ANT(2'') and seven different aminoglycoside antibiotics were studied by ITC. Titrations of the aminoglycosides to enzyme were performed in the absence (binary complex) and in the presence (quaternary complex) of the nonhydrolyzable substrate analogue AMPCPP in complex with  $\text{Mg}^{2+}$  (MgAMPCPP). Since binding of aminoglycosides to AGME is accompanied by protonation/deprotonation of functional groups (14), all titrations were performed in two different buffers to determine the intrinsic enthalpy of binding ( $\Delta H_{\text{int}}$ ). The observed enthalpy change ( $\Delta H_{\text{obs}}$ ) in an ITC experiment reflects contributions from both the intrinsic enthalpy of the reaction and the change in enthalpy due to release or uptake of protons by the buffer (20, 21).

Thermodynamic data for the formation of the binary enzyme–aminoglycoside and the quaternary enzyme–MgAMPCPP–aminoglycoside complexes are presented in Tables 1 and 2, respectively. For one of the aminoglycosides studied, amikacin, the affinity was too low to obtain accurate values for  $\Delta H$  and  $T\Delta S$ . All binding interactions were characterized by favorable enthalpy ( $-\Delta H$ ) and unfavorable entropy ( $-T\Delta S$ ), which is typical for carbohydrate–protein interactions (22–24). The binding was tighter in the presence of MgAMPCPP for all aminoglycosides studied with the exception of sisomicin. In the case of sisomicin the dissociation constant is similar in the presence and absence of metal–nucleotide. For sisomicin, a member of the gentamicin family of 4,6-disubstituted aminoglycosides, a slightly more negative enthalpy and slightly more negative entropy in the presence of MgAMPCPP results in a similar free energy of binding. For the members of the kanamycin family of 4,6-disubstituted aminoglycosides, kanamycin A, kanamycin B, and tobra-

Table 1: Thermodynamic Parameters for Aminoglycoside Binding to ANT(2'') (Binary Complex) at pH 7.5<sup>a</sup>

	buffer	$K_D$ ( $\mu\text{M}$ )	$\Delta H_{\text{obs}}^b$ (kcal/ mol)	$\Delta H_{\text{int}}$ (kcal/ mol)	$-T\Delta S$ (kcal/ mol)	$\Delta G$ (kcal/ mol)
kanamycin A	HEPES	2.6	−15.6	−22.4	8.1	−7.5
	PIPES	3.0	−18.7		11.3	−7.4
kanamycin B	HEPES	0.44	−21.4	−27.8	10.3	−8.5
	PIPES	0.62	−24.3		13.9	−8.3
tobramycin	HEPES	0.61	−18.2	−26.6	10.0	−8.2
	PIPES	0.80	−22.0		13.9	−8.1
sisomicin	HEPES	1.7	−14.3	−21.2	6.6	−7.7
	PIPES	1.2	−17.4		9.5	−7.9
paromomycin I	HEPES	11.5	−16.3	−19.2	9.7	−6.6
	PIPES	7.3	−17.6		10.7	−6.9
neomycin B	HEPES	0.53	−14.3	−18.3	5.9	−8.4
	PIPES	0.59	−16.4		8.1	−8.3
amikacin	HEPES	~60	ND <sup>c</sup>	ND	ND	−5.7

<sup>a</sup> Determined at 293 K. Error values:  $K_D$ , 4–13%;  $\Delta H$ , 1–5%. The stoichiometry of complex formation was  $1.0 \pm 0.1$  for all titrations.

<sup>b</sup> Intrinsic enthalpy change determined using eqs 5a and 5b. <sup>c</sup> ND, not determined.

Table 2: Thermodynamic Parameters for Aminoglycoside Binding to ANT(2'') in the Presence of MgAMPCPP and Excess  $\text{Mg}^{2+}$  (Quaternary Complex) at pH 7.5<sup>a</sup>

	buffer	$K_D$ ( $\mu\text{M}$ )	$\Delta H_{\text{obs}}^b$ (kcal/ mol)	$\Delta H_{\text{int}}$ (kcal/ mol)	$-T\Delta S$ (kcal/ mol)	$\Delta G$ (kcal/ mol)
kanamycin A	HEPES	1.1	−14.1	−17.6	6.1	−8.0
	PIPES	1.3	−15.7		7.8	−7.9
kanamycin B	HEPES	0.24	−13.1	−18.8	4.2	−8.9
	PIPES	0.37	−15.7		7.1	−8.6
tobramycin	HEPES	0.25	−12.6	−18.6	3.6	−9.0
	PIPES	0.32	−15.3		7.6	−8.7
sisomicin	HEPES	1.4	−16.9	−21.5	9.0	−7.9
	PIPES	1.1	−19.0		11.0	−8.0
paromomycin I	HEPES	1.9	−21.1	−21.1	13.5	−7.6
	PIPES	2.9	−21.9		14.5	−7.4
neomycin B	HEPES	0.27	−15.4	−19.8	6.5	−8.9
	PIPES	0.30	−17.4		8.7	−8.7
amikacin	HEPES	~40	ND <sup>c</sup>	ND	ND	−5.9

<sup>a</sup> Determined at 293 K. Error values:  $K_D$ , 3–15%;  $\Delta H$ , 1–5%. The stoichiometry of complex formation was  $1.0 \pm 0.1$  for all titrations.

<sup>b</sup> Intrinsic enthalpy change determined using eqs 5a and 5b. <sup>c</sup> ND, not determined.

mycin, the presence of MgAMPCPP resulted in a 2–3-fold decrease in the dissociation constant. For all three of these substrates a less favorable (less negative) enthalpy of binding was overcome by a decrease in the unfavorable entropic contribution resulting in overall greater affinity when ANT(2'') is saturated with metal–nucleotide. For the 4,5-disubstituted aminoglycosides tested, neomycin B and paromomycin I, the dissociation constant also decreased in the presence of MgAMPCPP. This increase in affinity, in contrast to the increase observed with the kanamycins, results from an increase in favorable enthalpy of binding.

There is a correlation between the data from ITC experiments and previously determined kinetic data (15) for the 4,6-disubstituted aminoglycosides. A decrease in  $K_d$  values is observed with decreasing  $K_m$  values. In the quaternary complex an increase in  $-\Delta H$  is observed with increasing  $k_{\text{cat}}$  values.

Differences in the free energy of binding between structurally different aminoglycosides and the enzyme showed the importance of several positions for affinity and specificity.

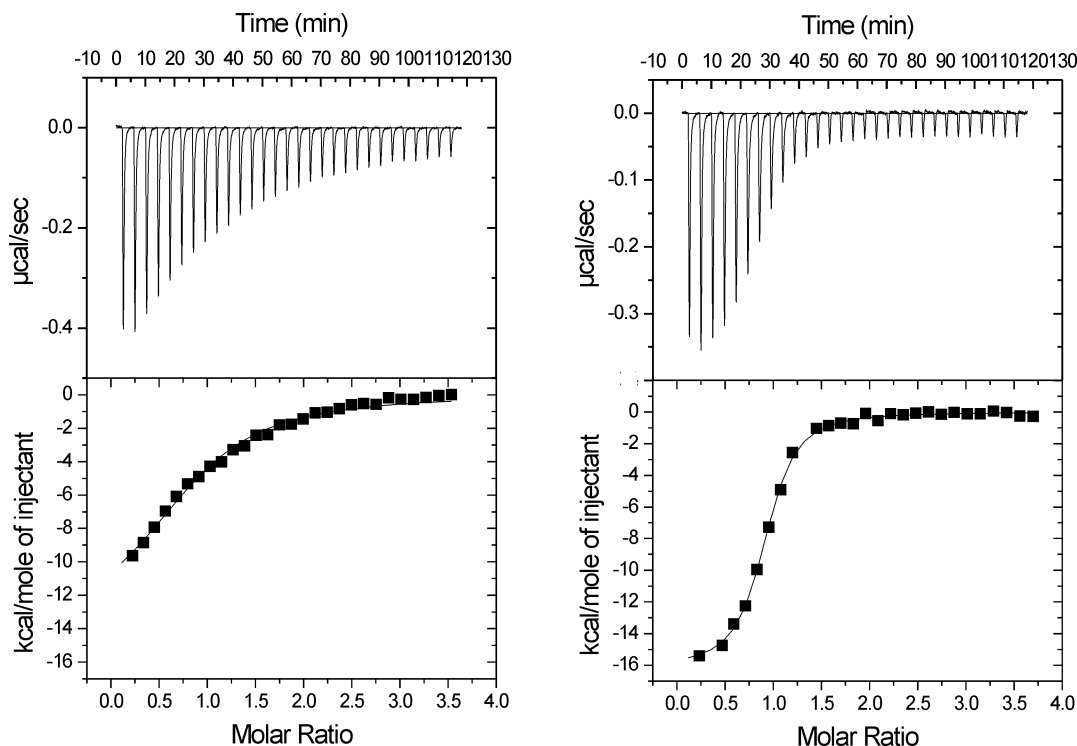


FIGURE 2: ITC profile of kanamycin A (left) and kanamycin B (right) binding to ANT(2'') (binary complex). Enzyme and ligand solutions contained 50 mM HEPES, pH 7.5, 50 mM KCl, and 2 mM TCEP. Identical ratios of titrant to protein were used.

Four pairs of the aminoglycosides tested differed only by the substituent at a single position. Comparison of the affinity within these sets of substrates was used to determine the role of each position in determination of specificity.

**Importance of the 1 Position.** The importance of position 1 on the deoxystreptamine ring is evident by comparing the binding affinities of amikacin and kanamycin A. These aminoglycosides are identical except that amikacin contains a 4-amino-2-hydroxybutyryl (AHB) attachment on the amine at position 1 (Figure 1). The acylation of the amine at position 1 results in an approximately 20-fold increase in the dissociation constant of the binary complex and an approximately 30-fold increase in the dissociation constant in the quaternary complex. This may, however, contain additional contributions and may cause overestimation of the effect of position 1 because the size of the AHB group may allow it to interfere with interactions with other positions on the aminoglycoside. The bulky AHB group may also provide a steric hindrance to aminoglycoside binding.

**Importance of the 2' Position.** Early kinetic studies already showed that the presence of an amino group at the 2' site, instead of a hydroxyl, makes aminoglycosides better substrates (12, 15). In this work, we demonstrate the significant effect of the presence of  $-\text{NH}_2$  vs  $-\text{OH}$  in the thermodynamic properties of enzyme–aminoglycoside complexes. Figure 2 illustrates the dramatic difference in the binding affinity of kanamycin B vs kanamycin A. Please note that the substrate-to-enzyme ratio has to be significantly increased with kanamycin A to achieve a similar binding curve observed with kanamycin B. These two aminoglycosides are identical with the exception of the substituent at the 2' position. Kanamycin A contains a 2'-OH while kanamycin B contains a 2'- $\text{NH}_2$ . We also note that this site is one of the most remote sites from the site of modification by this enzyme (2'') on aminoglycosides. Kanamycin B has an

approximately 5-fold higher affinity for the enzyme in both the binary and quaternary complexes. The thermodynamic basis for this difference in affinity is different for the two complexes. In the binary complex, the enthalpy of binding is about 5.5 kcal/mol more negative for kanamycin B than kanamycin A. This difference is partially diminished by a more unfavorable (more negative) entropic contribution with kanamycin B, resulting in an overall more favorable free energy of binding for kanamycin B compared to kanamycin A. In the quaternary complex the difference in enthalpy is much smaller. The intrinsic enthalpy of binding is only 1.2 kcal/mol more negative for kanamycin B, and the observed  $\Delta H$  values are similar. However, in the quaternary complex the entropy ( $T\Delta S$ ) is more negative for kanamycin A, resulting in weaker binding for kanamycin A compared to kanamycin B. The ability of an amino group to form more hydrogen bonds than a hydroxyl group may explain the greater affinity for kanamycin B. Hydrogen bonds are the major interactions governing affinity and specificity in enzyme–carbohydrate interactions (22, 23, 25). The change from an amino to a hydroxyl group can alter the number and geometry of hydrogen bonds formed in the complex. This change can not only have a direct effect but it can also alter the hydrogen-bonding network in the active site. Alternatively, a positively charged 2'-amino group ( $\text{p}K_a = 8.3$  in free kanamycin B) (26) may also be involved in electrostatic interactions with negatively charged side chains of ANT(2'').

**Importance of the 6' Position.** Neomycin B and paromomycin are not substrates for ANT(2''). These two compounds are competitive inhibitors of the enzyme with  $K_i$  values of  $7.0 \pm 3.1 \mu\text{M}$  for neomycin and  $78 \pm 24 \mu\text{M}$  for paromomycin at pH 7.5. These two 4,5-disubstituted aminoglycosides differ only at the 6' position. Neomycin B contains a 6'-amino while paromomycin I contains a 6'-hydroxyl. This



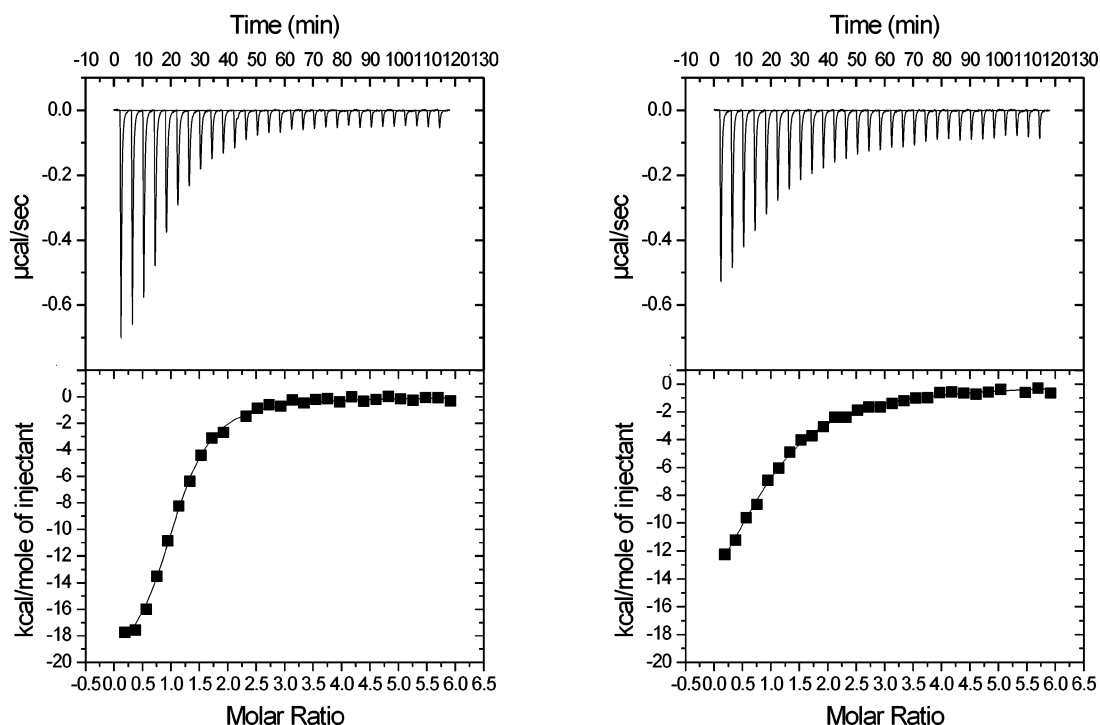


FIGURE 3: ITC profile of neomycin B (left) and paromomycin I (right) binding to ANT(2'') in the presence of excess MgAMPCPP (quaternary complex). Enzyme and ligand solutions contained 50 mM PIPES, pH 7.5, 50 mM KCl, and 2 mM TCEP. Identical ratios of titrant to protein were used. The experiments were performed under identical conditions.

site is also remote from the site of modification by ANT(2''). In the binary enzyme–aminoglycoside complexes the dissociation constant is at least 10-fold lower for neomycin than paromomycin. In the quaternary complexes the dissociation constant is 7-fold higher for paromomycin in HEPES and 10-fold higher in PIPES at pH 7.5. In both the binary and quaternary titrations paromomycin had more favorable observed enthalpy of binding. The weaker binding is evident in Figure 3, which shows titration of the two 4,5-disubstituted aminoglycosides. In both cases, though, paromomycin had a more disfavored entropy of binding leading to lower affinity compared to neomycin. Again, as discussed for kanamycin A and kanamycin B, the ability of the amino group to be involved in more hydrogen bonds or in electrostatic interactions ( $pK_a$  of the 6'-NH<sub>2</sub> is 8.6 in free neomycin) (27) is a possible reason for the observed tighter binding of neomycin to the enzyme compared to paromomycin. These findings clearly indicate that the 6'-amino group is important for binding to ANT(2'').

At first glance the explanation that improved hydrogen bonding or an electrostatic interaction may increase affinity in the enzyme–neomycin complex may seem counterintuitive since the change in enthalpy is more favorable in the enzyme–paromomycin complex. One must remember, however, that ITC-determined quantities reflect global thermodynamic changes. Therefore, the effect of an additional hydrogen bond or electrostatic interaction may not result in a more favorable enthalpy change. The number and geometry of hydrogen bonds between protein and ligand are directly related to affinity (25). However, the number of hydrogen bonds or electrostatic interactions is not directly correlated to the net enthalpy of the interaction (22). A notable example is the binding of ligands to FK506 binding protein. A point mutation on the protein which removes a hydrogen bond decreases affinity, but the enthalpy becomes more favorable

in the absence of the critical hydrogen bond (28). Similarly, on the basis of the crystal structure and the presence of a single hydrogen bond between the ligand and the enzyme in the hydrophobic active site pocket, an entropy-driven binding was anticipated with the major urinary protein (29). ITC studies, however, revealed an enthalpy-driven binding process. In the case of neomycin vs paromomycin binding to ANT(2'') a single difference exists on the ligand which results in increased affinity but a less favorable enthalpy.

Not every position on the aminoglycoside is important for binding to ANT(2''). Although the nature of the substituent at the 1, 2', or 6' position caused a significant change in affinity, a change at the 3' position did not effect binding. Kanamycin B and tobramycin are identical except for the 3' position. Kanamycin B contains a 3'-OH while tobramycin has a 3'-H. The thermodynamic parameters of the binding of these two aminoglycoside antibiotics to ANT(2'') are very similar to each other in both the binary and quaternary complexes. This suggest that the 3' position does not play an important role in determining specificity for ANT(2'')–aminoglycoside interactions. Interestingly, the 3' position is located between the 2' and the 6' positions, both of which affect the aminoglycoside binding.

*Role of Water in the Binding of Aminoglycosides to ANT(2'').* The effect of osmolytes on the affinity of a ligand for a macromolecule can be utilized to determine the difference between the number of water molecules associated with a protein–ligand complex compared to the total number of water molecules associated with the free protein and free ligand (19, 30, 31). In the present study ethylene glycol and glycerol are used to increase the osmotic pressure of the solution. Also, an antibiotic from each of the two major classes of aminoglycosides was used to compare the role of water in binding of 4,5- and 4,6-disubstituted aminoglycosides. The slope of the line in Figure 4 and eq 6 were used

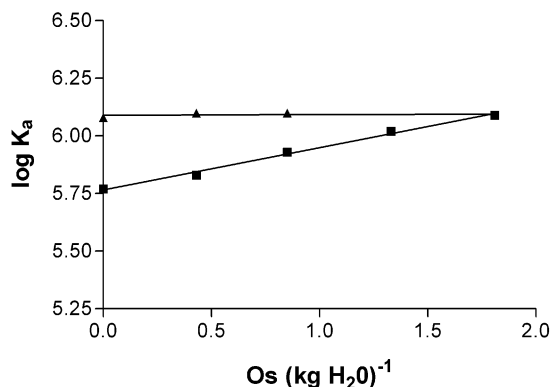


FIGURE 4: Change in binding affinity as a function of osmolality for neomycin ( $\blacktriangle$ ) and tobramycin ( $\blacksquare$ ). The line fit was determined by linear regression. The slopes of the lines are 0.18 for tobramycin and 0.003 for neomycin.

Table 3: Effect of Osmolytes on Binding of Tobramycin to ANT(2'') at pH 7.5<sup>a</sup>

	$K_D$ ( $\mu$ M)	$K_A \times 10^5$ ( $M^{-1}$ )	$\Delta H$ (kcal/mol)	$-T\Delta S$ (kcal/mol)	$\Delta G$ (kcal/mol)
no osmolyte	1.7	5.9	-10.7	3.0	-7.7
ethylene glycol, 2.5%	1.5	6.8	-11.1	3.3	-7.8
ethylene glycol, 5%	1.2	8.5	-11.5	3.6	-7.9
ethylene glycol, 7.5%	0.95	10.5	-11.9	3.8	-8.1
ethylene glycol, 10%	0.81	12.3	-12.2	4.0	-8.2
glycerol, 5%	1.2	8.1	-12.0	4.1	-7.9
glycerol, 10%	1.1	9.3	-12.6	4.6	-8.0

<sup>a</sup> Determined at 293 K. Error values:  $K_D$ , 2–8%;  $\Delta H$ , 1–4%. The stoichiometry of complex formation was  $1.0 \pm 0.1$  for all titrations.

to determine that a net release of  $4.4 \pm 0.2$  water molecules occurs when the 4,6-disubstituted aminoglycoside tobramycin binds ANT(2'') using ethylene glycol as the osmolyte. When glycerol is the osmolyte, the calculated loss of water molecules is  $4.0 \pm 0.7$ . For titrations in both glycerol and ethylene glycol the increased affinity is due to a more favorable enthalpic contribution (Table 3). In contrast to the results obtained using tobramycin, no change in affinity was observed under increasing osmotic stress in the case of neomycin binding to ANT(2''). The net change in bound water molecules is  $-0.1 \pm 0.01$  using ethylene glycol as the osmolyte (Figure 4) and  $0.0 \pm 0.03$  using glycerol. In the absence of a structure of ANT(2'') any interpretation of these results is subjective. The role of water at the interface of the protein and rings A and B is likely to be similar with tobramycin and neomycin. Therefore, the difference most likely arises from the other parts of the aminoglycosides. One possibility is that ring C of tobramycin is more complementary to the active site of the enzyme than ring C of neomycin so the interaction of ring C with the active site requires more water molecules in the case of neomycin. Another possibility is that the loss of water may result from the interactions of rings A and B in both cases; however, rings C and D of neomycin or paromomycin may protrude away from the enzyme into solution. Water associated with these rings would increase the net number of bound water molecules, which may cause coincidental compensation of the loss due to binding of rings A and B.

**Implications for Substrate Specificity.** At both the 2' and 6' positions the presence of an amine rather than a hydroxyl increases the affinity of the aminoglycoside for the enzyme.

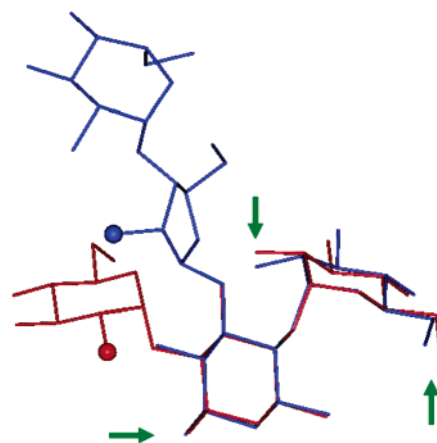


FIGURE 5: Kanamycin A (red) and neomycin B (blue) from the crystal structure of APH(3') (37) superimposed as described in ref 27. The A and B rings superimpose with enzyme-bound conformations determined by NMR spectroscopy with an RMSD of less than 0.18 Å. Positions (1, 2', and 6') determined in this study to be important for aminoglycoside binding to ANT(2'') are indicated with green arrows. The site of modification (2''-OH) on kanamycin A is shown as a red ball. The 2''-OH on neomycin B is indicated with a blue ball.

At position 1, modification of the amine leads to a dramatic decrease in affinity. These observations are similar to those made with two other AGMEs, the aminoglycoside phosphotransferase(3')-IIIa [APH(3')] and the aminoglycoside acetyltransferase(6')-Iy [AAC(6')], where the presence of an amino group in place of a hydroxyl at the 2' or 6' position increased the binding affinity of aminoglycosides to these enzymes. Similar preference shown by ANT(2'') is unusual in that it modifies a remote site from these positions while APH(3') and AAC(6') modify sites on the ring (A) containing these two positions. The results observed with ANT(2'') not only provide insight into the role of these functional groups in determining substrate specificity of this enzyme but also provide a rationale for the specificity for 4,6-disubstituted aminoglycosides as opposed to 4,5-disubstituted aminoglycosides when combined with studies determining the substrate conformations of aminoglycoside antibiotics. Earlier studies showed that the A and B rings of aminoglycoside antibiotics adopt similar conformations in the active sites of different AGMEs including ANT(2'') (32, 33), which led to a hypothesis that these two rings make the most important contacts with enzymes and RNA (34). Thermodynamic parameters of enzyme–aminoglycoside complexes determined in this work lend further support to this hypothesis and indicate that indeed several groups on rings A and B of aminoglycosides make the most important contacts with ANT(2''). These three positions anchor rings A and B in the active site. This “anchoring” positions the hydroxyl at the 2'' position suitably for a nucleophilic attack on the  $\alpha$ -phosphate of ATP. In the case of 4,5-disubstituted aminoglycosides rings A and B are also anchored in the active site, but ring C is not in position for the reaction to occur. As discussed earlier, water molecules may span the area between ring C and the active site, or rings C and D may point away from the enzyme into solution. Figure 5 shows kanamycin A and neomycin B superimposed at the primed and unprimed rings as described in a previous work (33). A full rotation of the glycosidic bond between the B and C rings will not bring any hydroxyls of neomycin B closer than

3.3 Å to the 2''-OH of kanamycin A. Thus, this explains why neomycins can bind to ANT(2'') competitively with substrates but are not modified by this enzyme.

The conclusions of this study are based solely on the importance of the individual substituents on the aminoglycoside molecule. Since no structure has yet been determined for ANT(2''), specific amino acid–aminoglycoside interactions are not known. Because of this limitation, the possibility of alternate modes of binding cannot be unequivocally ruled out. Specifically, there is the possibility that the aminoglycoside can bind in an inverted manner in which ring A can occupy the site normally occupied by ring C and ring C can occupy the site normally occupied by ring A. Such binding is observed with a nucleotidyltransferase that modifies 4' or 4'' positions on kanamycins (35). We note that this was observed for kanamycins only, which are more symmetric molecules and the 4'-OH and 4''-OH almost swap positions in the inverted binding mode. Thus, either can act as the attacking nucleophile. Such inverted binding with less symmetric neomycins would be less likely to position another hydroxyl group suitable for a nucleophilic attack on MgATP. Additionally, this binding mode would require the ribose moiety (ring C) of neomycin to make similar contacts with the enzyme as the 2-deoxystreptamine ring. It is unlikely that the ribose ring of neomycin could effectively bind at the deoxystreptamine binding site because of the importance of the amine at position 1. The amine at position 3 is also important for binding to many AGMEs. Ring C of neomycin (and paromomycin) does not contain any amine groups. Furthermore, there is no evidence for alternate modes of productive binding to ANT(2'') even for kanamycins. NMR spectra of the isolated product show only a single product, aminoglycoside-2''-AMP (32, 36). The existence of an alternate mode of binding which is nonproductive is a possibility, however, because of the substrate inhibition observed with ANT(2''). If this occurs, the affinity for both modes must be similar because only a single binding event is observed in the ITC titrations.

There is no structural information available for ANT(2''). Therefore, assigning specific interactions to the changes in enthalpy, entropy, and free energy is not possible. In addition, any interpretation of changes in enthalpy and entropy due to the change in one substituent on the aminoglycoside is complicated by the contribution of solvent, by enthalpy–entropy compensation, and by the effects of cooperative hydrogen bonding. However, the change in free energy due to the change in a single substituent on the aminoglycoside does reveal the molecular determinants of affinity and specificity for aminoglycoside binding to ANT(2''). Understanding the contributions of specific functional groups to the free energy of binding of different aminoglycoside antibiotics to this important resistance enzyme will be useful in designing new antimicrobial agents less susceptible to modification.

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BI060935D