

To the Graduate Council:

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**Isolation and Thermodynamic Characterization of
Aminoglycoside Nucleotidyltransferase(2'')-Ia**

**A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville**

**Roger Edward Wright
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Abstract

Aminoglycoside nucleotidyltransferase(2'')-Ia is one of the most important aminoglycoside-modifying enzymes. Because of the difficulties in isolating this enzyme with a high level of purity, very little work has been reported for this enzyme. A procedure for obtaining this enzyme with a purity level of greater than 95% from inclusion bodies was developed. The optimal conditions for isolating ANT(2'') were solubilizing the inclusion bodies in 8 M urea followed by direct dilution of the enzyme into 0.1 M Tris-HCl pH 8.5, 0.2 M KCl, 0.4 M L-arginine and 5 mM reduced glutathione at 4°C. The determination of an effective method of obtaining this enzyme allowed for characterization by thermodynamic and kinetic methods. Fluorescence spectroscopy titrations showed that ANT(2'') binds MgATP much tighter than free ATP and electron paramagnetic resonance (EPR) experiments showed that the enzyme binds a second divalent cation in addition to the one in the MgATP complex. Isothermal titration calorimetry (ITC) was used to show that binding of metal–nucleotide increases the affinity for the aminoglycoside substrate for all but one aminoglycoside. The binding of aminoglycosides to ANT(2'') occurs with a favorable negative enthalpy and unfavorable negative entropy. Molecular determinants of substrate specificity for aminoglycosides were also determined. An amino group at the 2' position is preferred over a 2'-hydroxyl in terms of greater catalytic efficiency ($k_{\text{cat}}/K_{\text{m}}$) and tighter binding. The same preference is observed at the 6' position where an amino group rather than a hydroxyl at that position leads to tighter binding. The 1 position on the deoxystreptamine ring is also shown to be important in terms of binding and catalysis.

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Part I. Introduction

Aminoglycoside antibiotics

Aminoglycoside-aminocyclitol antibiotics, commonly referred to as aminoglycosides, are broad-spectrum antibiotics that are effective against Gram-positive and Gram-negative bacteria. The first aminoglycoside used was streptomycin which was discovered in 1944 in a search to find antibiotics capable of treating Gram-negative infections (1). The introduction of streptomycin was followed by the discovery of several more antibacterials produced by organisms from the genera *Streptomyces* and *Micromonospora*. Later semi-synthetic aminoglycosides were produced by the modification of the naturally-occurring compounds. These semi-synthetic aminoglycosides were designed to thwart resistance mechanisms and to reduce harmful side effects associated with aminoglycosides.

The common feature of these molecules is an aminocyclitol ring. For most aminoglycosides this ring is 2-deoxystreptamine. One notable exception is streptomycin which has a streptidine ring. The 2-deoxystreptamine containing aminoglycosides can be further grouped into the 4,5-disubstituted and 4,6-disubstituted aminoglycosides. The nomenclature refers to the position of the amino sugar substituents linked to the 2-deoxystreptamine ring (Figure 1). Three methods of naming the rings are present in the literature. The conventions for referring to these rings are as follows: The amino sugar at position 4 is ring I, ring A or the primed ring. The deoxystreptamine ring is ring II ring B or the unprimed ring. The amino sugar at position 4 or 5 is ring III, ring C or the double primed ring. For aminoglycosides with more than three rings the additional rings connected to ring C are rings D and E which can also be referred to as rings IV and V or as the triple and quadruple primed rings.

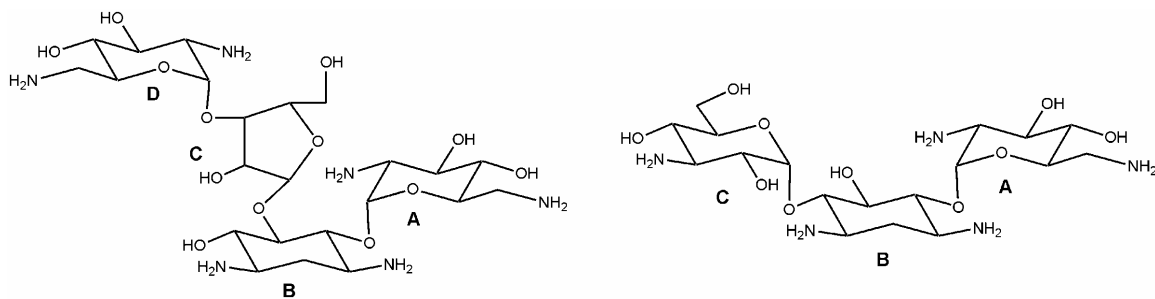


Figure 1. A 4,5-disubstituted aminoglycoside (neomycin B) is shown on the left and a 4,6-disubstituted aminoglycoside (kanamycin B) is shown on the right.

Aminoglycosides bind to the tRNA (A site) binding site of the 16S rRNA of the 30S ribosomal subunit of prokaryotic organisms leading to an increase in translational errors (2, 3). The initial entry of aminoglycosides into the cell is not well understood. Since anaerobic bacteria are unaffected by aminoglycoside antibiotics the entry is thought to be dependent on the electrical potential generated by the electron transport system (4). Further evidence supporting this theory is that inhibitors of electron transport also prevent the uptake of aminoglycosides (5, 6). This initial entry of a small amount of aminoglycosides causes mistranslation of proteins leading to a more permeable membrane (7). This increased permeability allows for the increased influx of aminoglycosides, leading to more mistranslated protein which leads to death of the organism (8, 9).

Aminoglycoside antibiotics continue to be useful in the treatment of several Gram-negative organisms. Specifically, enterobacteriaceae and *Pseudomonas aeruginosa* infections acquired in a clinical setting are usually treated with an aminoglycoside. Aminoglycosides are also often a component of multidrug treatment for difficult to treat infections (10, 11). An example of this strategy is the use of streptomycin in a multidrug treatment regimen to treat *Mycobacterium tuberculosis* which has acquired resistance to the newer treatment methods (12). Aminoglycosides also continue to be used in externally-applied antibacterials such as eye infection treatments.

Although these compounds are still widely used, the use of aminoglycosides has decreased because of two factors. First, the side effects associated with these drugs include damage to the ear and kidney. These side effects may be caused by aminoglycosides binding to human rRNA and affecting protein production.

Aminoglycosides have been shown to bind eukaryotic rRNA. However this affinity is tenfold lower than the affinity for prokaryotic rRNA (13, 14). Also aminoglycosides have been shown to inhibit ornithine decarboxylase and activate phospholipase C which are important signaling components in the cell (15, 16). Both of these factors may contribute to the side effects caused by these antibiotics. Second, resistance mechanisms have developed as a response to treatment with aminoglycosides. These mechanisms include drug efflux pumps and modified target ribosomal RNA (17, 18). However the most prevalent cause of resistance to aminoglycosides is the presence of enzymes which covalently modify aminoglycosides (19, 20).

Aminoglycoside-modifying enzymes

Over fifty enzymes have been identified which cause resistance to aminoglycoside antibiotics (21). These enzymes include *N*-acetyltransferases (AACs), *O*-phosphotransferases (APHs), and *O*-nucleotidyltransferases (ANTs). The covalent modification of aminoglycosides by these enzymes greatly reduces the ability of the antibiotic to bind the rRNA (22). Aminoglycoside acetyltransferases transfer the acetyl group from acetyl CoA to an amino group on aminoglycosides. The AACs are members of the Gcn5-related N-acetyltransferases (GNAT) superfamily of enzymes. This superfamily includes histone acetyltransferases and serotonin N-acetyltransferases. The APHs are closely related to kinases including eukaryotic protein kinases.

Aminoglycoside phosphotransferases transfer the γ -phosphate from ATP to a hydroxyl on an aminoglycoside. Aminoglycoside nucleotidyltransferases transfer the adenyl group from ATP to a hydroxyl on the aminoglycoside. The ANTs are members of the

nucleotidyltransferase superfamily. This superfamily includes DNA polymerases and RNA polymerases.

At least one crystal structure has been published for each of the three groups of aminoglycoside-modifying enzymes (23-25). In each case the overall fold of the aminoglycoside-modifying enzyme more closely resembles other members of its superfamily than enzyme structures from other classes of aminoglycoside-modifying enzymes. The same comparison holds true for primary sequences. The sequences of different classes of aminoglycoside-modifying enzymes are not closely related. These relationships suggest that aminoglycoside-modifying enzymes are an example of convergent evolution. These enzymes may have evolved from proteins which carry out different functions in the organism to provide antibiotic resistance to the bacteria.

The genes for most of these enzymes are carried on R-plasmids. These plasmids often carry more than one resistance enzyme and can be transferred between species. The prevalence of individual resistance enzymes is dependent on the usage patterns of aminoglycosides in a given area (26).

Each type of modifying enzyme contains several members each of which is specific for modifying primarily at one position on aminoglycoside molecules. Each enzyme is capable of several different aminoglycosides. Also, each aminoglycoside can be modified by several different enzymes (Figure 2).

The site of modification for each enzyme is indicated in parenthesis, a roman numeral follows to indicate the resistance profile conferred by the enzyme and finally a lower case letter is used for different primary sequences with the same resistance pattern.

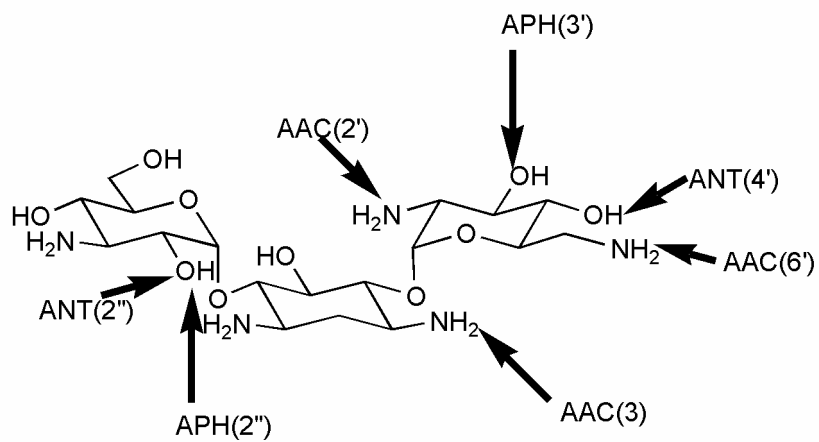


Figure 2. A single aminoglycoside can be modified by several different enzymes. The sites of modification of kanamycin B are indicated with arrows and the modifying enzymes for each position are indicated.

Aminoglycoside nucleotidyltransferase (2'')-Ia

One of the most important aminoglycoside modifying enzymes (AGMEs) is aminoglycoside nucleotidyltransferase (2'') (ANT(2'')). This enzyme was first identified in a clinical isolate of *Klebsiella pneumoniae* in 1971 (27). 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 (28, 29). 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 (30, 31). Also this enzyme is relatively unstable and has a low degree of solubility. ANT(2'') catalyzes the adenylation of aminoglycosides which contain a hydroxyl group at the 2'' position (Figure 1). The reaction is shown in figure 3.

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 (30-33). 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 (34). This work also showed that the reaction followed an ordered Theorell-Chance mechanism with the metal-nucleotide binding first, followed by the aminoglycoside substrate (35). These studies were conducted with a mixture of two enzyme variants and

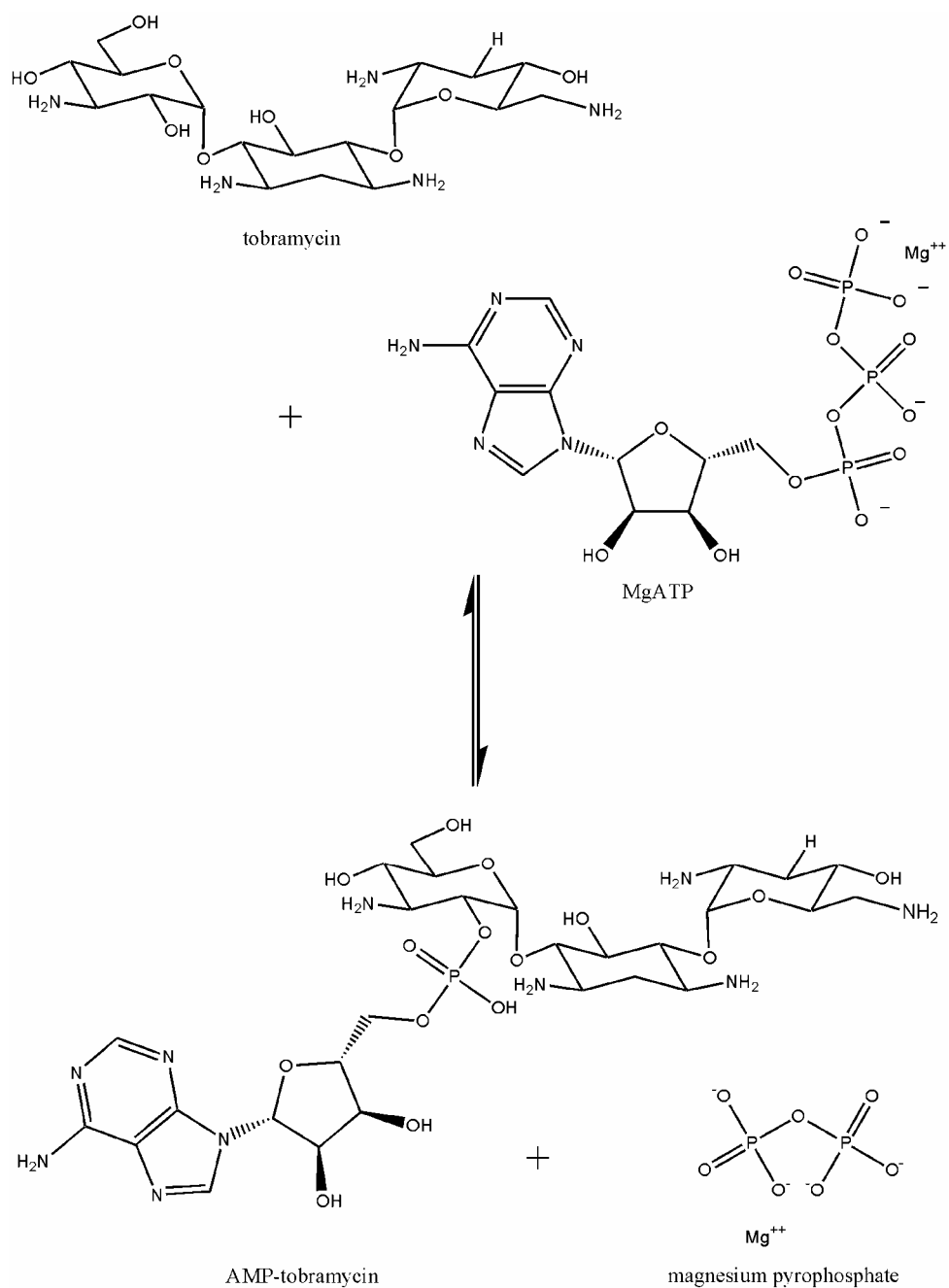


Figure 3. The reaction catalyzed by ANT(2'') is shown with tobramycin as the aminoglycoside substrate.

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 (36). The enzyme used by Gates and Northrop (34, 35) 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.

The amino acid sequence of ANT(2'') (figure 4) 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 (37, 38)). The acetyl- and phosphotransferases are even less similar to ANT(2''). Thus the few well-characterized other AGMEs (19, 39) 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) (21). Most AGMEs modify positions on the A or B ring.

A project was initiated to gain insight into ANT(2'') because of its clinical importance, its unique properties and the lack of biochemical data on this enzyme available in the scientific literature. This project resulted in the development of a method to isolate large quantities of this enzyme with a high degree of purity (Part II, (40)). This project also produced the first thermodynamic characterization of ANT(2'') including binding affinities of substrates and cofactors (Part III,(41)). Also determinants of specificity for ANT(2'') were established by comparing the affinities for different aminoglycoside antibiotics (Part IV). Finally the role of cysteine residues in this enzyme was investigated and one cysteine was found to be critical for enzyme activity (Part V).

MACYDCFFVQ SMPRASKQQA RYAVGRCLML
WSSNDVTQQG SRPKTKLGRM DTTQVTLIHK
ILAAADERNL PLWIGGGWAI DARLGRVTRK
HDDIDLTFPG ERRGELEAIV EMLGGRVMEE
LDYGFLAEIG DELLDCEPAW WADEAYEIAE
APQGSCPEAA EGVIAGRPVR CNSWEAIIWD
YFYYADEVPP VDWPTKHIES YRLACTSLGA
EKVEVLRAAF RSRYAA

Figure 4. The amino acid sequence of ANT(2'')-Ia is shown.

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Part II: Isolation of Aminoglycoside Nucleotidyltransferase(2'')-Ia from Inclusion Bodies as Active, Monomeric Enzyme

This section is a slightly revised version of a manuscript by Edward Wright and Engin H. Serpersu published in *Protein Expression and Purification* in 2004:

Wright, E., and Serpersu, E. H. (2004) Isolation of aminoglycoside nucleotidyltransferase(2'')-Ia from inclusion bodies as active, monomeric enzyme, *Protein Expression Purif.* 35, 373–380.

My contributions to this manuscript include performing all experimental procedures, conducting all data collection and data analysis, writing the initial draft of the manuscript and participating in all subsequent drafts including the final accepted manuscript.

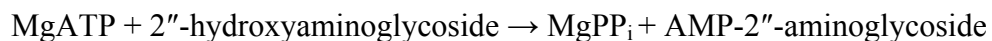
Abstract

Aminoglycoside nucleotidyltransferase(2'')-Ia (ANT(2'')) confers resistance to pathogenic bacteria against several aminoglycoside antibiotics including gentamicin, kanamycin and tobramycin. The gene for this aminoglycoside-modifying enzyme has been cloned from a clinical isolate of *Pseudomonas aeruginosa*. This gene was inserted into an overexpression vector, the vector was then transformed into *Escherichia coli* BL21(DE3) and the protein has been isolated in the form of inclusion bodies. Optimal refolding conditions have been determined to be direct dilution of solubilized inclusion bodies into 0.1 M Tris-HCl pH 8.5, 0.2 M KCl, 0.4 M L-arginine and 5 mM reduced glutathione at 4°C. The refolded enzyme is monomeric in solution and has similar kinetic properties and substrate selectivity to the enzyme isolated in soluble form.

Introduction

Aminoglycoside antibiotics bind to the 16S ribosomal RNA of prokaryotes increasing the frequency of translational errors. These errors decrease the integrity of the bacterial cell wall allowing the influx of more aminoglycoside molecules to interact with the

rRNA leading to the death of the organism (1-4). ANT(2'') catalyzes the covalent attachment of adenosine monophosphate (AMP) to the 2'' position of 4,6-disubstituted aminoglycoside antibiotics (Figure1). The overall reaction is



The modified aminoglycoside is no longer able to bind effectively to the ribosomal RNA. Thus bacteria which possess the gene for this enzyme are resistant to 4,6-disubstituted aminoglycosides which contain a hydroxyl group at the 2'' position.

Over fifty enzymes have been identified which covalently modify aminoglycosides (5). These include acetyltransferases and phosphotransferases as well as two other nucleotidyltransferases, ANT(3'') and ANT(4',4''). Of all the aminoglycoside-modifying enzymes ANT(2'') is the most prevalent in North America (5,6). Although several of these enzymes have been well characterized (7-9), very little data exists for ANT(2'') due to the difficulties in purification of the soluble enzyme, the relative instability of the enzyme and the low yield of soluble enzyme from culture (10-12).

Overexpression of ANT(2'') even at lower temperatures and minimal levels of IPTG leads to less than 5% of the enzyme present in the soluble fraction of the cell lysate (Figure 2A). The remaining >95% is present in the insoluble fraction in the form of inclusion bodies. Since the refolding of proteins isolated from inclusion bodies vary among individual proteins, a test to find the optimal conditions for refolding ANT(2'') in active form was devised based on commonly used refolding protocols (13-15).

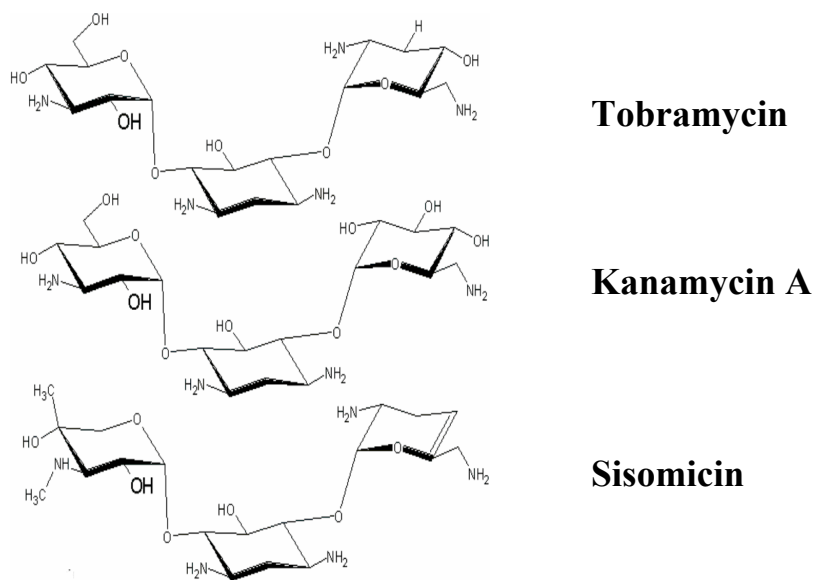


Figure 1. Aminoglycoside substrates of ANT(2'') used in this work. The hydroxyl group which is the site of adenylation is in bold.

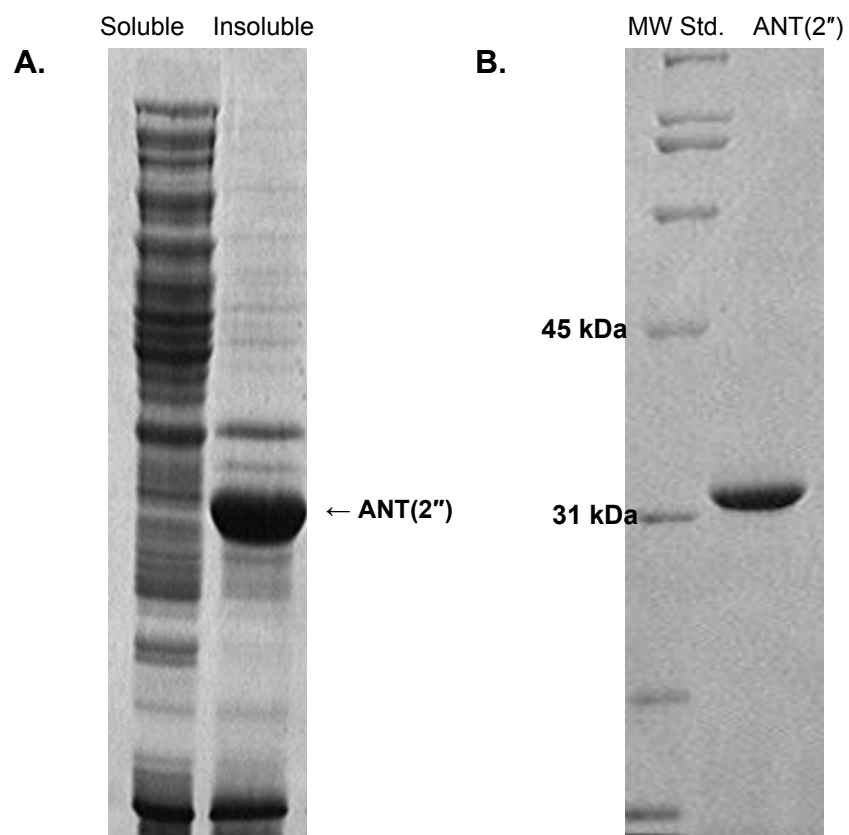


Figure 2. SDS-PAGE of ANT(2'')-Ia showing majority of the enzyme in insoluble fraction after cell lysis (A) and purity after refolding (B).

Materials and Methods

Materials. *E. coli* BL21(DE3) and pET 22b(+) were purchased from Novagen (Madison, WI). Trizma[®] base, reduced glutathione (GSH), oxidized glutathione (GSSG), L-arginine, kanamycin A, tobramycin and sisomicin were purchased from Sigma (St. Louis, MO). All restriction enzymes and potassium chloride were purchased from Fisher Scientific (Fair Lawn, NJ). Isopropylthiogalactopyranoside (IPTG) and dithiothreitol (DTT) were purchased from Inalco Pharmaceuticals (Milan, Italy). Inorganic pyrophosphatase was purchased from Roche Diagnostics (Indianapolis, IN).

Cloning and Expression. The gene for ANT(2'')-Ia was cloned as described previously from the R-plasmid of a clinical isolate of *Pseudomonas aeruginosa* kindly provided by Dr. Karen Shaw at Schlering-Plough (16). The gene was inserted between the NdeI and BamHI sites in the multiple cloning region of pET 22b(+). The plasmid was transformed into *E. coli* BL21(DE3). The cells containing the recombinant plasmid were grown at 37°C on an LB plate containing 50 µg/ml ampicillin and 20 µg/ml tobramycin. A single colony was inoculated into 10 ml LB medium containing 50 µg/ml ampicillin. After overnight growth this culture was transferred to 1L of LB containing 50 µg/ml ampicillin. Cells were grown with vigorous shaking at 37°C. When the culture reached an OD₆₀₀ of 0.4, IPTG was added at a final concentration of 0.5 mM to induce overexpression. After induction, the cells were grown an additional 6 h and harvested by centrifugation at 6000g for 15 min at 4°C. The pellet was washed with STE (10 mM Tris-HCl pH 8.0, 0.1 M NaCl and 1 mM EDTA) and the centrifugation was repeated. The cells were resuspended in 50 mM Tris-HCl, 0.1 M NaCl and 1 mM EDTA and lysed by two passes

through a French press at 18,000 lb/in². The soluble and insoluble fractions of the cell lysate were separated by centrifugation at 12,000g for 15 min at 4°C.

Inclusion body preparation. The insoluble fraction of the cell lysate containing the ANT(2") inclusion bodies was washed two times in 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 10 mM DTT and 0.5% Triton X-100. The wash was repeated two additional times with the same buffer lacking Triton X-100. After each wash the inclusion bodies were pelleted by centrifugation at 20,000g for 20 min at 4°C. The inclusion bodies were solubilized by incubation in 8 M urea, 0.1 M Tris-HCl pH 8.0 and 10 mM DTT at a concentration of 25-35 mg/ml for 1 h at room temperature. The remaining insoluble material was removed by centrifugation at 30,000g for 30 min.

Determination of optimal refolding conditions. Seventy-two sets of refolding conditions were tested. A matrix was devised varying temperature, pH, salt concentration, anti-aggregation agents and reducing agents. Each refolding buffer was prepared and used at both 4°C and 25°C. Each buffer contained 0.1 M Tris-HCl at pH 8.0, 8.5 or 9.0. The concentrations of KCl used to test the effect of salt concentration on refolding were 0 M, 0.05 M and 0.2 M. Either 0.4 M L-arginine and 2% glycine or 10% glycerol and 5% ethylene glycol were used to reduce aggregation during refolding. Also, either 5 mM DTT or 10 mM reduced glutathione and 1 mM oxidized glutathione were used to determine the optimal reducing agent or reduction–oxidation pair. Initial screens also tested 2-mercaptoethanol as a reducing agent, but in all cases the results were inferior to those obtained using DTT or glutathione.

To 10 ml of each refolding buffer 0.25 mg of inclusion bodies solubilized in 8 M urea, 0.1 M Tris-HCl pH 8.0 and 10 mM DTT were added. After 8 h the solution was

concentrated to 2 ml under nitrogen in a stirred cell ultrafiltration unit with a modified polyethersulfone membrane (Pall Gelman). Each set of conditions was evaluated based on protein recovery and enzymatic activity. The samples showing highest recovery and activity were subjected to analytical gel filtration to determine the relative amount of monomer and dimer compared to higher order oligomers.

Protein concentration was determined by the absorption at 280 nm and confirmed by the method of Bradford using BSA as the standard (17).

Activity assay. The activity assay utilized was described previously (16). The assay is based on the conversion of inorganic pyrophosphate to two molecules of inorganic phosphate by inorganic pyrophosphatase. The amount of inorganic phosphate is determined by the method of Ames (18). The assay mixture included 50 mM TAPS pH 8.5, 50 mM KCl, 5 mM MgCl₂, 0.5 mM ATP, 0.01 mM – 0.2 mM aminoglycoside and 2.0 units of inorganic pyrophosphatase except in the V_{max} and K_m determinations. In these assays ATP was varied from 0.1 mM - 2.0 mM and MgCl₂ was varied accordingly to maintain a constant free magnesium concentration. Kanamycin A was varied from 0.01- 0.15 mM in the K_m and V_{max} determinations. One unit of activity represents the production of 1.0 μmole of product per minute.

Analytical gel filtration. Aliquots of 50 μl containing refolded ANT(2'') were analyzed on a Zorbax GF-250 4.6 × 250 mm gel filtration column (Agilent) attached to an Agilent 1100 series HPLC system equipped with a UV/vis detector. The mobile phase used was 0.1 M Tris-HCl pH 8.2, 0.1 M KCl and 1.0 mM DTT.

Large scale refolding. For larger scale refolding experiments 5 mg of solubilized inclusion bodies were diluted into 250 ml of refolding buffer. The solution was kept at 4°C for 12-16 h and then concentrated to 3-4 ml by ultrafiltration.

Results

Cloning and expression. In earlier work with ANT(2''), two forms of the enzyme were isolated from bacteria harboring the original plasmid taken from clinical isolates. These proteins had apparent molecular weights of 35,000 Da and 29,000 Da (10). Analysis of the DNA sequence suggested the two forms could be due to two start sites, one coding for a polypeptide of 249 amino acids and the other coding for a polypeptide of 226 amino acids (19). Both forms were cloned and expressed. Each form was found almost entirely in the insoluble portion of the cell lysate. Both the 226 and 249 amino acid versions of ANT(2'') refolded optimally under similar conditions. However, the shorter version consistently had higher levels of expression, a greater percentage recovery from refolding and higher specific activity (Table 1). Therefore the data in the remainder of this manuscript will refer to the 226 amino acid version of this protein unless otherwise noted.

Determination of optimal refolding conditions. The best results were obtained when the refolding buffer consisted of 0.1 M Tris-HCl pH 8.5, 0.2 M KCl, 0.4 M L-arginine, 2% glycine, 10 mM glutathione (reduced) and 1 mM glutathione (oxidized). The use of this refolding buffer resulted in $\geq 96\%$ recovery of protein and a specific activity of 0.23 units mg^{-1} . The complete tables of results for the thirty-six conditions tested at both 4°C and 25°C are shown in tables 3 and 4 in the appendix.

Table 1: Expression and purification of ANT(2'') from two different start sites

	Percentage of Total Protein Expressed	Yield ^a (mg)	Percent recovery ^b (%)	Purity After Refolding from Inclusion Bodies	Activity units/mg
First start site 249 amino acids	15-20%	47-55	60-64	≥ 85%	0.17
Second start site 226 amino acids	25-30%	74-91	68-77	≥ 95%	0.23

^aMilligrams of refolded protein per liter culture (1 liter = 7.1-7.6 g wet weight cells)

^bPercent of total ANT(2'') recovered after refolding

Several trends were apparent in testing the refolding buffers. For most conditions refolding at 4°C and 25°C yielded similar percent recovery of ANT(2"). However the specific activity was almost always greater when the enzyme was refolded at 4°C. Refolding at pH 8.0 or 8.5 was always better than refolding at pH 9.0. Although the use of 10% glycerol and 5% ethylene glycol resulted in soluble, active protein, ANT(2") refolded in buffers containing this set of anti-aggregation agents had a much greater tendency to form tetramers and higher order oligomers than protein refolded in buffers containing arginine and glycine. The results using DTT and glutathione were similar in most cases. DTT can be substituted for glutathione with only a slight decrease in protein recovery and similar specific activity.

After establishing the optimal set of refolding conditions in small scale experiments, a larger scale refolding was undertaken. Five mg of ANT(2") was diluted into 250 ml of buffer. After concentration to 4 ml, 73% of the original protein was recovered with a specific activity of 0.20 units mg⁻¹. The diminished recovery is due to increased losses incurred during concentration of a larger volume of protein solution.

After establishing this base set of conditions, studies were conducted to determine the optimal concentration of each refolding buffer component. Varying the glycine concentration from 0-10% (w/v) had no significant effect on refolding efficiency (data not shown). Therefore glycine was omitted from the refolding buffer. At arginine concentrations above 0.4 M a decrease in specific activity was observed (Figure 3A).

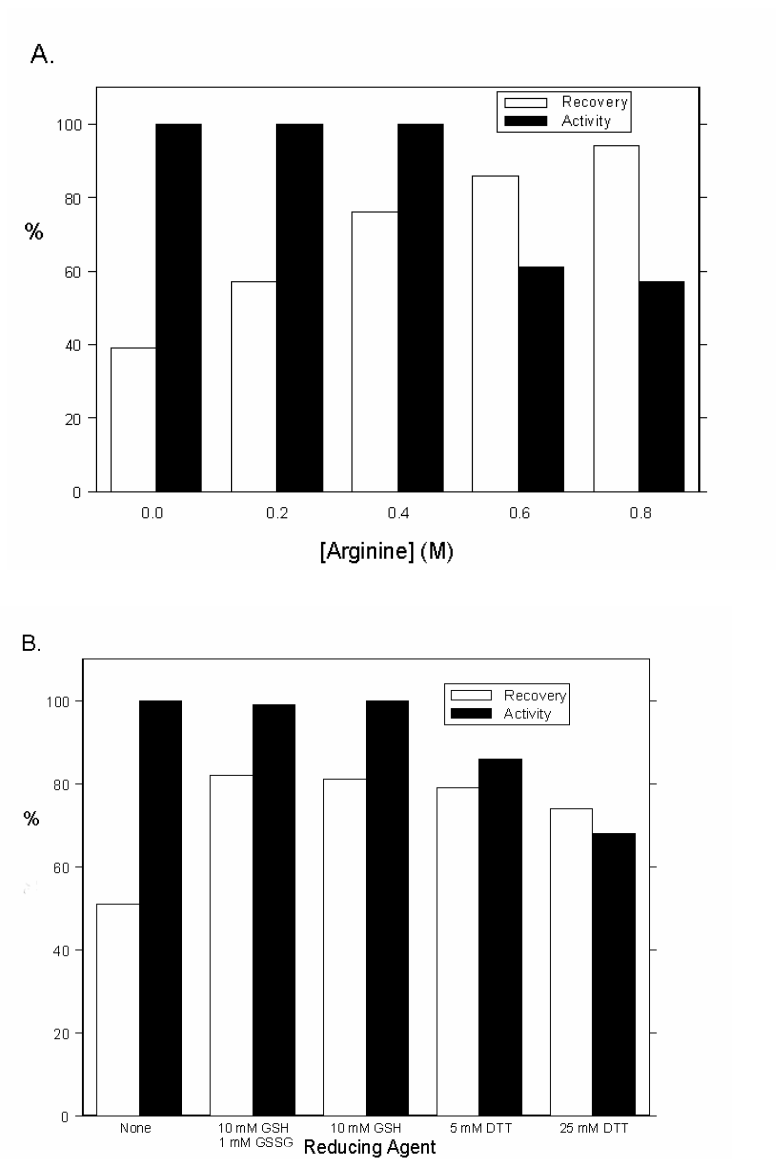


Figure 3. Effect of concentration of arginine and reducing agents on refolding. Activity under the base set of refolding conditions (0.23 units mg^{-1}) was used as 100 % activity.

Therefore the arginine concentration was kept at 0.4 M throughout. The omission of a reducing agent in the refolding buffer resulted in a significant decrease in protein recovery with no loss of specific activity. However, increasing the concentration of DTT above 5 mM or reduced glutathione above 10 mM led to a decrease in activity (Fig 3B). The use of oxidized glutathione had no significant effect on recovery or activity so it was omitted from the refolding buffer. The detergent CHAPS, and cosolvents polyethylene glycol and magnesium salts were also tested. None of these additives improved the recovery or activity of the enzyme (data not shown). After these changes the refolding buffer consisted of 0.1 M Tris-HCl pH 8.5, 0.2 M KCl, 0.4 M arginine and 5 mM glutathione (reduced) prepared and used at 4°C. Under these conditions the purity of the refolded ANT(2'') is greater than 93% (Fig 1B) and primarily monomeric when refolded in this buffer (Figure 4).

Activity and substrate specificity. The substrate rate profile of ANT(2'') refolded from inclusion bodies is in agreement with previous studies using the enzyme purified in soluble form (Table 2). The only group to do a comprehensive kinetic study on ANT(2'') used a slightly different form of the enzyme (22). However the values for K_m and V_{max} obtained using ANT(2'') refolded from inclusion bodies are similar to the values reported for enzyme isolated in soluble form. Gates and Northrup reported a K_m of 126.0 μ M and V_{max} of 5.3 units mg^{-1} for kanamycin A (21). ANT(2'') refolded from inclusion bodies using the methods described in this paper has a K_m of 155 μ M and V_{max} of 3.0 units mg^{-1} using kanamycin A as the aminoglycoside substrate.

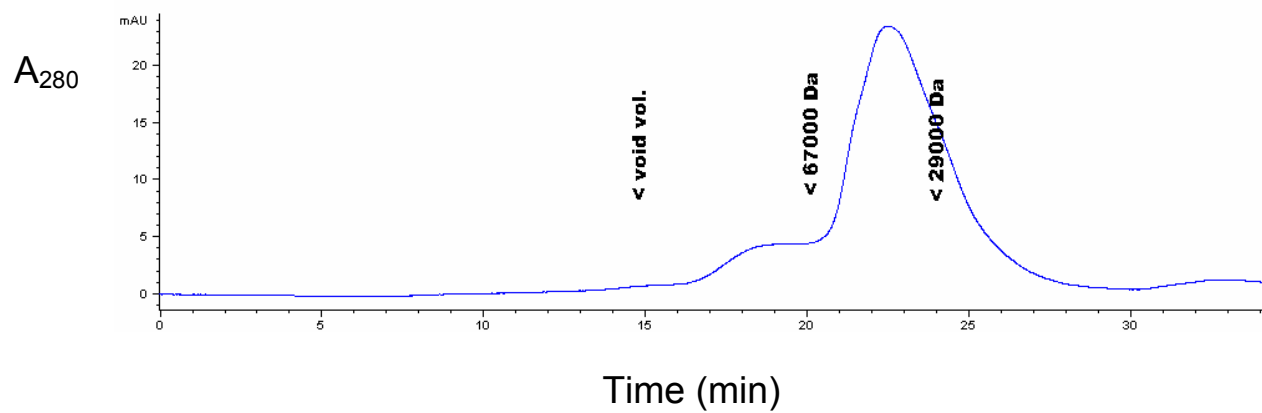


Figure 4. Gel filtration profile of refolded ANT(2'') isolated primarily as a monomer. Elution times of molecular weight standards BSA (67000 Da) and carbonic anhydrase (29000 Da) are indicated.

Table 2: Substrate specificity of ANT(2'')

	Refolded from Inclusion Bodies	Bongaerts and Molenijk(12)	Devaud et al(20)	Gates and Northrup(21) ^b	Smith and Smith(10)
Sisomicin	166	292	284	99	NT
Tobramycin ^a	100	100	100	100	100
Kanamycin A	63	56	NT	52	51

^a Rate using tobramycin for each study set to 100 for comparative purposes

^b ANT(2'') used in this study is a different isoform of the enzyme

NT: not tested

Discussion

Earlier protocols for obtaining ANT(2'') from soluble fractions required several chromatographic steps. The time required to isolate the protein in this manner reduces the time available to perform experiments with this unstable enzyme. Also since this enzyme is known to bind cellulose and dextran, significant losses occur when columns containing these materials are employed (12,22). Previous work with ANT(2'') isolated from inclusion bodies resulted in an active oligomeric form of the enzyme which retained most of the properties of the monomeric species (16). However since ANT(2'') isolated from pathogenic bacteria has been shown to be a monomer, working with an oligomeric species is not ideal. The specific activity of the monomer is almost twice the activity observed utilizing the oligomeric species. Also, although the aminoglycoside substrates adopt similar conformations in the active sites of different aminoglycoside-modifying enzymes, (23) ANT(2'') has a low degree of sequence homology with these other enzymes (24). So the more well-studied aminoglycoside-modifying enzymes are not a reliable model for the structural and activity-related aspects of this enzyme. Refolding the enzyme from inclusion bodies using the methods described herein will facilitate more detailed structural and biochemical studies with ANT(2'').

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Appendix

Table 3: Refolding of ANT (2'') at 4°C

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 8.0 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	48	0.10
pH 8.0 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	60	0.15
pH 8.0 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	56	0.16
pH 8.0 0 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	60	0.086
pH 8.0 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	80	0.14
pH 8.0 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	72	0.18
pH 8.0 0 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	60	0.11

Table 3 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 8.0 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	48	0.10
pH 8.0 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	60	0.15
pH 8.0 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	56	0.16
pH 8.0 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	60	0.086
pH 8.0 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	80	0.14
pH 8.5 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	72	0.18
pH 8.5 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	60	0.11

Table 3 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 8.5 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	56	0.17
pH 8.5 0 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	60	0.082
pH 8.5 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	88	0.16
pH 8.5 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	76	0.20
pH 8.5 0 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	56	0.092
pH 8.5 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	96	0.18
pH 8.5 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	> 98	0.23

Table 3 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 8.5 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	56	0.17
pH 8.5 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	60	0.082
pH 8.5 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	88	0.16
pH 9.0 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	76	0.20
pH 9.0 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	56	0.092
pH 9.0 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	96	0.18
pH 9.0 0 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	> 98	0.23

Table 3 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 9.0 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	36	0.10
pH 9.0 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	44	0.13
pH 9.0 0 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	52	0.11
pH 9.0 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	64	0.13
pH 9.0 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	68	0.13
pH 9.0 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	32	0.057
pH 9.0 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	40	0.062

Table 3 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 9.0 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	40	0.064

^a All buffers contain 0.1 M Tris-HCl

Table 4: Refolding of ANT (2'') at 25°C

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 8.0 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	56	0.094
pH 8.0 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	60	0.14
pH 8.0 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	60	0.14
pH 8.0 0 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	60	0.068
pH 8.0 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	76	0.092
pH 8.0 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	72	0.11
pH 8.0 0 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	64	0.091

Table 4 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 8.0 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	80	0.13
pH 8.0 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	92	0.17
pH 8.0 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	48	0.074
pH 8.0 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	64	0.12
pH 8.0 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	80	0.13
pH 8.5 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	52	0.080
pH 8.5 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	56	0.10

Table 4 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 8.5 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	56	0.10
pH 8.5 0 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	56	0.11
pH 8.5 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	88	0.14
pH 8.5 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	56	0.17
pH 8.5 0 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	36	0.070
pH 8.5 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	80	0.16
pH 8.5 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	76	0.20

Table 4 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 8.5 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	52	0.12
pH 8.5 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	60	0.15
pH 8.5 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	72	0.086
pH 9.0 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	40	0.074
pH 9.0 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	44	0.13
pH 9.0 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM DTT	60	0.057
pH 9.0 0 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	32	0.038

Table 4 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 9.0 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	40	0.060
pH 9.0 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM DTT	44	0.057
pH 9.0 0 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	60	0.063
pH 9.0 0.05 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	68	0.094
pH 9.0 0.2 M KCl 0.4 M Arginine 2% Glycine 5 mM GSH, 1 mM GSSG	68	0.089
pH 9.0 0 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	44	0.061
pH 9.0 0.05 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	64	0.072

Table 4 cont.

Condition tested ^a	% Recovery	Specific Activity (units mg ⁻¹)
pH 9.0 0.2 M KCl 10% Glycerol 5% Ethylene Glycol 5 mM GSH, 1 mM GSSG	60	0.070

^a All buffers contain 0.1 M Tris-HCl

**Part III: Enzyme–Substrate Interactions with an Antibiotic Resistance Enzyme:
Aminoglycoside Nucleotidyltransferase(2'')-Ia Characterized by Kinetic and
Thermodynamic Methods**

This section is a slightly revised version of a manuscript by Edward Wright and Engin H. Serpersu published in *Biochemistry* in 2005.

Wright, E. and Serpersu, E.H. (2005) Enzyme–Substrate Interactions with an Antibiotic Resistance Enzyme: Aminoglycoside Nucleotidyltransferase(2'')-Ia Characterized by Kinetic and Thermodynamic Methods, *Biochemistry* 44, 11581-11591.

My contributions to this manuscript include performing all experimental procedures, conducting all data collection and data analysis, writing the initial draft of the manuscript and participating in all subsequent drafts including the final accepted manuscript.

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'-NH₂ are better substrates (higher $k_{\text{cat}}/K_{\text{m}}$) than ones with 2'-OH when magnesium (II) is used as the catalytically-required divalent cation. The activity is reduced ten-fold for substrates with 2'-NH₂ 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 three-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 two 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.

Introduction

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



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).

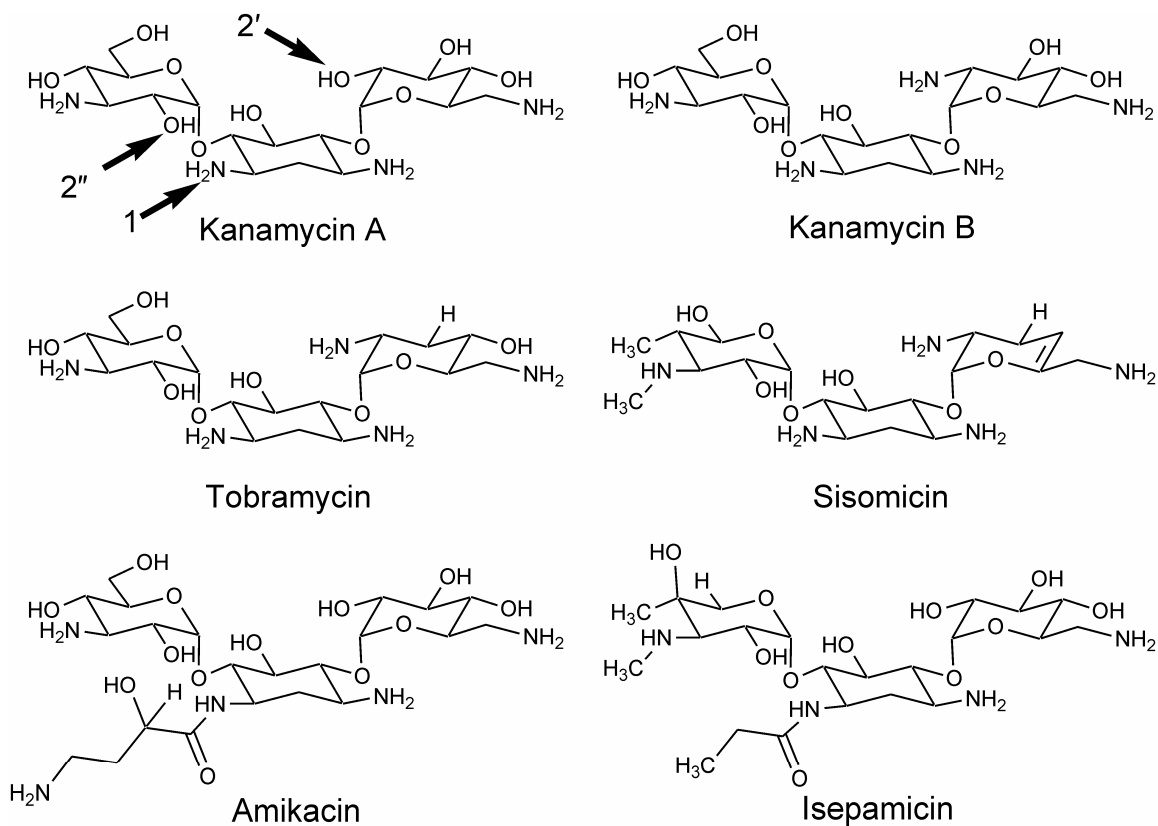


Figure 1. The aminoglycosides used in this study are shown. The site of modification (2'') and positions important in enzyme–substrate interactions (1,2') are indicated on the kanamycin A structure. In all the structures the deoxystreptamine ring (ring B) is in the center with ring C which contains the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

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 phosphotransferases 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).

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 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 $15,400 \text{ M}^{-1} \text{ cm}^{-1}$ (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 x 1.0 cm) (17). For all kinetic assays and circular dichroism experiments the final buffer is 50 mM TAPS¹ 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 μ M. Each assay mixture contained 0.15–0.35 μ M 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 equation (1), R is the universal gas constant ($1.987 \text{ cal mol}^{-1} \text{ K}^{-1}$) and T is the temperature in degrees Kelvin. The activation enthalpy, activation entropy, and Gibbs' free energy of activation were determined according to the equations:

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

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

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

where k_B is the Boltzmann constant, h is Plank's constant and A is the preexponential factor determined using equation (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):

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

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

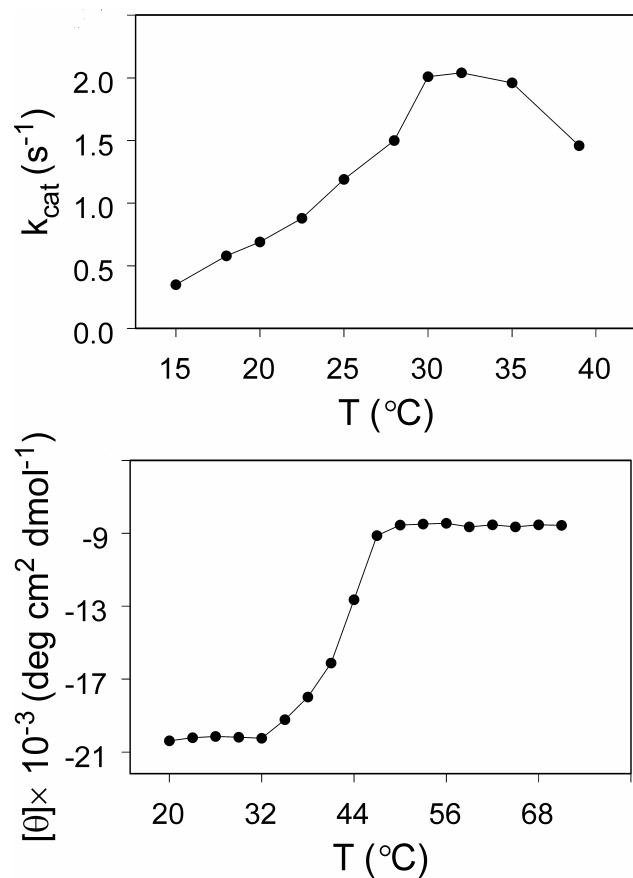


Figure 2. The temperature dependence of the reaction catalyzed by ANT(2'') is shown in the top panel. The assays were performed at pH 8.5 and contained 2.0 mM MgATP. The free magnesium concentration was 5 mM and the tobramycin concentration was varied from 10 to 500 μM . The k_{cat} values were calculated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

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 μ L and were separated by 240 s. Cell stirring speed was 300 rpm. Each titration contained 5–10 μ M enzyme in the sample cell. For binary enzyme:tobramycin² and quaternary enzyme–MgAMPCPP:tobramycin complexes, the tobramycin concentration was 60–120 μ M in the injection syringe. For the quaternary titrations the sample and ligand solutions each contained 2.0 mM MgCl₂ 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 MgCl₂. 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 MgCl₂ 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 MgAMPCPP binding was similar to MgATP binding to enzyme. Titrations of ATP into the enzyme solution in the absence of Mg²⁺ 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 μ L aliquots from a concentrated stock solution of ATP or MgATP was added to 2.0 mL of 1.0 μ M 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:

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

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 two-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 two 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 similar substrate dependence 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 four-fold

Table 1: Kinetic parameters of selected aminoglycoside substrates of ANT(2'')

Substrate	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)	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.

^bSubstrate inhibition not detected at substrate concentrations up to 0.5 mM.

higher than 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 pattern and has a K_i value very similar to 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-NH₂ 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 were 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 4, A and 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 aminoglycosides when

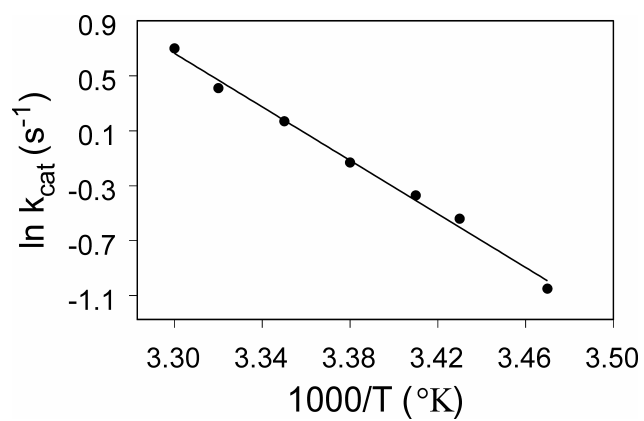


Figure 3. An Arrhenius plot constructed from the data for temperatures 15 to 30°C in figure 2 is shown. The slope of the linear fit of the data was used to calculate the activation energy.

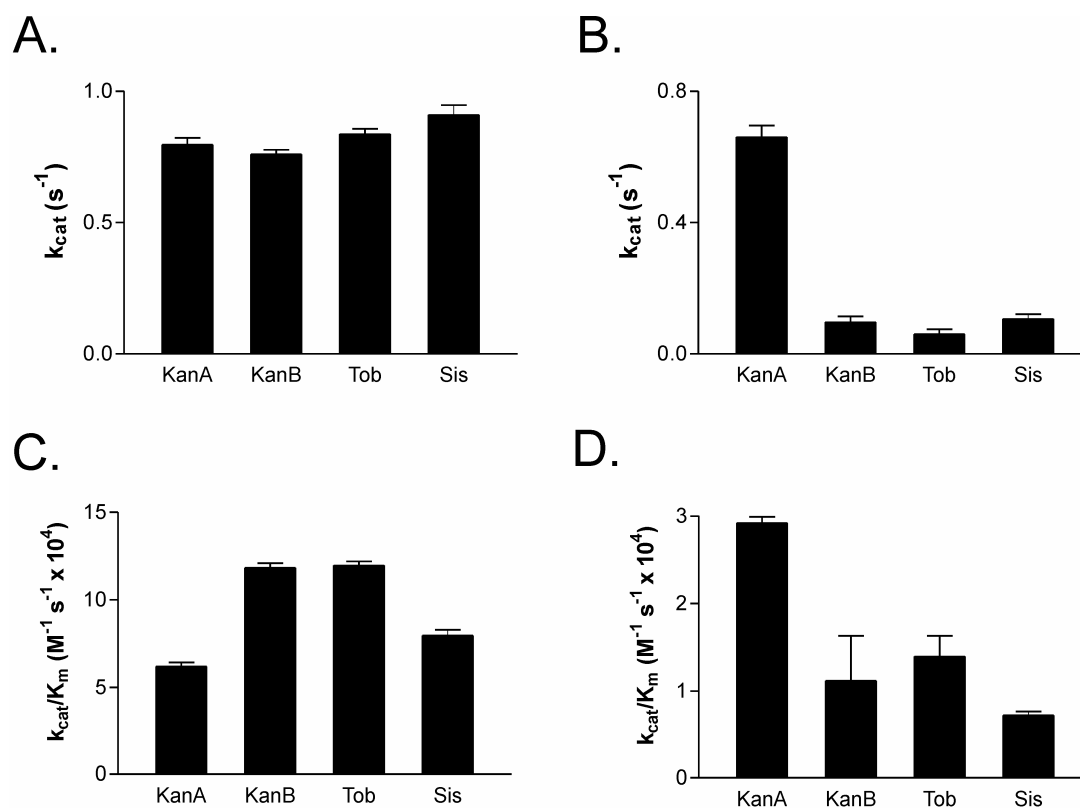


Figure 4. The substitution of manganese for magnesium as the required divalent cation alters the substrate specificity of ANT(2''). Panels A and C show the kinetic data for magnesium as the required metal. Panels B and D show the kinetic data for manganese as the required metal. The assays were performed 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 μ M and the data fit to equation 1.

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 of the 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 5 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 $23 \pm 3 \mu\text{M}$. 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 $111 \pm 24 \mu\text{M}$ 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

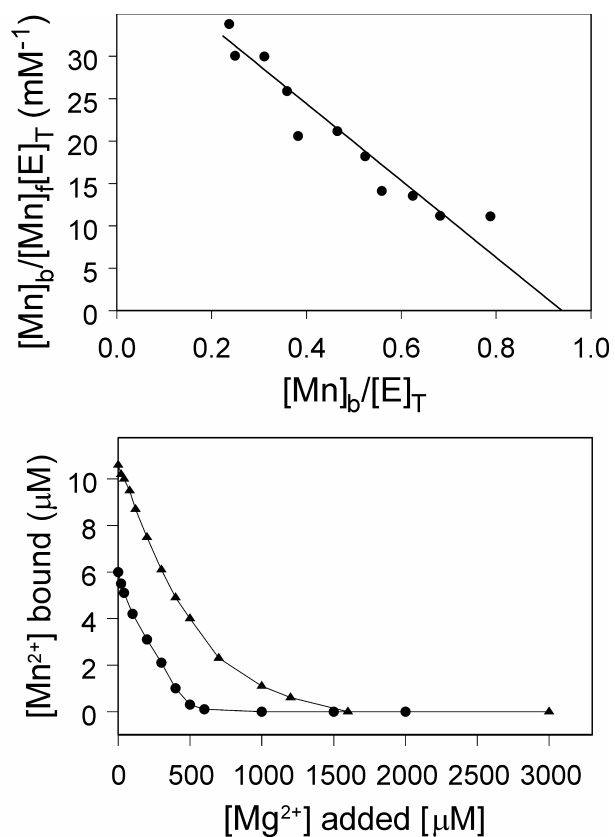


Figure 5. Scatchard analysis shows that one manganese ion binds to the enzyme with a dissociation constant of $23 \pm 3 \mu M$ (top panel). The displacement of manganese by magnesium in solutions containing 20 μM (●) and 50 μM (▲) manganese(II) and 15 μM enzyme. This data is used to determine the binding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

both divalent cations of similar size, manganese binds to the enzyme four to five times tighter than magnesium. The tighter binding by manganese to enzyme has been observed in several other 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 $8 \pm 3 \mu\text{M}$. 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 ($8 \mu\text{M}$ vs. $23 \mu\text{M}$). 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

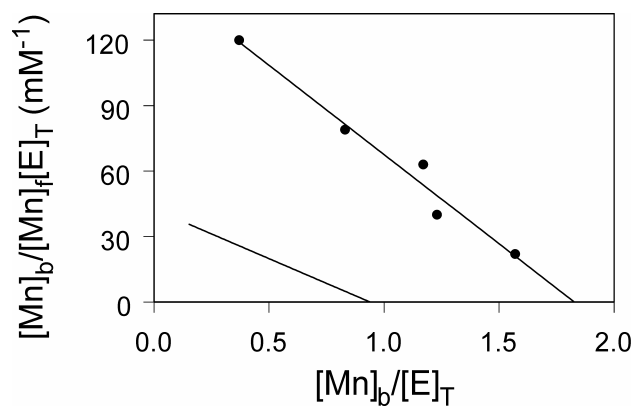


Figure 6. ATP changes the the stoichiometry from 0.94 ± 0.09 to 1.8 ± 0.3 divalent cations per molecule of enzyme. The linear curve fit for binding in the absence of nucleotide (from Figure 6, top) without data points is shown for comparative purposes.

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, can not 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. Interestingly, 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 weaker 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

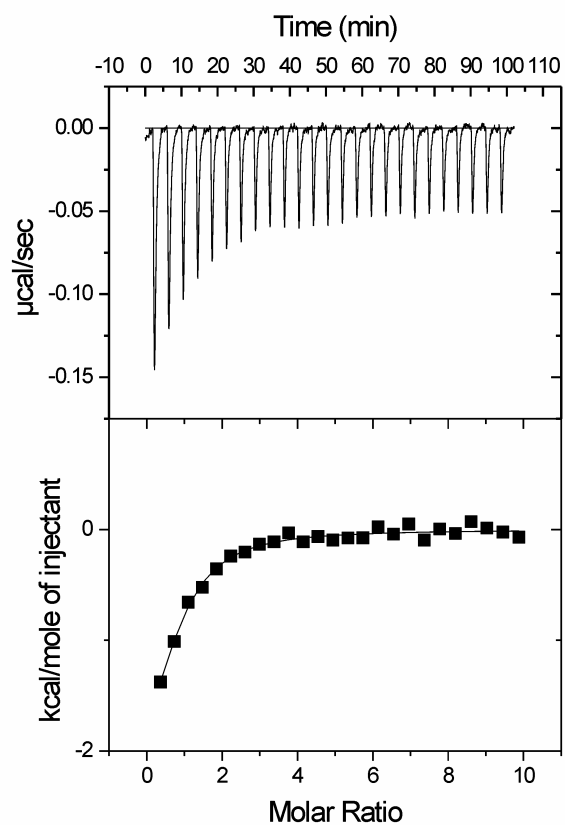


Figure 7. The binding isotherm from the titration of MgATP to enzyme in the presence of excess magnesium is shown. Reproducible results were obtained with only 5-10 μM enzyme in the sample cell to obtain thermodynamic parameters for this interaction.

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

^aContained 5 mM excess Mg^{2+}

^bContained minimal free Mg^{2+} (less than 25 μM)

^cNo heat change was observed using 2–10 mM ATP titrated into 10 μM enzyme in the absence of Mg^{2+} at 20°C.

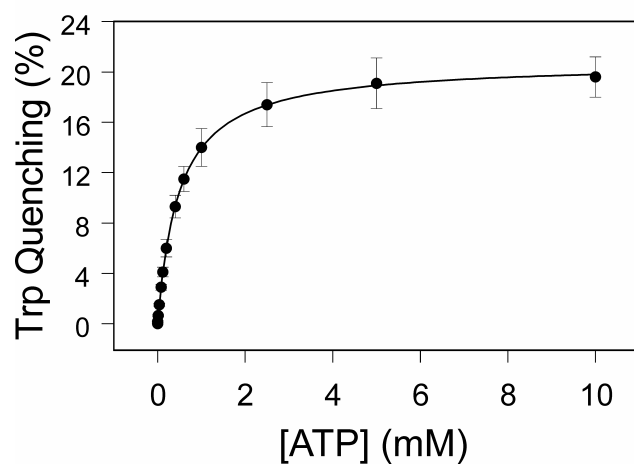
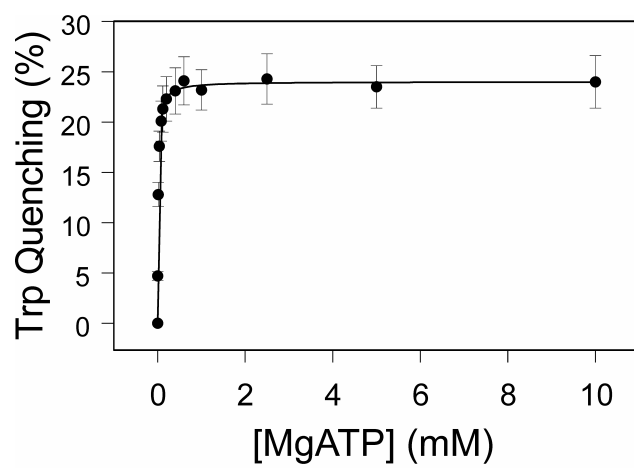


Figure 8. The change in intrinsic tryptophan fluorescence of ANT(2'') as a result of nucleotide binding shows that MgATP (top) has a much higher affinity for enzyme than free ATP (bottom).

could be attributed to the metal–nucleotide binding and not binding of free magnesium to the enzyme. A dissociation constant of $15 \pm 3 \mu\text{M}$ 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 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, the binding of tobramycin to the enzyme is entropically disfavored, which was compensated for 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 $0.64 \mu\text{M}$ was determined for the enzyme:tobramycin complex. This value is two orders of magnitude lower than the kinetically determined K_i value. The dissociation constant was reduced three-fold in the presence of saturating metal–nucleotide in the form of the non-hydrolyzable ATP analog, 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

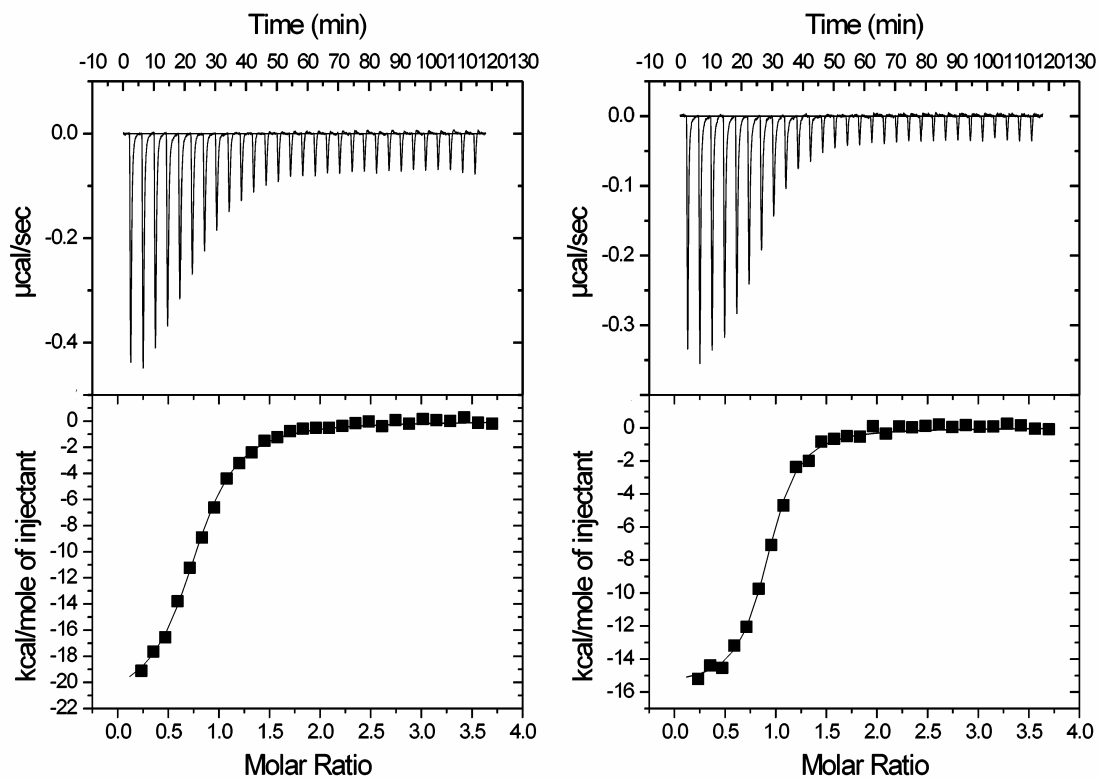


Figure 9. The binding of the tobramycin to ANT(2'') in the absence(left) and presence (right) of MgAMPCPP. The presence of metal–nucleotide increases the affinity of aminoglycoside to enzyme.

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 like kanamycin A (2' -OH) compared to tobramycin or kanamycin B (2' -NH₂). 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 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 analogs 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).

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 tighter 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). A model for the role of the two metals for ANT(2") is presented in figure 10.

The binding of metal-nucleotide also makes the binding of aminoglycoside more favorable. The presence of MgAMPCPP reduces the enthalpic contribution, however, the entropic contribution becomes more favorable and yields a slightly more favorable ΔG

Scheme I.

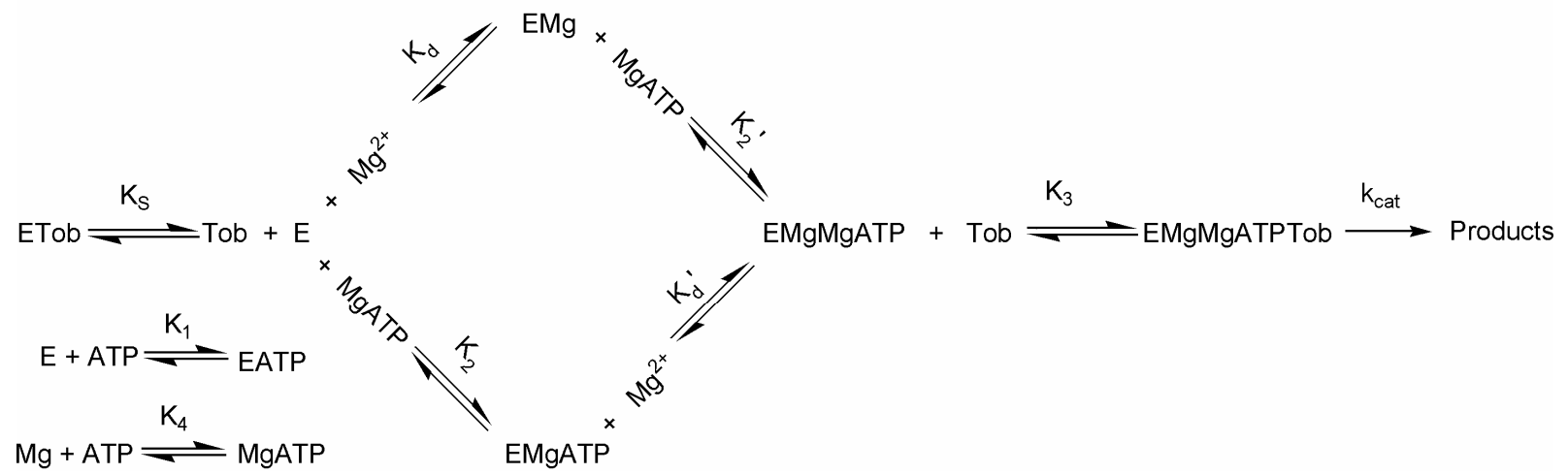


Table 3: Dissociation constants of complexes shown in Scheme 1

Parameter	Definition	K_D (μM)
K_d	$(E)(\text{Mg}^{2+}) / (E\text{Mg})$	111 ± 24^b
K_d'	$(E\text{MgATP})(\text{Mg}^{2+}) / (E\text{MgMgATP})$	38 ± 9^c
K_2	$(E)(\text{MgATP}) / (E\text{MgATP})$	18.4 ± 5.0^d
K_2'	$(E\text{Mg})(\text{MgATP}) / (E\text{MgMgATP})$	12.6 ± 3.6^d
K_3	$(E\text{MgMgAMPCPP})(\text{Tob}) / (E\text{MgMgAMPCPPTob})^a$	0.21 ± 0.08^d
K_s	$(E)(\text{Tob}) / (E\text{Tob})$	0.64 ± 0.17^d
K_1	$(E)(\text{ATP}) / (E\text{ATP})$	530 ± 40^e

^aThe ATP analog AMPCPP was used to estimate K_3

The dissociation constants were determined by ^bEPR, ^dITC, and ^efluorescence.

^cCalculated from the relation $K_2K_d' = K_4K_d$

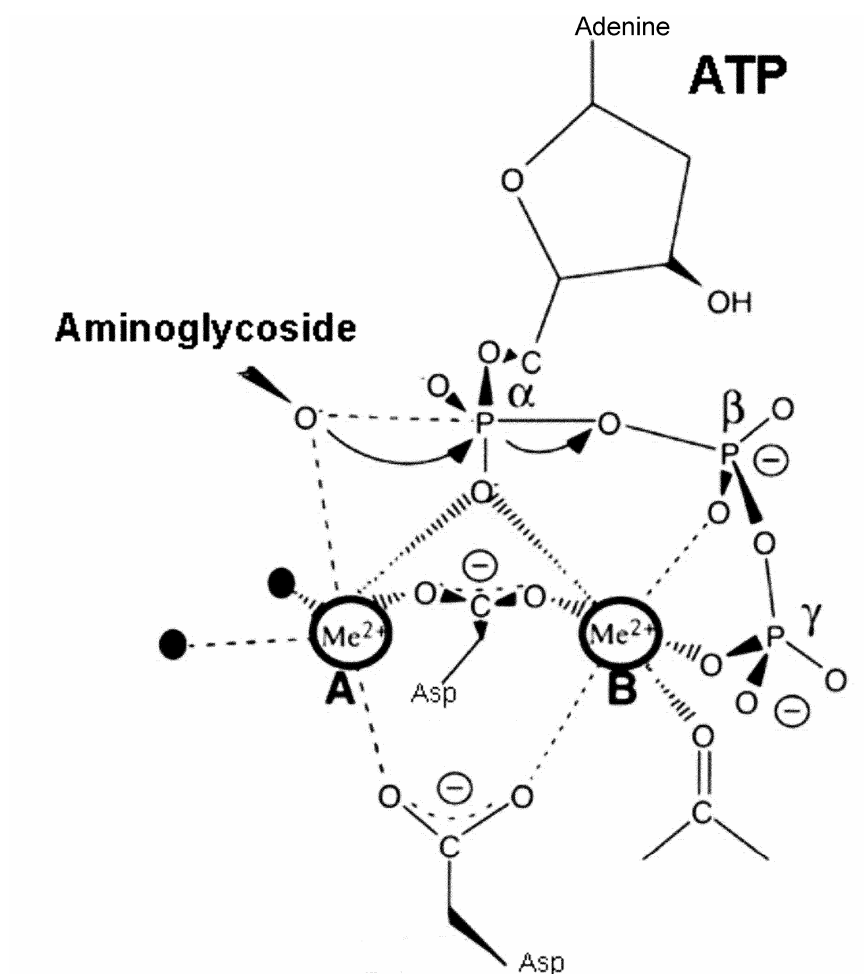


Figure 10. A model for the role of the two metals in the nucleotidyl transfer catalyzed by ANT(2''). Metal A facilitates the nucleophilic attack on the α -phosphate of ATP and metal B aids the pyrophosphate leaving group. Both metals stabilize the transition state. This figure is adapted from references 39 and 56.

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 rather 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 μ M concentration. 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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**Part IV: Thermodynamics of Aminoglycoside Binding to the Aminoglycoside
Nucleotidyltransferase(2'')-Ia**

This chapter is a slightly revised version of a manuscript submitted to *Biochemistry* in 2005.

Wright E. and Serpersu, E.H. (2005) Thermodynamics of Aminoglycoside Binding to the Aminoglycoside nucleotidyltransferase(2'')-Ia, *Biochemistry* submitted.

My contributions to this manuscript include performing all experimental procedures, conducting all data collection and data analysis, writing the initial draft of the manuscript and participating in all subsequent drafts including the final submitted manuscript

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 importance of these three positions on rings A and B of may

also provide the rationale for the ability of ANT(2'') to confer resistance against kanamycins but not neomycins.

Introduction

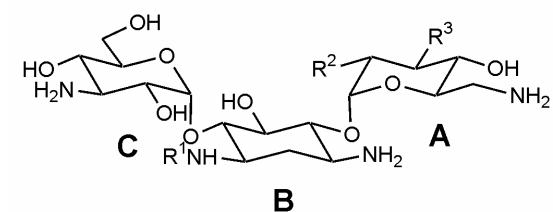
The increasing incidence of bacteria resistant to treatment with antibiotics is a major concern for the medical community. One class of antibiotics particularly susceptible to resistance are the aminoglycoside antibiotics. This group of antibiotics is important because they are often used to treat hospital-acquired 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) (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

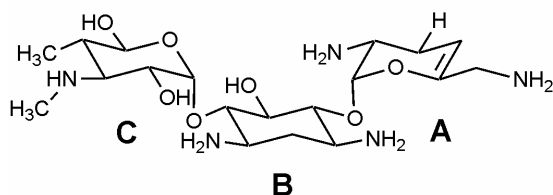
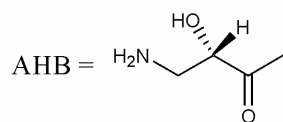
on the α -phosphate of ATP producing AMP–Aminoglycoside.(5) This enzyme provides resistance against tobramycin, kanamycin, gentamicin and other aminoglycoside antibiotics. ANT(2'') is one of the most frequently occurring AGMEs. This enzyme has been detected in a large percentage of resistant Gram-negative 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 (4,6-disubstituted). ANT(2'') is unusual in that it is one of only two clinically relevant AGMEs that modify predominantly on ring C of 4,6-disubstituted 2-deoxysreptamines (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 AAC(6')-APH(2''). This enzyme is different than ANT(2''), however in that it can also modify 4,5-disubstituted 2-deoxystreptamines. APH(2'') is capable of phosphorylating 4,5-disubstituted aminoglycosides (8). ANT(2'') is only capable of modifying 4,6-disubstituted aminoglycosides.

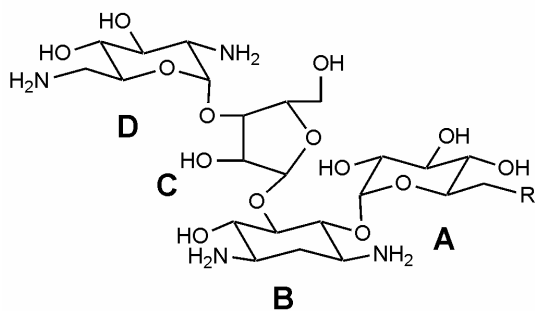
Previous studies determining the substrate specificity of ANT(2'') have used kinetic studies to compare the aminoglycoside substrates (9-12). In this work we use isothermal titration calorimetry (ITC) to directly measure the binding affinity of various aminoglycosides for ANT(2''). Thermodynamic parameters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



	R ¹	R ²	R ³
Kanamycin A	NH ₂	OH	OH
Kanamycin B	NH ₂	NH ₂	OH
Tobramycin	NH ₂	NH ₂	H
Amikacin	AHB	OH	OH



Sisomicin



	R
Neomycin B	NH ₂
Paromomycin I	OH

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.

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 MgCl₂ and 1.0 mM ATP. The concentration of tobramycin was varied from 10 to 500 μ M. The concentrations of inhibitor were 0, 0.375, 0.75 and 1.5 mM. Kinetic data were fit to equation (1) to determine the kinetic parameters and type of inhibition. Data were fit to equation (2) to determine the inhibition constants for neomycin B and paromomycin:

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

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

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 $K_{i,sub}$ is the inhibition constant for substrate inhibition. $K_{m,app}$ is the apparent K_m in the presence of competitive inhibitor and $K_{i,comp}$ 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. Both enzyme and ligand solutions were degassed under vacuum for 10 min at 15°C. Titrations consisted of 29 injections of 10 μ L and were separated by 240 s. Cell stirring speed was 300 rpm. Each titration contained 5–20 μ M enzyme in the sample cell. For binary enzyme–aminoglycoside and quaternary enzyme MgAMPCPP–aminoglycoside complexes, the aminoglycoside concentration was 60–150 μ M in the injection syringe. For the quaternary titrations the sample and ligand solutions each contained 5.0 mM $MgCl_2$ and 0.8 mM AMP-CPP. 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 0.2–0.5.

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 (n) (17). The free energy (ΔG) and entropy (ΔS) 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:

$$\Delta H_{obs1} = \Delta H_{int} + \Delta H_{ion1} \Delta n \quad (5a)$$

$$\Delta H_{obs2} = \Delta H_{int} + \Delta H_{ion2} \Delta n \quad (5b)$$

where the subscripts 1 and 2 refer to different buffers, ΔH_{obs} is the observed enthalpy change upon binding, ΔH_{ion} is the heat of ionization of the buffer and Δn 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).

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 alone (binary complex) were performed. Also, titrations of aminoglycosides to enzyme saturated with MgAMPCPP, an MgATP analog, were performed. All titrations were performed in two different buffers to determine the intrinsic enthalpy of binding (ΔH_{int}). The observed enthalpy change (ΔH_{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 (19, 20).

The results are presented in tables 1 and 2. For one of the aminoglycosides studied, amikacin, the affinity was too low to obtain accurate values for ΔH and $T\Delta S$. All binding interactions were characterized by favorable enthalpy ($-\Delta H$) and unfavorable entropy ($-T\Delta S$). 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 tobramycin, the presence of MgAMPCPP resulted in a two to threefold 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-

Table 1: Thermodynamic parameters for aminoglycoside binding to ANT(2'') (binary complex) at pH 7.5^a

	Buffer	K _D (μM)	ΔH _{obs} ^b (kcal/mol)	ΔH _{int} (kcal/mol)	-TΔS (kcal/mol)	ΔG (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	0.64	-18.2	-26.6	10.0	-8.2
	PIPES	0.83	-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 ^c	ND	ND	-5.7

^aDetermined at 293 K. Error values: K_D, 4-13%, ΔH, 1-5%. The stoichiometry of complex formation was 1.0 ± 0.2 for all titrations.

^bIntrinsic enthalpy change determined using equations 5a and 5b.

^cND, not determined.

Table 2: Thermodynamic parameters for aminoglycoside binding to ANT(2'') in the presence of MgAMPCPP and excess Mg^{2+} (Quaternary complex) at pH 7.5^a

	Buffer	K_D (μM)	ΔH_{obs}^b (kcal/mol)	ΔH_{int} (kcal/mol)	-T ΔS (kcal/mol)	ΔG (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	0.22	-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 ^c	ND	ND	-5.9

^aDetermined at 293 K. Error values: K_D , 3-15%, ΔH , 1-5%. The stoichiometry of complex formation was 1.0 ± 0.2 for all titrations.

^bIntrinsic enthalpy change determined using equations 5a and 5b.

^cND, not determined.

disubstituted aminoglycosides tested, neomycin B and paramomycin 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_{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. 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 twentyfold increase in the dissociation constant of the binary complex and an approximately thirtyfold increase in the dissociation constant in the quaternary complex. This may, however, contain additional contributions and may cause overestimation of the 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. The importance of this position for catalysis of the modification of aminoglycosides by ANT(2'') has been demonstrated previously.(12, 15) The importance of this position for binding to ANT(2'') is illustrated by comparing the ITC-determined binding parameters of kanamycin A and kanamycin B (Tables 1 and 2, Figure 2). 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'-NH₂. Kanamycin B has an approximately fivefold 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) entropy for 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 ΔH 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 (21-23). The change from an amino to hydroxyl group can alter the number and geometry of hydrogen bonds formed in the complex. This change can not only have a direct effect, it can also alter the hydrogen-bonding network in the active site. Alternatively, a positively charged 2'-amino group ($pK_a = 8.3$ in free kanamycin B) (24)

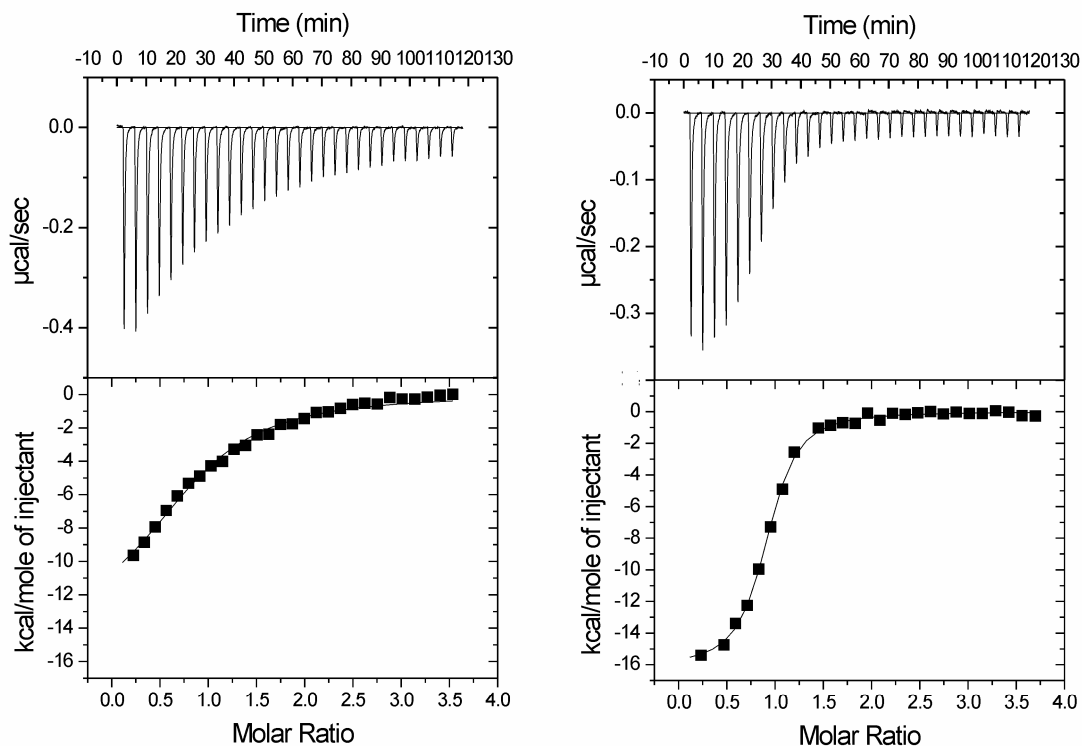


Figure 2: ITC profile of ANT(2'') in the presence of excess MgAMPCPP with Kanamycin A(left) and Kanamycin B(right). Enzyme and ligand solutions contained 50 mM HEPES pH 7.5, 50 mM KCl and 2 mM TCEP. Identical ratios of titrant to protein were used.

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. In the binary enzyme–aminoglycoside complexes the dissociation constant is at least tenfold lower for neomycin than paromomycin. In the quaternary complexes the dissociation constant is sevenfold higher for paromomycin in HEPES and tenfold 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 more disfavorable 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'-NH₂ is 8.6 in free neomycin) (25) are possible reasons 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'').

Although a change in the substituent at the 1, 2' or 6' 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

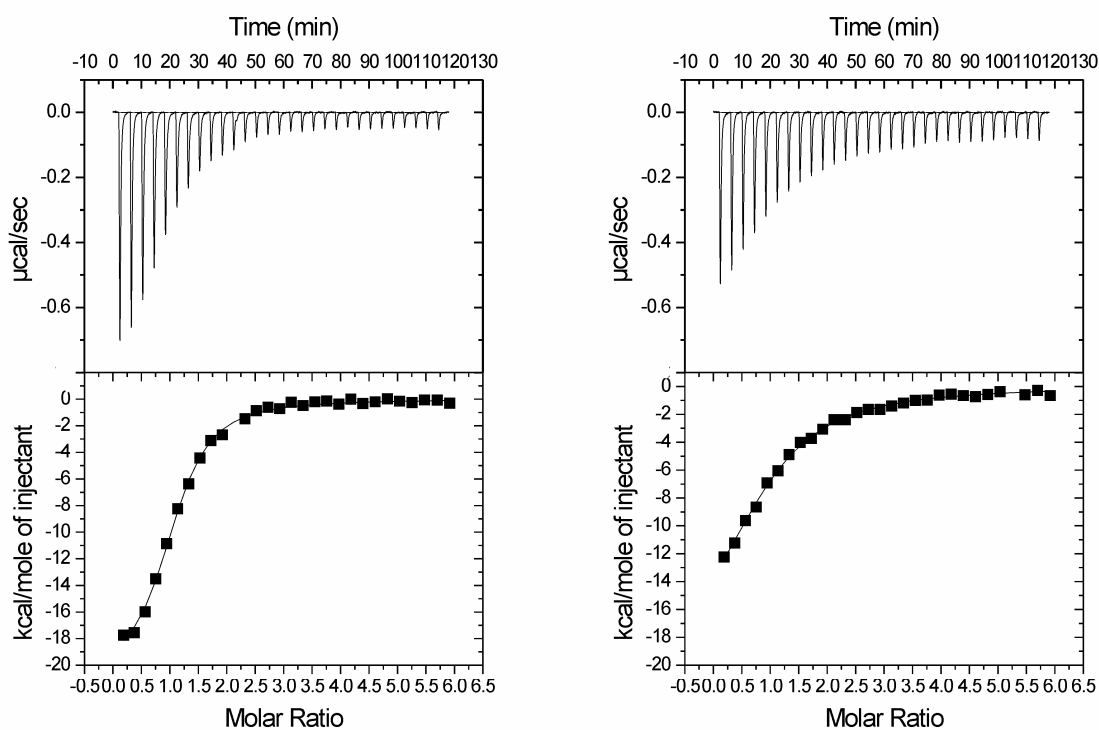


Figure 3: ITC profile of ANT(2'') in the presence of excess MgAMPCPP with neomycin B (left) and paromomycin I (right). Enzyme and ligand solutions contained 50 mM PIPES pH 7.5, 50 mM KCl and 2 mM TCEP. Identical ratios of titrant to protein were used. The experiments were performed under identical conditions.

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.

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. 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 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 they 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'') (26, 27) which led to a hypothesis that these two rings make the most important contacts with enzymes and RNA (28). Thermodynamic parameters of enzyme–aminoglycoside complexes determined in this work lend further support to this hypothesis and indicate that indeed several groups on the rings A and B of aminoglycosides make the most important contacts with ANT(2''). Functional groups on

rings A and B determine substrate specificity despite the fact that this enzyme modifies a site on ring C. Figure 4 shows kanamycin A and neomycin B superimposed at the primed and unprimed rings as described earlier.⁽²⁷⁾ The sites of interaction on these rings are indicated by arrows. Thus, even though the site of modification for ANT(2'') is on ring C, these interactions anchor these two rings in the active site. In the case of 4,6-disubstituted aminoglycosides, this positions the hydroxyl at the 2'' position for nucleophilic attack on the α -phosphate of ATP. For 4,5-disubstituted aminoglycosides, no hydroxyl is close enough for interaction with ATP (Figure 4). 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.

There is no structural information available for ANT(2''). Therefore, assigning specific interactions to the changes in enthalpy, entropy and free energy are not possible. Also, 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.

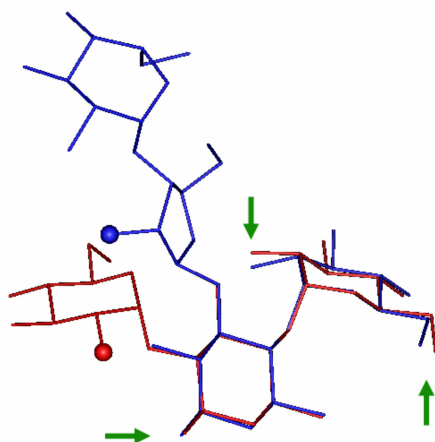


Figure 4: Kanamycin A (red) and neomycin B (blue) from the crystal structure of APH(3')(29) superimposed as described in reference 27. The A and B rings superimpose with enzyme-bound conformations determined by NMR spectroscopy with an RMSD of less than 0.18 Å. Positions (1-, 2'- and 6') determined in 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.

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Part V. Effects of Mutation and Modification of Cysteine Residues

Abstract

Stepwise removal of the cysteine residues in aminoglycoside nucleotidyltransferase (2'')-Ia by site-directed mutagenesis was done in an attempt to improve the folding characteristics of this protein. Also the importance of the cysteine residues for folding and activity were assessed. Six mutants of ANT(2'') containing five, four, three, two, one and no cysteines were compared to the wild type enzyme. Removal of cysteines did not increase the level of ANT(2'') found in the soluble fraction of cell lysate when the protein was expressed in *E. coli* BL21(DE3). Circular dichroism spectra indicated that all mutants had a similar fold to wild type. However, mutants lacking cysteine at position 136 had less than 10% of wild type activity. The use of reducing agents had only a minor effect on wild type enzyme activity while the effect of sulfhydryl alkylating agents was comparable to the effect of mutation of residue 136. These results suggest that C136 is involved in substrate binding or catalysis rather than a disulfide bond.

Introduction

Aminoglycoside nucleotidyltransferase contains seven cysteine residues of 226 in the amino acid sequence (Figure 1). In an attempt to improve the solubility and stability of the protein for structural studies the cysteine residues were replaced with either alanine or serine. The rationale for this approach is twofold. First, the aggregation of folded ANT(2'') could be a result of the formation of intermolecular disulfide bonds. Second, the initial folding of the enzyme in vivo may be slowed by the presence of cysteines. This slow rate of folding coupled with overexpression of the protein could be the cause of

³MAC⁶CYDCFFVQ SMPRASKQQA RTAVGR²⁷CLML WSSNDVTQQG SRPKTKLGRM
 DTTQVTLIHK ILAAADERNL PLWIGGGWAI DARLGRVTRK HDDIDLTFPG
 ERRGELEAIV EMLGGRVMEE LDYGFLAEIG DELLD¹³⁶CEPAW WADEAYEIAE
¹⁵⁶APQGSCPEAA EGVIAGRPVR ¹⁷¹CNSWEAIIWD YFYYADEVPP VDWPTKHIES
²⁰⁵YRLACTSLGA EKVEVLRAAF RSRYAA

Figure 1: The amino acid sequence for ANT(2'')-Ia. Cysteine residues are underlined and in bold type.

inclusion body formation. Also the efficiency of folding from inclusion bodies could be improved by removing cysteine residues. A more rapid rate of refolding could decrease the incidence of aggregation caused by hydrophobic interaction between hydrophobic portions of the protein which are buried in the native structure.

Also because the cellular location of aminoglycoside-modifying enzymes is still controversial (1-3) the possibility of disulfide bonding has to be considered. Whether the enzymes are present in the cytoplasm where the reducing environment would inhibit disulfide formation or in the periplasmic space where disulfide formation can occur has not been resolved. However because of the limited availability of ATP in the periplasmic space ANT(2") is probably located in the cytoplasm.

The mutations were done in a stepwise manner instead of individually because the initial aim was to generate an active ANT(2") removing all non-essential cysteines. The first two cysteines (residues no.3 and 6) were changed to alanines creating an ANT(2") with only five cysteine residues. One by one each remaining cysteine was changed to a serine residue. This process ultimately resulted in an ANT(2") containing no cysteines. The individual mutants and the nomenclature used to describe each are outlined in table 1.

Materials and Methods

Materials. All chemicals were of the highest purity commercially available. All reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO) except for inorganic pyrophosphatase purchased from Roche Diagnostics Corp. (Indianapolis, IN), dithiothreitol (DTT) and isopropylthiogalactopyranoside (IPTG) purchased from Inalco

Table 1: Nomenclature used for the cysteine mutants discussed in this section

Name	Mutation
5 cys	C3A C6A
4 cys	C3A C6A C27S
3 cys	C3A C6A C27S C136S
2 cys	C3A C6A C27S C136S C156S
1 cys	C3A C6A C27S C136S C156S C171S
No cys	C3A C6A C27S C136S C156S C171S C205S

Pharmaceuticals (Milan, Italy) and *E. coli* BL21(DE3) and pET 22b(+) purchased from Novagen (Madison, WI).

Cloning and expression. The original cloning of ANT(2") has been described previously(4, 5). The original pET22b(+) vector engineered to produce ANT(2") was mutated to replace cysteine residues with either alanine or serine residues by Bio S&T, Inc. (Montreal, Canada). These constructs were transformed into *E. coli* BL21(DE3). The cells containing recombinant plasmid were selected on LB plates containing 100 mg/L ampicillin. A single colony from each construct was grown 12 h in 10 mL of LB containing 100 mg/L ampicillin. These cultures were then transferred to 1 L of LB medium and grown to an OD₆₀₀ of 0.4. After induction the cells were harvested as described previously (5). All mutants of ANT(2"), similar to wild type, were found to predominantly in the insoluble fraction of the cell lysate as inclusion bodies. The protein for each mutation was isolated as described previously(5).

Enzyme activity assays. Activity assays for ANT(2") were performed as described previously. For determination of the concentration dependent inactivation of ANT(2") by iodoacetamide, *N*-ethylmaleimide (NEM) or dithiothreitol (DTT) the enzyme was incubated for 30 min with either iodoacetamide, NEM or DTT at 30°C. The enzyme was then kept on ice for 15 min prior to the start of the assay. For determination of kinetic parameters for ANT(2") with extensive alkylation of cysteine residues, 25 µM protein was incubated with 50 mM iodoacetamide for 1 h under nitrogen at room temperature. The enzyme was dialyzed 12 h against 50 mM HEPES pH 8.0 to remove excess iodoacetamide.

Circular dichroism spectroscopy. All circular dichroism (CD) spectra were collected on an Aviv (Lakewood, NJ) model 202 spectrometer. All spectra were collected at 25°C. The CD signal was monitored from 260 nm to 190 nm. All data represent the average of five scans. The data was converted to percentage α -helix by CDNN CD spectra deconvolution software version 2.0.

Results and Discussion

The mutants all expressed primarily as insoluble inclusion bodies (figure 2). After refolding from inclusion bodies each mutant had comparable CD spectra. Deconvolution of these spectra indicates that each mutant contained a similar percentage of α -helix (table 2). The similar CD spectra suggest that all mutants have a overall structure. All mutants, however, were not capable of enzymatic modification of aminoglycosides. The 5 cys and 4 cys mutants displayed activity similar to wild type enzyme. The 3 cys, 2 cys, 1 cys and no cys mutants all displayed less than 10% of wild type activity (figure 3). From these results cysteine 136 is necessary for the activity of ANT(2"). There are two possible reasons for the requirement of cysteine 136. First, this residue could be necessary for a required disulfide bond. Second, this residue could directly be involved in binding of substrates or catalysis. To test these two possibilities activity assays of the wild type protein under reducing conditions to reduce any disulfides and under conditions in which free cysteines would be alkylated were performed.

First ANT(2") activity in the presence of increasing concentrations of the reducing agent, DTT, was tested. Increasing the concentration of DTT from 0 to 5 mM caused an initial decrease in activity (figure 4). However, increasing the concentration from 5 to

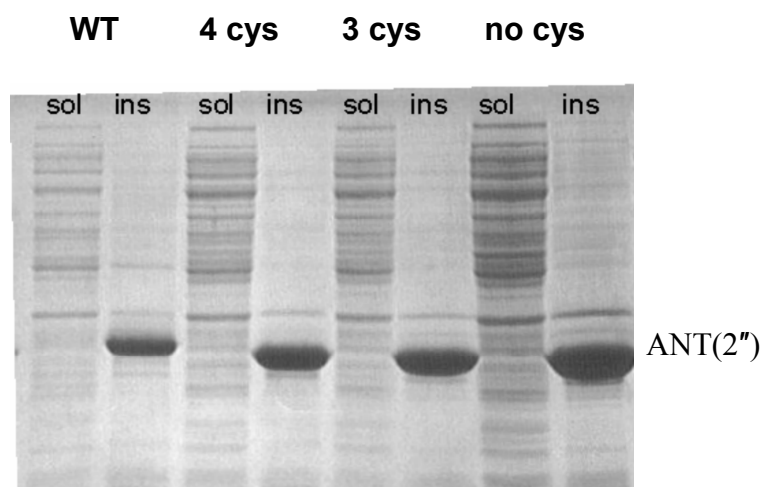


Figure 2: The majority of the protein expressed for all mutants and wild type is recovered in the insoluble fraction of cell lysate. The soluble (sol) and insoluble (ins) fractions were run on a 10% SDS-PAGE after separation by centrifugation for 15 min at $12,000 \times g$.

Table 2: Percentage α -helix in wild type and mutant ANT(2'') as determined by circular dichroism

	Percentage α -helix ^a
Wild Type	38
5 cys	34
4 cys	41
3 cys	40
2 cys	33
1 cys	42
No cys	38

^aDetermined as described in materials and methods. The standard error is $\pm 10\%$.

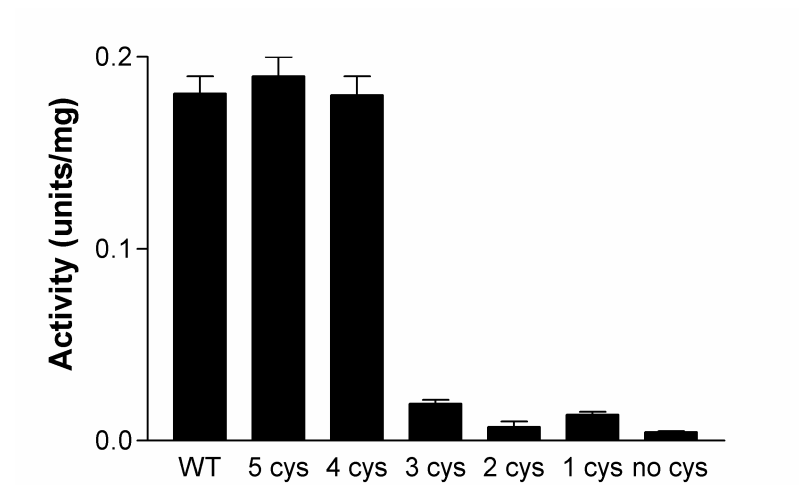


Figure 3: The activity of cysteine mutants determined at pH 8.0. Each assay contained 1.0 mM MgATP and 40 μ M tobramycin.

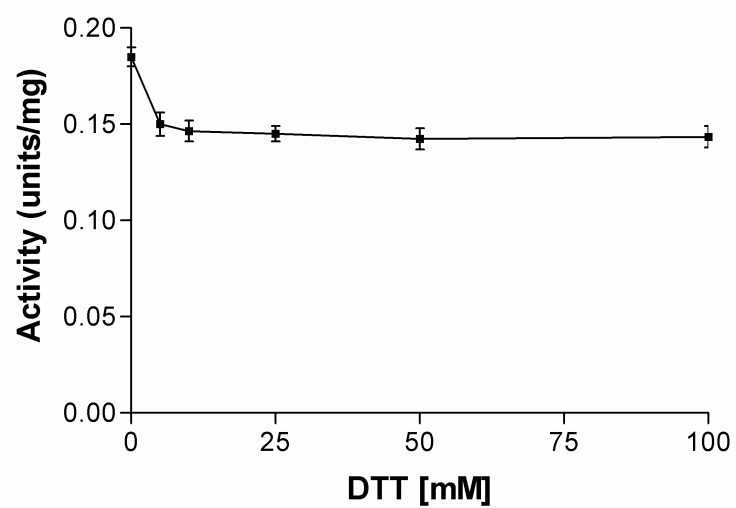


Figure 4: The activity of wild type ANT(2'') after incubation with indicated concentration of DTT for 30 min at 30°C. Activity determined at pH 8.0. Each assay contained 1.0 mM MgATP and 40 μ M tobramycin. DTT was added to assay mixture to maintain concentration at indicated levels.

100 mM caused no further loss of activity. These results lead to the conclusion that the loss in activity in the 3 cys mutant is not due to the inability to form an essential disulfide bond. If that were the case, the loss of activity under extreme reducing conditions (100 mM) would be comparable to the loss of activity in the 3 cys mutant. To eliminate the unlikely possibility of the existence of a buried disulfide inaccessible to reducing agent the protein was refolded in 100 mM DTT after denaturation with 8 M urea. This refolded protein retained 75% of activity compared to standard assay conditions (1 mM DTT). This result compared favorably with the results shown in figure 4 in which the addition of 100 mM DTT to folded protein led to approximately 80% of normal activity. These results provide more evidence that the loss of activity in the 3 cys mutant is not a consequence of the loss of an essential disulfide bond.

To test whether the loss of activity in the 3 cys mutant results from involvement of a cysteine residue in substrate binding or catalysis two sulfhydryl-modifying reagents were employed, iodoacetamide and NEM. First alkylation of cysteine residues was performed by incubation with a large excess (>250-fold) of iodoacetamide followed by removal of unreacted iodoacetamide by dialysis. This process reduced activity ($V_{\max}$) from 0.18 units/mg to less than 0.01 units/mg. Because iodoacetamide at very high concentrations can modify other residues the concentration dependence was investigated. The loss in activity due to alkylation by iodoacetamide was found to be concentration dependent with a 50% reduction at approximately 5 mM iodoacetamide (figure 5, top). Another alkylating agent, NEM, also inactivated ANT(2") in a concentration dependent manner (figure 5, bottom). Finally to determine the role of cysteine in ANT(2") catalysis,

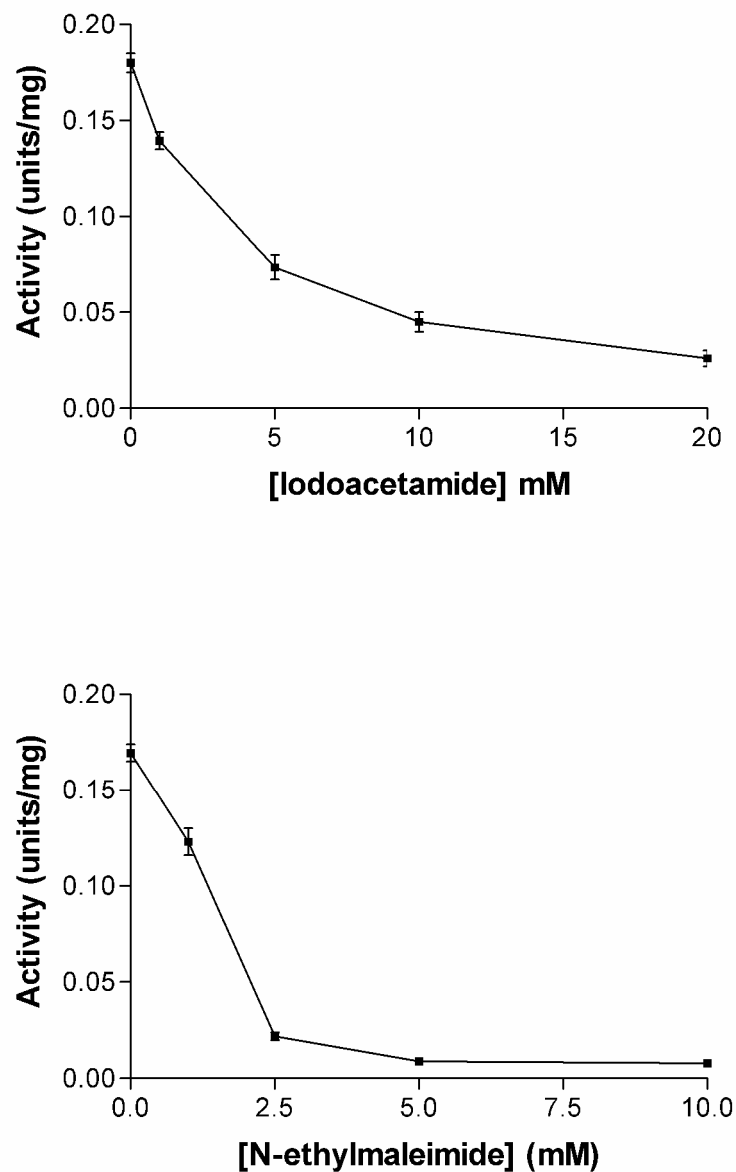


Figure 5: The activity of wild type ANT(2'') after incubation with indicated concentration of iodoacetamide (top) or *N*-ethylmaleimide (bottom) for 30 min at 30°C. Activity determined at pH 8.0. Each assay contained 1.0 mM MgATP and 40μM tobramycin.

the substrates were tested for the ability to protect against inactivation by iodoacetamide. Preincubating the enzyme with increasing concentrations of Mg^{2+} had no effect on the inactivation by iodoacetamide. Preincubation with increasing concentrations of MgATP, however, did protect against inactivation by iodoacetamide (Figure 6). Data from protection assays preincubation with aminoglycosides was not possible to interpret because of substrate inhibition at aminoglycoside concentrations necessary for the experiment. This data suggest that cysteine 136 is located in the MgATP binding site. However binding of MgATP may cause a conformational change in the enzyme which could also explain the protection against inactivation.

Conclusions

Although this data indicates that C136 is important for MgATP binding further work is necessary to confirm the role of this residue. Binding experiments using wild type ANT(2") and the cysteine mutants need to be performed. Isothermal titration calorimetry experiments to study binding of MgATP and aminoglycosides need to be undertaken to directly determine the effect of the mutations. Also electron paramagnetic resonance to observe metal binding and change in fluorescence to observe ATP binding should also be completed. If binding of all substrates and cofactors is diminished in the inactive mutants then the global fold of the enzyme is probably defective. If a single binding event is affected in the inactive mutant the role of C136 can be explained.

If it is determined that the no cys mutant is capable of some functions (e.g. aminoglycoside binding) then more detailed work will be necessary such as ^{15}N -HSQC NMR experiments to determine if it has similar structural properties to wild type

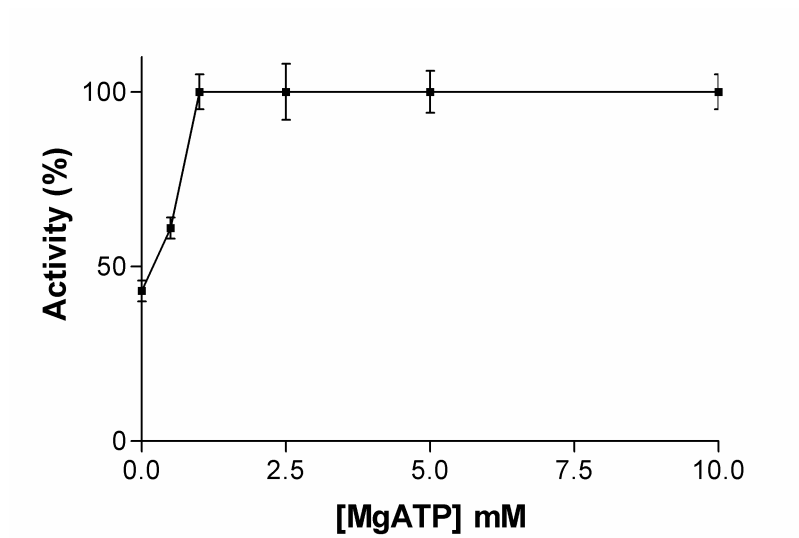


Figure 6: The activity of wild type ANT(2'') after incubation with indicated concentration of MgATP for 5 min followed by 20 min with 5 mM iodoacetamide at 30°C. Activity determined at pH 8.0. Each assay contained 1.0 mM MgATP and 40μM tobramycin.

ANT(2"). If the no cys mutant has improved solubility and stability it may be a better candidate for structure determination than wild type ANT(2"). Also the solubility and stability of the 4 cys mutant should be determined relative to wild type ANT(2"). This mutant removes the most cysteines while retaining activity. This mutant may also be a better option than wild type for structural studies. Finally if removal of cysteines does improve solubility and stability one should consider reintroducing C136 into the no cys mutant. The resulting mutant may have improved properties for structure determination while retaining enzymatic activity.

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Part VI: Conclusion

Overview

Previous work with ANT(2'') included determination of the resistance profile, monitoring the frequency and spread of this enzyme, kinetic characterization with impure or not well-defined forms of the enzyme and an enzyme-bound substrate conformation using isepamicin. We have developed an isolation technique to obtain enzyme with a high level of purity. This has led to the first kinetic studies with a well-defined form of the enzyme and the first thermodynamic characterization of ANT(2''). The work presented in this thesis should form a basis for future thermodynamic and structural studies to better understand the interactions between ANT(2'') and aminoglycoside antibiotics.

Future Work

The next stage of this project should consist of further thermodynamic characterization of this enzyme using ITC and development of a method to increase the solubility of ANT(2'') so that structural and dynamic studies are feasible. ITC has proven to be a reliable technique to obtain thermodynamic data with ANT(2''). Further characterization of interactions with aminoglycosides should include studies of binding as a function of pH, temperature, ionic strength and osmotic stress.

Binding as a function of pH can be used not only to determine the pH optimum for binding, but also can be used to determine any changes in pK_a 's of important ionizable groups upon binding (*I-4*). The change in binding enthalpy as a function of temperature can be used to determine the heat capacity change (ΔC_p) due to binding of ligand. This change in heat capacity is thought to originate primarily from the change in solvent

accessible surface area (5) . Binding as a function of ionic strength can be used to determine the importance of ionic interactions. The number of groups participating in ionic interactions can also be estimated using this method (6). Finally binding affinity as a function of osmolality can be used to evaluate the role of water in binding interactions by determining the net uptake or release of water (7, 8).

For many of the experimental methods necessary for characterization of this enzyme the concentration of enzyme needs to be greater. Characterization of this enzyme should include structure determination by X-ray crystallography or NMR. Another goal of this project is to study the dynamics of ANT(2") by NMR. Both of these aims will require a higher protein concentration than is currently obtainable. Also the protein is relatively unstable and begins to lose activity after 60 h at 4°C and even more quickly at higher temperatures. Two approaches should be considered to improve these properties.

First the buffer which provides maximum stability and solubility should be determined in a quantitative manner. A screening technique similar to the one employed for determination of the optimal refolding buffer should be used to find the best storage buffer. Variables such as pH, salt composition and concentration and reducing agent composition and concentration should be tested. Additionally agents known to increase protein stability such as sugars and alcohols should also be tested.

Another approach is to find conditions to produce soluble protein *in vivo* rather than isolating ANT(2") from inclusion bodies. Several cell lines designed to control expression were tested, induction using different levels of IPTG (0-1 mM w/v), and expression at different temperatures (10-30°C). These conditions altered the level of total protein but did not change the ratio of insoluble to soluble protein recovered from the cell

lysate. The expression vector, pET22b(+), has not been changed. Cloning the gene for ANT(2'')-Ia into other expression vectors should be explored. Expression vectors which produce the protein of interest as a fusion protein with maltose-binding protein should be tested. This method has been shown to increase production of soluble protein in some cases (9, 10).

Implications for Combatting Antibiotic Resistance

The results from kinetic and thermodynamic studies show that the functional groups at the 1, 2' and 6' positions are important for aminoglycoside binding to ANT(2''). These three positions are also important for binding to the other two aminoglycoside-modifying enzymes that have thermodynamic data available, AAC(6')-Iy and APH(3')-IIIa (11, 12). This information in addition to the previous observation that the A and B rings of aminoglycosides adopt the same conformation in the active site of different aminoglycoside-modifying enzymes suggest a common recognition motif for aminoglycoside binding (13). The structural and thermodynamic aspects of rRNA–aminoglycoside interactions have been described extensively (14-20). Rings A and B of aminoglycosides adopt a similar conformation bound to either RNA or AGMEs. Positions 2' and 6' also make important contacts with RNA. Therefore generating new aminoglycosides lacking the functional groups necessary for tight binding to resistance enzymes will not be effective antibiotics (21). Another option to combat resistance due to ANT(2'') is modification of the 1 position. However, the introduction of amikacin, which has an alkyl group attached to position 1 has led to the emergence of resistance enzymes which modify this aminoglycoside (22). So the introduction of semi-synthetic

aminoglycosides which are not modified by the more prevalent resistance enzymes is an ineffective long-term strategy for preventing AGME-mediated resistance. The best strategy may be to produce inhibitors of aminoglycoside antibiotics to be co-administered with aminoglycosides or to develop novel antibiotics based on the mode of action of aminoglycosides but lacking the aminoglycoside framework. The first non-carbohydrate inhibitor of ANT(2'') competitive with the aminoglycoside substrate has already been produced using this strategy (23). The information from aminoglycoside interactions with ANT(2'') as well as thermodynamic and structural information from other aminoglycoside-modifying enzymes can be used to design inhibitors effective against different classes of AGMEs or to design new antibiotics that bind to bacterial rRNA and disrupt translation but are not susceptible to resistance enzymes.

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Vita

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