

Comparison of Activation Enthalpies for Aminoglycoside Modification Reactions

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DEDICATION

This Thesis is dedicated to my parents, Andrea-Lee Friedman and Ron Soto, who have provided a wealth of love and support throughout my academic career. Along with my parents, this Thesis is dedicated to George Ashison, my former childhood soccer coach, and Janet Bayes, a family friend. These two individuals have had a profound impact on my personal development and have been long standing role models throughout my life.

“An investment of Knowledge pays the best interest”

-Aristotle

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ABSTRACT

At highly elevated temperatures, many biological reactions can proceed spontaneously from the ground state to the transition state. However, due to the long half-life of these reactions, catalysts are required to catalyze these reactions at modern day temperatures by lowering the activation energy. Wolfenden et al. has previously shown that catalysts enhance the rate of the reaction by reducing the enthalpy of activation. Therefore, the activation energies have been determined for three aminoglycoside modifying enzymes, APH(3')-IIIa, AAC(3)-IIIb, and AAC(3)-VIa, to determine whether these three enzymes distinguish between the two classes of aminoglycoside antibiotics by reducing the enthalpy of activation during catalysis.

Aminoglycosides are broad-spectrum antibiotics active against Gram-positive and Gram-negative organisms. These antibiotics contain a 2-deoxystreptamine ring and are classified into two classes: the 4,6-di-substituted kanamycin-like aminoglycosides and the 4,5-di-substituted neomycin-like aminoglycosides. Since their discovery in the 1940s, bacteria have shown a high level of resistance to these antimicrobial agents mainly due to the emergence of aminoglycoside modifying enzymes (AGMEs). AGMEs are classified into three main families based on the type of modification reaction: (a) *O*-Nucleotidyltransferases (ANT) catalyze the adenylation reaction of hydroxyl groups in the presence of ATP; (b) *O*-Phosphotransferases (APH) phosphorylate hydroxyl groups in the presence of ATP; (c) and *N*-Acetyltransferases (AAC) which acetylate an amino group on the 2-deoxystreptamine ring or aminohexose sugar rings in the presence of acetyl coenzyme A as the acetyl donor.

APH(3')-IIIa and AAC(3)-IIIb are two very promiscuous AGMEs while AAC(3)-VIa has a limited substrate profile. In general, there were no observed trends in the activation energies that distinguished between the two classes of aminoglycoside antibiotics for APH(3')-IIIa and AAC(3)-IIIb. When studying the activation energies for a more hydrophobic aminoglycoside, gentamicin C₁ [C1] and C₂ [C2], AAC(3)-IIIb appeared to favor the more methylated gentamicin component, gentamicin C₁ [C1], whereas AAC(3)-VIa was shown to have a lower activation energy for gentamicin C₂ [C2].

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CHAPTER I: INTRODUCTION

A. Temperature-Dependent Rate Enhancements Produced by a Catalyst

I. Chemical Reaction Pathway

Spontaneous chemical reactions proceed from the initial collision of molecules at the ground state to the transition state, the highest energy state, and proceed to product formation. Shown in figure 1, an uncatalyzed reaction (red line) must overcome a substantially high activation energy (kinetic barrier) before reaching the transition state, thereby having an overall unfavorable reaction pathway. Due to an extremely high kinetic barrier, many biological reactions require a catalyst to reduce the activation energy (green line). Without the presence of catalysts, many necessary biological reactions will spontaneously occur but not within a relevant time frame due to a high half-life for the uncatalyzed reaction. Such reactions have been shown with peptide hydrolysis, which has a half-life of ~500 years or α -glucosidase, which has a half-life of 10^{14} years^{1,2,3,4}.

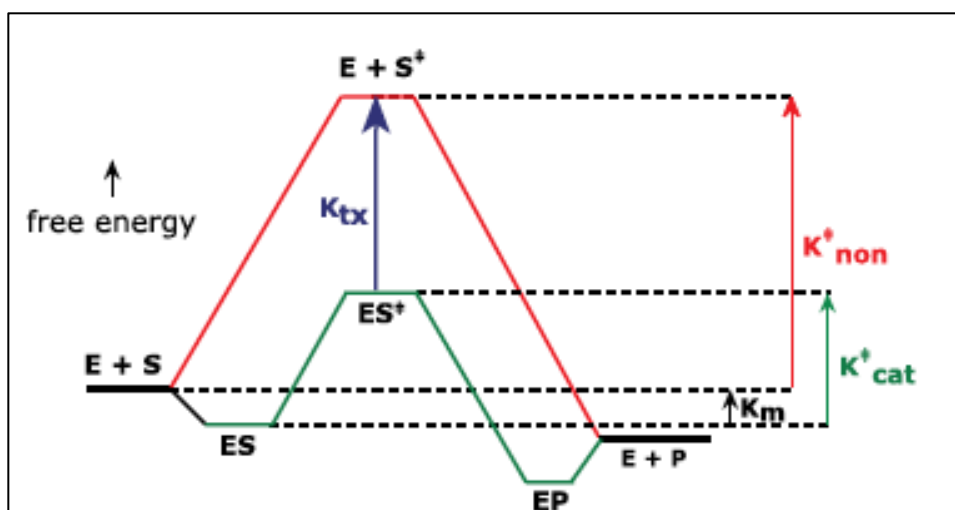


Figure 1: Free energy diagram for enzyme catalysis, showing the activation energy to the transition state required for a nonenzymatic reaction (red) and in the presence of a catalyst (green)².

II. Rate Enhancements produced by Catalysts

Two generalizations about rate enhancements produced by catalysts have been summarized by Wolfenden et al.: (a) the rates of chemical reactions increase as temperature increases; (b) the rate of product formation from the transition state doubles as temperature increases by 10°C. For these generalizations to hold true, catalyzed and uncatalyzed reactions would exhibit similar heats of activation, mainly associated with a more favorable entropy of activation. Therefore, Wolfenden et al. conducted a series of studies with biological catalysts to examine the role of enthalpy and entropy of activation in enzyme catalysis¹. When the enthalpy and entropy of activation values were plotted for numerous spontaneous biological reactions, the spontaneous reactions were observed to have a significant change in the enthalpy of activation while the entropy of activation remained fairly constant. These observations suggested that primordial enzymes enhanced the rate of the reaction by lowering the enthalpy of activation thereby improving the effectiveness of the catalyst. Furthermore, it has been proposed that the rate enhancement corresponded with an increase in affinity to the transition state and that the effectiveness of primordial enzymes improved as the earth cooled. When the earth first formed ~4.6 million years ago, biological reactions may have proceeded spontaneously due to extremely high temperatures. As the earth cooled to modern day temperatures, enzymes required for biological reactions may have increased their efficiencies to catalyze the reactions by reducing the enthalpy of activation. One example that supports this hypothesis is the temperature dependence of glycoside hydrolysis, where the rate enhancement was achieved by reducing the enthalpy of activation for the catalyzed reaction. In the presence of α -glucosidase, the rate enhancement for the catalyzed reaction was much faster at ordinary temperatures compared to the spontaneous reaction. Illustrated in figure 2, the rate enhancement for the catalyzed reaction improved as temperatures decreased. Intuitively, the increase in rate enhancement at lower temperatures would be consistent with the cooling of the earth to present day temperatures^{1,2,3}. An exception to the enzymes that reduced the enthalpy of activation was peptidyl transferase, an enzyme that is specific to the ribosome for peptide bond formation in protein synthesis^{1,2}. For this catalyst, the rate enhancement was achieved solely by a favorable entropy of activation rather than a favorable enthalpy of activation. This phenomenon appeared to be consistent with substrate specific

enzymes whereas the rate enhancement produced by catalysts was more associated with the reduction of activation enthalpy. Overall, catalysis appears to be driven by the enthalpy of activation, which may provide further support to the thermodynamic behavior of enzymes that bind tightly to the active site by inducing conformational changes and formation of new electrostatic interactions and hydrogen bonds in the transition state^{1,2,3,4}.

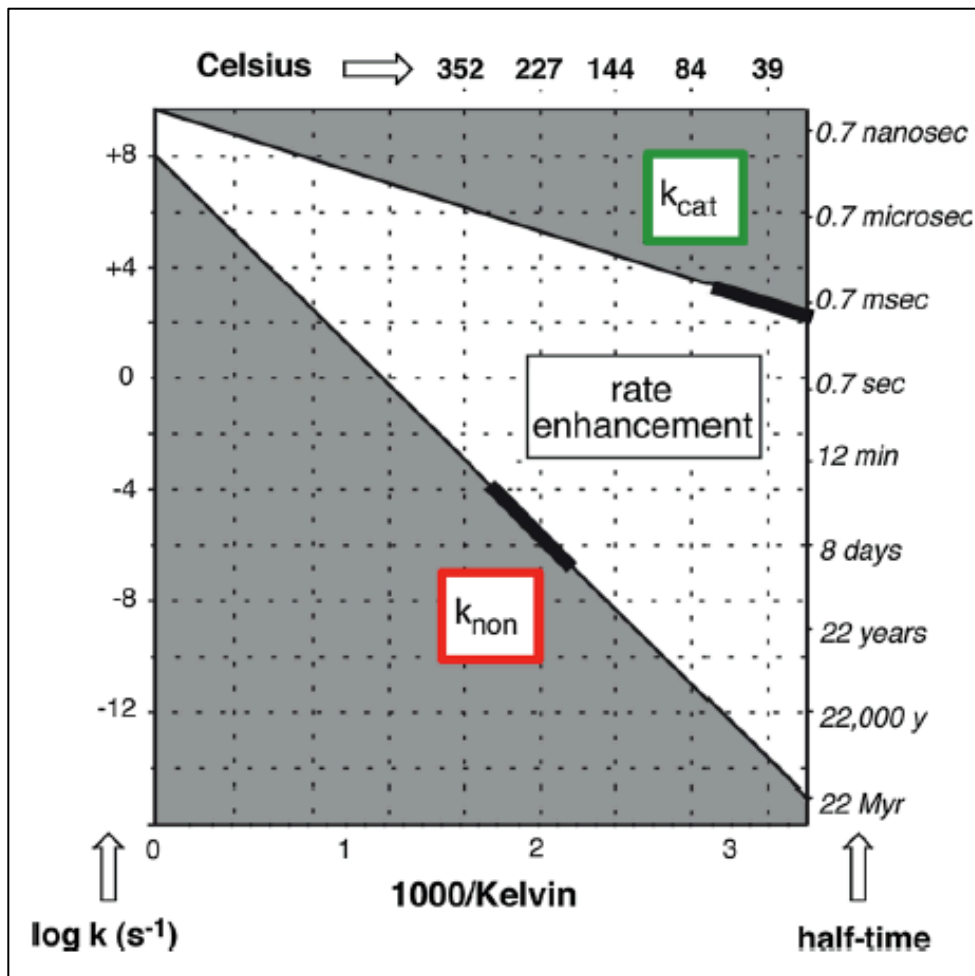


Figure 2: The effect of temperature on the rate enhancement for the catalyzed and uncatalyzed reaction for glucoside hydrolysis. The rate enhancement by α -glucosidase was achieved by reducing the enthalpy of activation and was more pronounced at lower temperatures (white wedge). The bold lines indicate the temperature range at which the reactions were measured^{1,2}.

B. Aminoglycoside Antibiotics

I. Discovery of Aminoglycosides and Chemical Structure

Aminoglycoside antibiotics are broad-spectrum antibacterial agents used mainly to treat bacterial infections caused by Gram-positive and Gram-negative bacteria. They have also been shown to be effective against ameboid or protozoal infections and are inhibitors of HIV-1 production. Aminoglycosides are a large family of hydrophilic, highly polar, polycationic molecules comprised of multiple amino sugar rings possessing several amino and hydroxy functional groups. Many of these compounds are natural products produced by bacteria. The antibiotics synthesized by *Streptomyces* are denoted by –mycin whereas the antibiotics synthesized by *Micromonospora* are denoted by –micin. Streptomycin was isolated from *Streptomyces griseus* in 1944 by Selman Waksman and was the first aminoglycoside to be used in clinical treatments against tuberculosis. Since the discovery of this antibiotic, aminoglycosides have been vital broad-spectrum antibacterial agents for treatments against numerous infections and are critically important to present day drug discovery^{5,6}.

Aminoglycosides are classified into two main structural classes, those that contain a 2-deoxyamine ring and those that do not. Further classification of aminoglycoside antibiotics containing the 2-deoxyamine ring can be subdivided into the kanamycin and neomycin groups based on their substitution patterns on the 2-deoxyamine ring. The kanamycin-like class of aminoglycosides are di-substituted at the 4,6-position on the 2-deoxystreptamine ring, while the neomycin-like class of aminoglycosides are di-substituted at the 4,5-position on the 2-deoxystreptamine ring (figure 3). For the neomycin class, the number of amino modified sugar rings range from two to six. The conventional numbering for each amino sugar ring is determined at the 6-aminohexose ring linked to position 4 on the 2-deoxyamine ring, denoted as prime (‘), whereas the hexose or pentose ring linked to position 5 or 6 on the 2-deoxyamine ring is denoted as double prime (‘‘)^{5,6}.

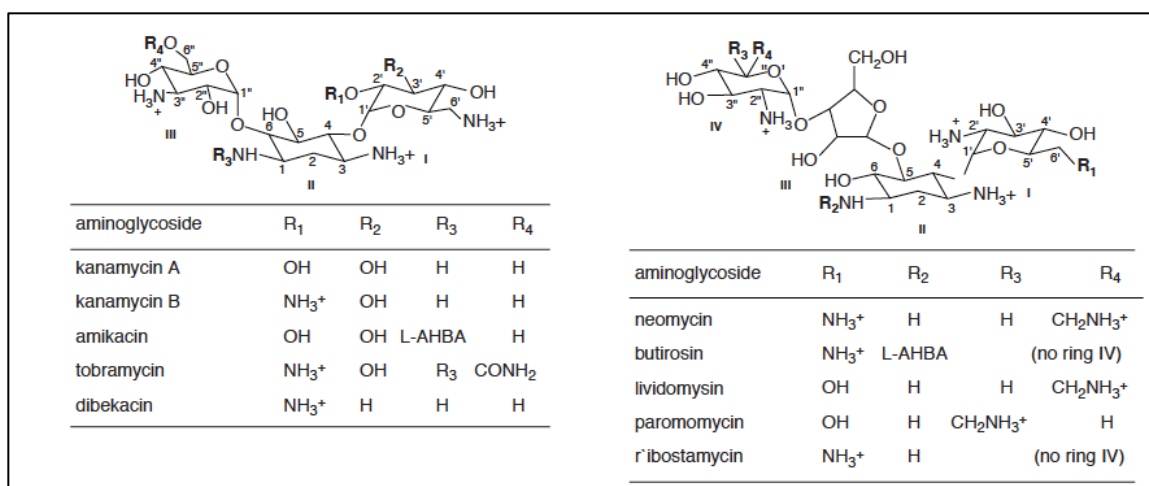


Figure 3: Aminoglycoside antibiotics are classified by structural differences: the kanamycin-like aminoglycosides are disubstituted at the 4,6-position on the 2-deoxystreptamine ring and are composed of 3 modified amino sugar rings (left), while the neomycin-like aminoglycosides are disubstituted at the 4,5-position on the 2-deoxystreptamine ring and range from 2 to 6 modified amino sugar rings (right).

II. Mode of Action

Aminoglycoside antibiotics have a high affinity for prokaryotic ribosomes by binding to the tRNA binding region (A-site) of the 16S rRNA of the 30S ribosomal subunit. The uptake of aminoglycosides within the cell is a multiphase process divided into two energy-dependent phases, EDP-I and EDP-II. Aminoglycosides first gain entry into the cell by an energy independent, drug-induced, disruption of Mg²⁺ bridges between lipopolysaccharides in Gram-negative bacteria or by passing through the cell wall in Gram-positive bacteria. The transport of aminoglycosides across the cell membrane is an energy-dependent process (EDP-I) by electron transport. Once in the cytosol, the aminoglycoside antibiotics rapidly and reversibly bind to the 30S ribosomal subunit in an energy-dependent manner (EDP-II). The binding of aminoglycosides to the 30S subunit does not appear to prevent the formation of the ribosomal complex; rather it impairs the proofreading process and translational accuracy resulting in misfolded, non-functional proteins. These proteins can interact with the cell membrane causing a loss in membrane integrity and increase membrane permeability for an influx of aminoglycoside antibiotic to saturate all of the ribosomes in an irreversible manner, resulting in cell death^{6,7,8}.

The degree of antibiotic activity has been shown to be dependent upon structural characteristics. Previous studies have shown that the hydroxyl groups have little effect on antibiotic activity compared to the amino groups. The number of amino groups and positions at which they are located play a significant role in activity shown in decreasing order of 2', 6'-diamino > 6'-amino > 2' amino > absence of amine^{9,10}.

C. Aminoglycoside Modifying Enzymes

I. Nomenclature and Mechanism of Modification

Since the discovery of aminoglycoside antibiotics, bacterial resistance has emerged mainly due to the presence of aminoglycoside-modifying enzymes. Once modified, aminoglycosides have a low binding affinity for the 30S ribosomal subunit allowing the bacteria to survive in the presence of the drug. To date, there are over 50 different modifying enzymes classified into three main families based on the type of functional group transferred to the aminoglycoside structure and inactivating the antibiotic's activity. The three groups are: (a) *O*-Nucleotidyltransferases (ANT) which utilize ATP as a second substrate to catalyze an adenylation reaction at the hydroxyl groups; (b) *O*-Phosphotransferases (APH) which catalyze the ATP-dependent phosphorylation of hydroxyl groups; and (c) *N*-Acetyltransferases (AAC) which acetylate an amino group on the 2-deoxystreptamine ring or aminohexose sugar rings in the presence of acetyl coenzyme A as the acetyl donor. Each family of aminoglycoside modifying enzymes contains numerous genes differing in substrate regiospecificity and promiscuity (figure 4)^{6,11,12}.

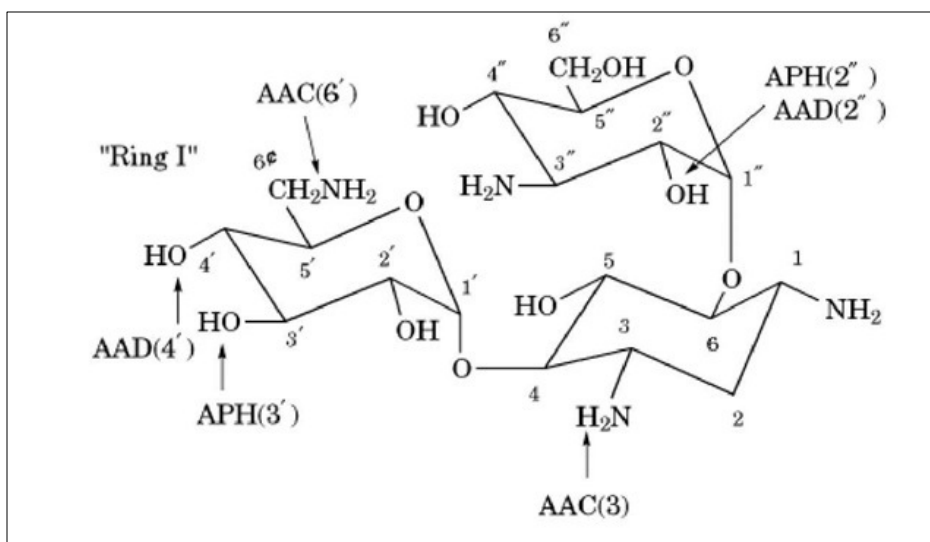


Figure 4: Site of modification for kanamycin B by aminoglycoside modifying enzymes.

D. Introduction to Aminoglycoside -3'-Phosphotransferase-IIIa

The APH family of enzymes is one of the best studied of the aminoglycoside modifying enzymes. This family shows sequence homology at the carboxy end of the protein and contains four subfamilies based on their site of modification: -2'', -3', 3'', -6, -4. Of the APHs, the APH(3') subfamily has at least seven different genes (I-VII) that have been identified. APH(3')-IIIa is in particular interest due to its broad substrate profile capable of modifying both classes of aminoglycosides and at the 3'- and 5''-OH groups: kanamycin A, kanamycin B, neomycin B, paromomycin, ribostamycin, lividomycin, butirosin, amikacin, and isepamicin^{11,12}.

I. Structure of APH(3')-IIIa

APH(3')-IIIa is a 31kDa enzyme produced by *Escherichia coli* and *Staphylococcus aureus*, both Gram-positive organisms. The gene encoding for APH(3')-IIIa was cloned from plasmid pJH1, a 72.6kb R plasmid isolated from a clinical isolate. This enzyme is purified as a monomer in the presence of dithiothreitol (DTT) and can dimerize without the presence of a reducing agent. Head to tail covalent bonds connected by two disulfide bonds between Cys19

and Cys156 links the homodimer. The dimer contains two active sites, both equally capable of phosphorylating aminoglycosides^{13,14}. Each monomer consists of two lobes containing five anti-parallel β -sheets and two α -helices on the N-terminus and six α -helices and four β -strands on the C-terminus (figure 5). APH(3')-IIIa resembles traditional protein kinases by utilizing the transfer of the γ -phosphate of ATP to modify aminoglycosides¹⁵. The nucleotide binding pocket is formed by a cleft between the N- and C-termini and contains six conserved residues across the APH(3') family of enzymes: Ser27, Lys44, Glu60, Asp190, Asn195, and Asp208. Ser27 and Lys44 interact with the γ -phosphate of ATP and increase binding affinity. Asp190 plays a critical for binding of the aminoglycoside while Asn195 and Asp208 stabilize the Mg^{2+} ions in the transition state to initiate the release of ADP during the rate-limiting step^{15,16,17,18,19,20,21}. The aminoglycoside-binding site is lined with acidic, highly negatively charged residues, which increase the affinity for positively charged aminoglycosides. Mutagenesis studies confirmed the critical role of Asp190, which is suggested to be the general base and forms hydrogen bonds with the 3'-OH group on aminoglycosides, the primary site of modification (figure 6)^{20,21,22}.

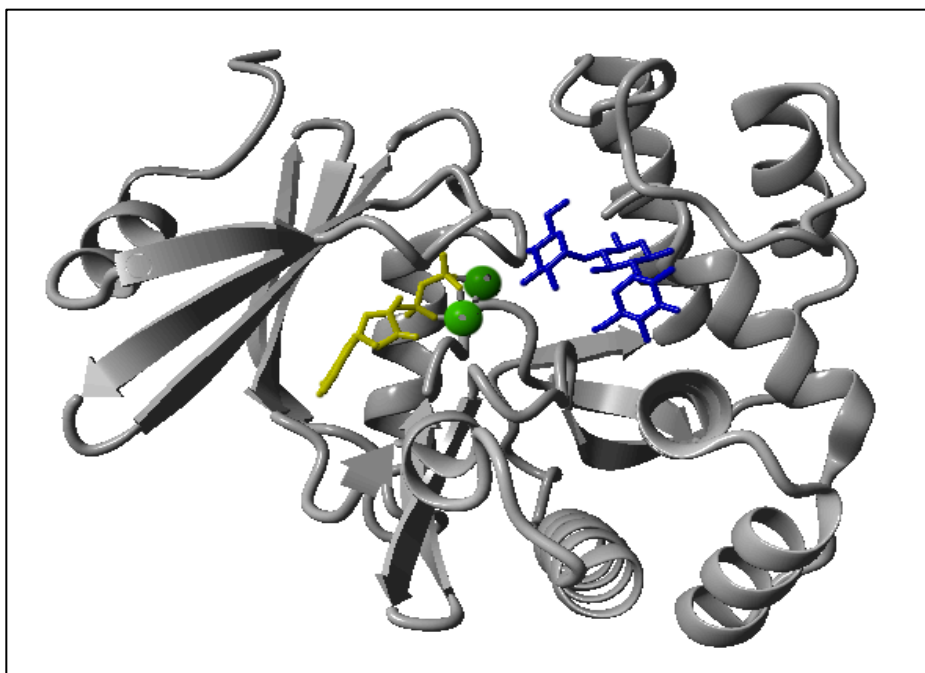


Figure 5: Cartoon representation of the structure of APH(3')-IIIa in complex with ADP (yellow), Mg^{2+} (green) and kanamycin A (blue) (PDB: 1L8T)²². The structural representation of APH(3')-IIIa was created using YASARI software.

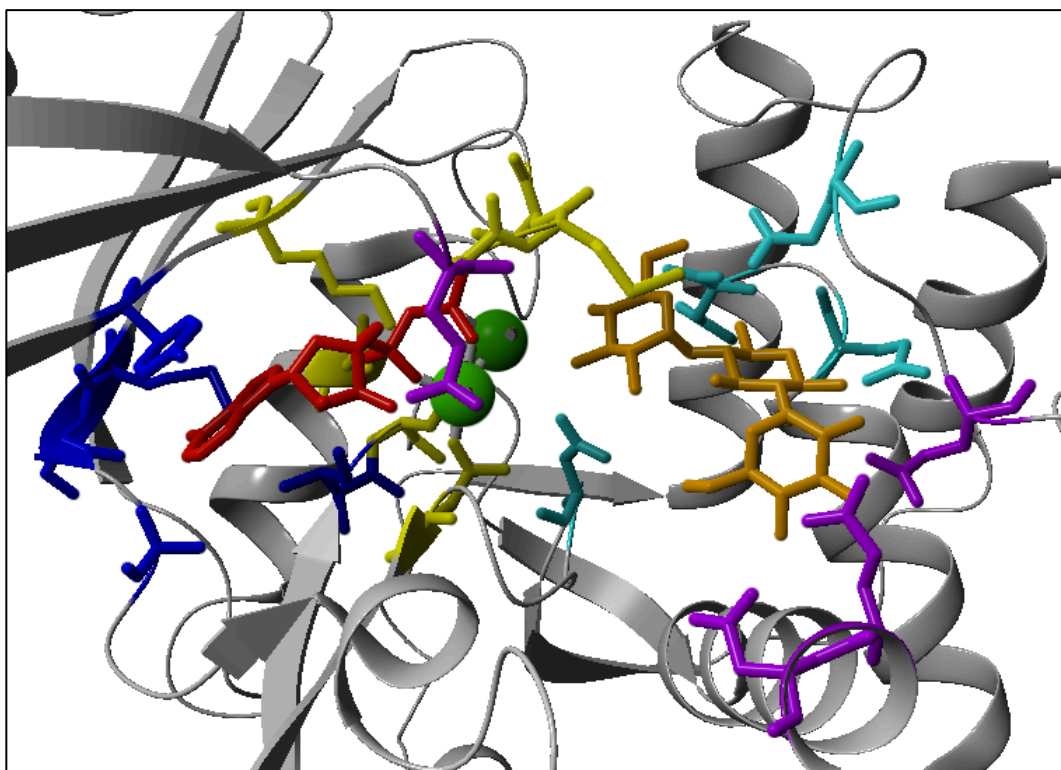


Figure 6: A cartoon representation of the active site for AHP(3')-IIIa (PDB: 1L8T) in complex with ADP (red), Mg^{2+} (green), and kanamycin A (orange). The residues participating in binding are shown in color coded manner: the nucleotide binding region (blue), triphosphate binding pocket (yellow), specificity pocket (purple) and aminoglycoside catalytic binding site (cyan) are highlighted²⁰. The representation of the active site for AHP(3')-IIIa was created in YASARI software.

II. Protein Dynamics in Solution

The dynamic behavior of AHP(3')-IIIa has been studied for the apo-enzyme, binary complex with aminoglycoside, and ternary complex with the nucleotide and aminoglycoside. For the apo-enzyme, the Heteronuclear Single Quantum Correlation (HSQC) NMR spectrum contained a majority of resonances overlapping between ~ 7.5 ppm and 8.8 ppm in the H^1 dimension. Although these spectra are similar to those of denatured or partially unfolded proteins, secondary structure does form in the AHP (3')-IIIa apoenzyme but most likely contains weaker hydrophobic interactions and hydrogen bonds. Therefore, in the absence of ligand, AHP(3')-IIIa was suggested to be highly flexible and dynamic, capable of sampling

several conformational states exhibited by the broadened, overlapping amide resonances (figure 7A)²³.

In the binary complex with only the antibiotic, APH(3')-IIIa – aminoglycoside, the HSQC spectrum undergoes a drastic change in proton chemical shifts between 6 ppm and 11 ppm for amide resonances. The shift in resonances indicates that APH(3')-IIIa adopts a well-defined structure upon aminoglycoside binding. In the ternary complex, ~30 cross-peaks exhibited line broadening associated with amino acids located near the nucleotide binding site that were present in the binary complex. In other words, the APH-nucleotide-aminoglycoside complex gains more flexibility as compared to the binary enzyme–aminoglycoside complex. . The solvent accessibility however increased by >20 unbroadened residues (figure 7C)²³. This is consistent with the fact that the binary enzyme–nucleotide complex is as flexible as the apo-enzyme.

In both the APH-neomycin and APH-kanamycin A ternary complexes, the Lys44 peak was absent in the HSQC spectra. Lys44 is situated within the nucleotide-binding site and was suggested to make direct contact with this ligand. Val57 and Glu60 also showed increased H-D exchange in the neomycin ternary complex but exhibited line broadening in the kanamycin A ternary complex. All of these residues are located within an α -helix and participate in hydrogen bonding with the five stranded, antiparallel β -sheet at the nucleotide-binding cleft^{17,22}. In particular, Glu60 forms electrostatic interactions to stabilize Lys44, allowing Lys44 to be more stable when making contacts with the nucleotide. Furthermore, it was proposed that ATP binding slightly destabilizes APH-(3')-IIIa by weakening or breaking hydrogen bonds formed between the α -helix and β -sheet at the nucleotide-binding interface²³. Further evidence for the destabilization of the β -sheet was shown in the binary complex in the presence and absence of β - γ -methylene 5'-triphosphate (AMPPCP) and MgCl₂, an MgATP analogue. In the absence of Mg-AMPPCP, slow H-D exchange (strong protection of solvent) was exhibited compared to the fast exchange observed when Mg-AMPPCP was present. This was observed in both kanamycin A- and neomycin B ternary complexes, with the exception that the neomycin B ternary complex has less broadened residues, suggesting that neomycin B induces greater protein stability than kanamycin A²³.

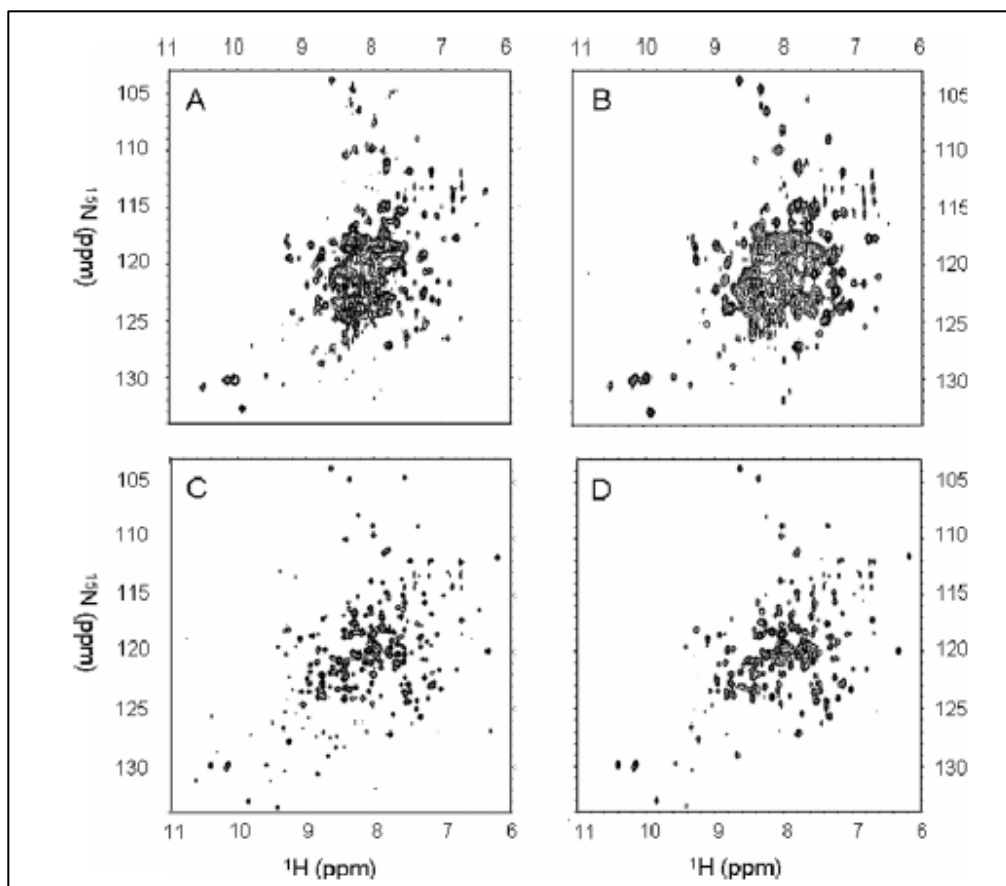


Figure 7: HSQC NMR spectrum for APH(3')-IIIa in apo-form (A), in complex with MgATP (B), in complex with tobramycin (C), and tobramycin-MgATP complex (D). Tobramycin is a competitive inhibitor for kanamycin-phosphate. In the binary complex (C), APH undergoes significant structural changes compared to the apo-enzyme. In the ternary complex, APH(3')-IIIa exhibits a less structured spectrum than the binary complex²³.

III. Theorell-Chance Kinetic Mechanism

APH(3')-IIIa follows a Theorell-Chance kinetic mechanism, a rare form of an ordered bi-bi kinetic mechanism. The Theorell-Chance mechanism follows an ordered substrate binding and product release with a very small accumulation of the enzyme-substrate complex. This specific mechanism contains a ternary complex but does not contribute to the overall rate of the reaction. As the concentration increases for the first substrate, initial rates increase with the concentration of the second substrate below the K_m ^{15,24,25}.

For APH(3')-IIIa, the chemical step of the reaction is defined as the binding of ATP (first substrate bound), followed by the binding of an aminoglycoside. Phosphorylated aminoglycoside is then rapidly released followed by the rate-limiting, slow release of ADP (figure 8). These findings were observed with both classes of aminoglycoside antibiotics, which is consistent with the Theorell-Chance mechanism where different substrates have little effect on the overall rate of reaction considering that the phosphorylated aminoglycoside is released prior to the rate-limiting step. At high substrate concentrations, substrate inhibition was observed suggesting that nonproductive binding to the enzyme-ADP complex contributed to the rate-limiting, ADP product release. Similarly, inhibition of ATP by kanamycin phosphate was abolished by increased concentrations of kanamycin A²⁵.

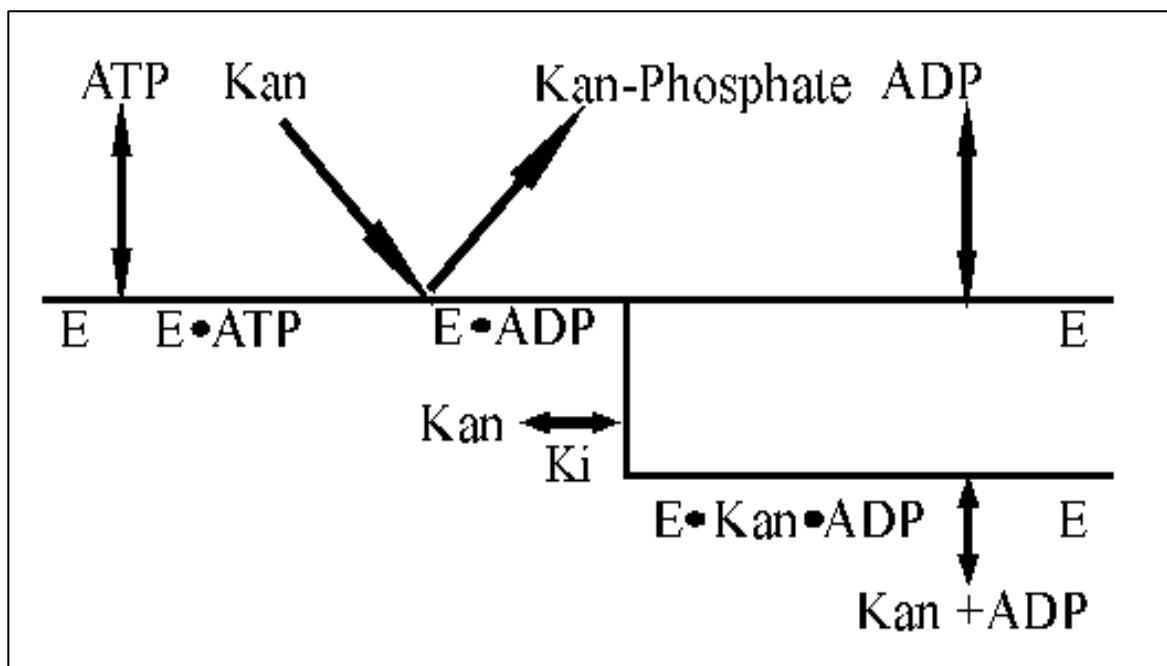


Figure 8: Theorell-Chance Mechanism for APH(3')-IIIa²⁵.

IV. Kinetic and Thermodynamic Properties

Isothermal Titration Calorimetry (ITC) studies have been conducted for APH(3')-IIIa with several 4,5- and 4,6-disubstituted 2-deoxystreptamine aminoglycosides. In general, APH(3')-IIIa binds to aminoglycosides with a favorable enthalpy and an unfavorable entropy

yielding an overall favorable Gibbs energy for the reaction. Both binary and ternary complexes exhibit different thermodynamic properties with none correlating with the kinetic parameters K_m , k_{cat} , or k_{cat}/K_m . In the binary complex, binding of an aminoglycoside is mainly driven by an exothermic reaction with an unfavorable entropic contribution. The unfavorable entropy of binding is likely to be due to the reduced conformational freedom shown by the HSQC spectrum, where binding of aminoglycoside to APH(3')-IIIa becomes highly structured. This was shown to be the case for all aminoglycosides with no clear trends distinguishing a difference between the 4,5- and 4,6-disubstituted aminoglycosides^{14,23}.

Similar to the binary complex, formation of the ternary complex, APH(3')-IIIa-CaATP-aminoglycoside, exhibited exothermic heat and favorable Gibbs free energy for all aminoglycosides. However, the ternary complex was observed to have a significantly lower enthalpic contribution and a less unfavorable entropy contribution. These observations are in agreement with the NMR data, where binding of a nucleotide increases the solvent accessibility and weakens the bonds between the α -helix and β -sheet at the nucleotide-binding interface. In general, the presence of CaATP induces tighter or unchanged binding of aminoglycosides to APH(3')-IIIa, which is in agreement with the suggested Theorell-Chance kinetic mechanism^{24,23,26}.

The kinetic parameters for APH(3')-IIIa varied among aminoglycosides. In general, the K_m values were lower for 4,6-disubstituted aminoglycosides with the exception of neomycin B and ribostamycin. General trends between the 4,5-disubstituted aminoglycosides differing in the addition or absence of a functional group were observed. Butirosin and ribostamycin only differ in their structure by the presence of a (S)-4-amino-2-hydroxybutyryl group at the aminocyclitol N-1 (butirosin). The k_{cat}/K_m was shown to have a 3.4-fold decrease for butirosin suggesting that the aminocyclitol ring plays a role in substrate orientation when binding to APH(3')-IIIa. It was also shown that the substitution of an amino group for a hydroxyl group for kanamycin A and B affected the kinetic parameters. This further supported the thermodynamic data by Ozen et al., where kanamycin B exhibited a 15-fold increase in affinity. These data suggested that the 2'-amino group on kanamycin B may participate in cooperative hydrogen bonding^{13,14,26}.

At high aminoglycoside concentrations, substrate inhibition was exhibited and more pronounced for 4,5-disubstituted aminoglycosides. The large K_i values for all aminoglycosides that exhibited substrate inhibition were suggested to have no biological significance. Tobramycin (3-deoxykanamycin B) was shown to not be a substrate for APH(3')-IIIa but was a competitive inhibitor for kanamycin A^{13,14,26}.

E. Introduction to Aminoglycoside -3-*N*-Acetyltransferase-IIIb

Aminoglycoside acetyltransferases (AAC) are some of the most diverse modifying enzymes. *N*-acetyltransferases contain four classes based on regiospecificity of modification on aminoglycosides at the 1-, 3-, 6'-, and 2'-amino group positions. These enzymes range in size from 15-22kDa and catalyze the transfer of an acetyl group from Acetyl coenzyme A to the respective amino groups, producing CoASH and *N*-Acetyl-aminoglycoside (Scheme 1). The AAC(3) class is one of the largest groups of enzymes, approximately 30kDa, which display significant homology in their N-terminal region. For this particular class, there are eight genes (I-IX), each varying in substrate specificity across the kanamycin and neomycin classes. AAC(3)-III contains three gene clusters one isolated from *Pseudomonas aeruginosa* and two isolated from *Pseudomonas* strains. This subfamily acetylates the amino group at the 3-carbon position on the 2-deoxystreptamine ring (2-DOS). AAC(3)-IIIb is a homo-dimer consisting of two active sites. This protein is one of the most promiscuous modifying enzymes as it modifies both 4,5- and 4,6-disubstituted 2-deoxystreptamine aminoglycosides, including tobramycin, kanamycin A, kanamycin B, gentamicin C, sisomicin, neomycin B, paromomycin A, ribostamycin, and lividomycin^{6,12,27}.

I. Protein Dynamics in Solution

A series of ¹H-¹⁵N HSQC experiments were conducted for AAC(3)-IIIb apo-enzyme and ligand-bound forms. In both forms, AAC(3)-IIIb exhibited a well defined structure. In the apo-form, ~40-50 resonances were missing in the spectrum located near the antibiotic-

binding loop. These data suggested that AAC(3)-IIIb has a well defined protein structure with a highly flexible antibiotic binding loop. The binding of CoASH to the first binding site exhibited a small shift in peaks without broadening. Upon CoASH binding to the second site, peak intensities increased but did not shift. Therefore, the vast majority of conformational changes to AAC(3)-IIIb are mainly due to aminoglycoside binding (figure 9)^{27,28,29}.

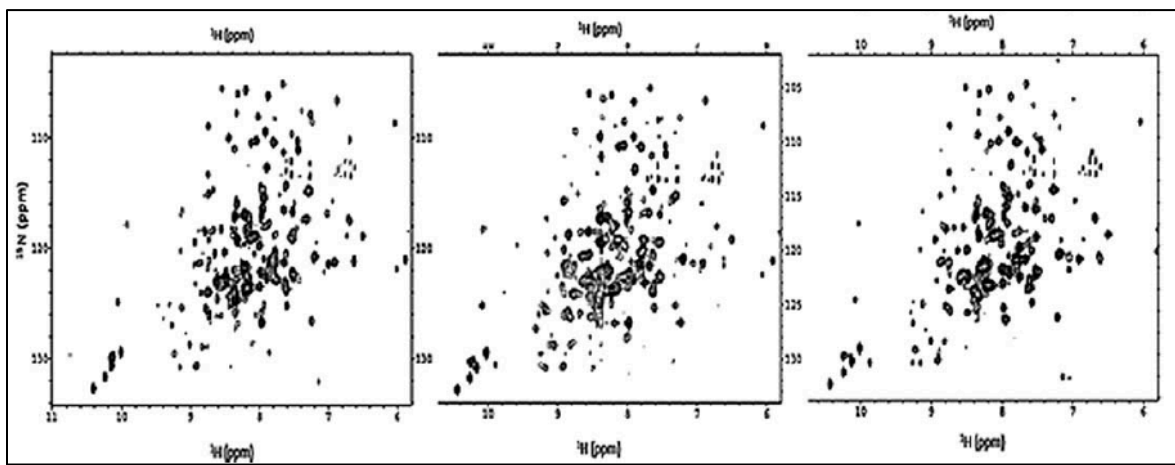


Figure 9: ^1H - ^{15}N HSQC NMR spectra for AAC(3)-IIIb in the apo-form (left), AAC(3)-IIIb-tobramycin complex (middle), and AAC(3)-IIIb-CoASH complex (right)³⁰.

II. Kinetic and Thermodynamic Properties

From what is previously known about AGMEs, AAC(3)-IIIb is one of the few enzymes that discriminate between the 4,5- and 4,6-disubstituted 2-DOS aminoglycosides. The 4,6-disubstituted aminoglycosides have turnover rates (k_{cat}) between 46s^{-1} and 55s^{-1} whereas the 4,5-disubstituted aminoglycosides have rates between 10s^{-1} and 34s^{-1} . Therefore, the kanamycin-like aminoglycosides are bound, acetylated, and released much faster than the neomycin-like aminoglycosides. It was suggested that the difference in catalytic turnover rates may be due to the orientation of the antibiotic within the active site of AAC(3)-IIIb. Although amikacin and butirosin, both substituted at the N-1 position, can bind to the enzyme, their k_{cat} values did not show measurable rates. Bulky substitutions at the N-1 position were thereby suggested to distort the orientation of the aminoglycoside at the antibiotic binding

loop and hinder proper alignment of the N-3 amine group for acetylation. Overall, the catalytic turnover rates for AAC(3)-IIIb with all substrates are among the highest observed for AGMEs. In particular, aminoglycosides containing a 2'-amine group (tobramycin, kanamycin B, and sisomicin) exhibited the highest specificity^{27,28}.

At high concentrations of aminoglycosides, AAC(3)-IIIb exhibited substrate inhibition with no correlation between K_i and K_m values. In general, smaller and/or less constrained aminoglycosides (kanamycin A, kanamycin B, ribostamycin, tobramycin) exhibited the lowest substrate inhibition compared to larger or more sterically hindered aminoglycosides (neomycin B, paromomycin, lividomycin A, and sisomicin)^{27,28}.

Unlike the kinetic parameters that differentiated between the 4,6-disubstituted and 4,5-disubstituted 2-DOS aminoglycosides, the thermodynamic parameters did not differentiate between the two classes. Also, AAC(3)-IIIb was shown to have no correlation between the thermodynamic and kinetic parameters. For all substrates, binding to AAC(3)-IIIb was shown to be exothermic with a favorable intrinsic enthalpy and unfavorable intrinsic entropy yielding an overall favorable Gibbs free energy. However, binding of kanamycin A in the binary complex exhibited a less favorable intrinsic enthalpy and a more favorable intrinsic entropy. In the presence of CoASH bound to one binding site in the ternary complex, a more favorable enthalpy and less favorable entropy contributed to an overall increase of 1.0 kcal/mol - 1.9 kcal/mol in the Gibbs free energy. These data suggest that the binding of the cofactor to one active site stabilizes the protein conformational state to position the antibiotic binding loop for greater aminoglycoside binding. In general, binding of CoASH to one binding site increases the binding affinity for aminoglycosides by 5-20 fold and is accompanied by a more favorable intrinsic enthalpy and a less favorable entropy. The more negative enthalpic contribution has been suggested to be caused by an increase in contacts made between the enzyme and aminoglycoside^{27,28}.

In the binary complex, AAC(3)-IIIb - aminoglycoside, smaller antibiotics bind to the active site with a stoichiometry of 1.5-2 molecules per enzyme, while aminoglycosides with 4 or 5 modified amino sugar rings were shown to have a stoichiometry of 1 molecule per

enzyme. This is likely to be the consequence of the large size of the aminoglycoside binding pocket to which more than one of the smaller aminoglycosides can fit in various binding modes. In the presence of CoASH, the stoichiometry for binding of all aminoglycosides to AAC(3)-IIIb was shown to be one molecule per enzyme regardless of size of the substrate²⁷.

Although there were no clear trends to distinguish between the 4,5- and 4,6-disubstituted 2-DOS aminoglycosides, substitution patterns for aminoglycosides differed in affinity for AAC(3)-IIIb. Aminoglycosides containing an amine group at the 2'-carbon (kanamycin B, tobramycin, sisomycin) were shown to be more favorable than a hydroxyl group at this position (kanamycin A). The presence of an amine group (neomycin B) versus a hydroxyl group (paromomycin A) at the 6'-carbon was preferred for aminoglycosides of equal size. The size of the aminoglycoside appeared to take precedent over the 6'-amine group where lividomycin (5 rings) and ribostamycin (3 rings) were observed to have weaker affinity to AAC(3)-IIIb when compared to neomycin B (4 rings). This trend also held true for 4,6-aminoglycosides (3 rings) containing a 6'-amine group, which was observed to also have a decrease in affinity compared to aminoglycosides containing 4 rings^{27,29,31}.

III. Proposed Orientation for Aminoglycosides in the Active Site

The orientations of 2-DOS aminoglycoside substrates in the active site of AAC(3)-IIIb have been proposed from manual docking and homology modeling with crystal structures of two proteins in complex with acetyl coenzyme A, both from *Bacillus* and members of the antibiotic_NAT superfamily, YokD and AAC (3) (PDB 2NYG and 3IJW, respectively). Within the antibiotic binding pocket, negatively charged side chains are positioned to interact with amine groups of the antibiotic, placing the N-3 amine in a proximal position next to the acetyl group of acetyl coenzyme A. The fourth ring of neomycin B may attract a carboxyl side chain, thereby blocking other antibiotics from binding to the active site. Kanamycin A appears to make similar contacts as neomycin B but the third ring may not be able to interact as favorably as aminoglycosides with four or more rings. Therefore, it was suggested that the weaker contacts made by kanamycin A would leave the antibiotic binding loop more exposed

allowing similar structured aminoglycosides to bind. This hypothesis would provide an explanation for the binding stoichiometry of >1 for smaller aminoglycosides^{27,28,30}.

F. Introduction to Aminoglycoside -3-*N*-Acetyltransferase-VIa

AAC(3)-VIa is a rare isolate found in Gram-negative organisms, mainly in *Escherichia coli*. This enzyme is 32kDa with a limited substrate profile capable of modifying gentamicin C, sisomicin, 6'-*N*-ethylmetilmicin, tobramycin, netilmicin, and kanamycin B at the N-3 position on the 2-deoxystreptamine ring. AAC(3)-VIa shows 48% sequence homology to AAC(3)-IIa. Little characterization has been conducted for this enzyme and studies are currently underway in the lab^{12,32}.

G. Enzyme Characteristics for APH(3')-IIIa, AAC(3)-IIIb, and AAC(3)-VIa

APH(3')-IIIa, AAC(3)-IIIb, and AAC(3)-VIa exhibit significantly different protein characteristics (table 1). In general, AAC(3)-IIIb has lower K_m values and much greater turnover rates compared to APH(3')-IIIa. APH(3')-IIIa binds to all aminoglycosides with a stoichiometry of 1:1 whereas AAC(3)-IIIb varies in stoichiometry between 1-2 depending on the aminoglycoside with the exception of kanamycin A that has a stoichiometry of 2.8. Both APH(3')-IIIa and AAC(3)-IIIb are highly promiscuous whereas AAC(3)-VIa has a limited substrate profile and unable to bind to neomycin^{13,14,23,27,28,29}.

Thermodynamically, APH and AAC(3)-IIIb both exhibit exothermic binding reactions with a favorable enthalpy and unfavorable entropy contribution yielding an overall favorable net free energy. The ternary complex for APH differs from AAC(3)-IIIb, where the observed intrinsic enthalpy contribution is less favorable with a more favorable intrinsic entropy contribution. The binding of cofactors to each enzyme exhibited similar trends but had a more profound effect for AAC(3)-IIIb. When MgATP is bound to APH, there was a slight increase in the affinity for aminoglycosides, whereas the binding of acetyl coenzyme A to AAC(3)-IIIb greatly increased the affinity of substrates^{14,23,27,28,29}.

Protein dynamics for APH and AAC(3)-IIIb exhibit various differences in the apo-enzyme, binary, and ternary complex. APH was observed to be highly dynamic in the apo-form and became structured when bound to an aminoglycoside. The presence of MgATP did not significantly alter the structure of APH when the ternary complex was formed. Unlike APH, the AAC(3)-IIIb apo-enzyme was well structured with a highly flexible antibiotic binding loop. Titration with ligands cause small shifts in several resonances and changed dynamics of several amino acids such that their resonances became visible in the spectrum^{23,27,28,30}.

While APH(3')-IIIa and AAC(3)-IIIb are two highly promiscuous AGMEs with similar substrate profiles, AAC(3)-IIIb catalyzes the modification reaction faster than APH(3')-IIIa. As stated above, Wolfenden et al. described in several studies that the rate enhancement produced by catalysts is improved at lower temperatures and has a significantly less positive $\Delta H^\ddagger$. Therefore, the purpose of this study is to determine the temperature dependence of catalysis in the three enzymes. One caveat is whether the transition state for the chemical step will be measured, as for example, the rate determining step in APH(3')-IIIa is release of the ADP product. The rate determining steps in the other two enzymes still need to be determined. If chemistry is rate determining in any of the enzymes, we can examine whether the kinetic barrier is lowered by the enthalpy of activation during the reaction. Also of interest, the enthalpy of activation will also be studied with AAC(3)-VIa, a less promiscuous enzyme, and AAC(3)-IIIb bound to gentamicin C₁ and C₂, two antibiotics with the same structure that only vary by one methyl group. Here we propose that the activation energy will differ between the two gentamicin components for both enzymes, where AAC(3)-IIIb will favor the more hydrophobic gentamicin C₁ while AAC(3)-VIa will favor gentamicin C₂.

Table 1: Comparison of protein characteristics for APH(3')-IIIa, AAC(3)-IIIb, and AAC(3)-VIa.

Enzyme	^b Modification Reaction	^a Substrates	Crystal Structure	K _m (μM)	k _{cat} (s ⁻¹)	k _{cat} /K _m (M ⁻¹ s ⁻¹)
APH(3')-IIIa ¹³	ATP·AG·APH(3')-IIIa ↔ ADP·phospho-AG·APH(3')-IIIa	KanA, KanB, NeoB, Prm, Amk, Isep, Ribo, But, Liv	1L8T – KanA 3TMO – But 2B0Q - NeoB 1J7I – Apoenzyme 1J7L – ADP 1J7U - AMPPNP	kan A – 12.6±2.6 kanB – 19.4±2.2 amk – 245±27 neoB – 7.72±0.9 prm – 19.5±3.5 liv – 31.6±5.1 ribo – 9.30±1.8 but – 34.3±3.7	kanA – 1.79±0.09 kanb – 3.51±0.19 amk – 2.46±0.11 neoB – 2.08±0.07 prm – 3.62±0.25 liv – 3.97±0.25 ribo – 1.89±0.10 but – 2.02±0.07	kanA – 1.43 x 10 ⁵ kanB – 1.81 x 10 ⁵ amk – 1.00 x 10 ⁴ neoB – 2.69 x 10 ⁵ prm – 1.86 x 10 ⁵ liv – 1.26 x 10 ⁵ ribo – 2.03 x 10 ⁵ but – 5.87 x 10 ⁴
From AAC(3)-IIIb ²⁷	AG+AcCoA $\xrightarrow{\text{AAC}}$ AG-C(O)CH ₃ +CoASH	kanA, kanB, gnt, siso, neoB, tob, prm, ribo, liv		kan A – 4.8±0.5 kan B – 2.6±1.6 neoB – 1.2±0.2 prm – 3.3±0.6 liv – 3.6±0.1 tob – 1.3±0.8 siso – 1.5±0.4	kan A – 51.4±4.4 kan B – 54.5±3.6 neoB – 9.5±1.8 prm – 33.6±1.4 liv – 33.8±1.4 tob – 47.8±4.8 siso – 46.3±2.2	kan A – 11.2±2.3 x10 ⁶ kan B – 34.4±19.8 x10 ⁶ neoB – 9.1±0.2 x10 ⁶ prm – 10.8±2.5 x10 ⁶ liv – 9.5±0.8 x10 ⁶ tob – 60.5±34.6 x10 ⁶ siso – 33.4±9.5 x10 ⁶
AAC(3)-VIa ³²	AG+AcCoA $\xrightarrow{\text{AAC}}$ AG-C(O)CH ₃ +CoASH	kanB, gnt, siso, tob, ntl, 6'ntl		NA	NA	NA

^aAbbreviations for aminoglycoside antibiotics: kan A – kanamycin A; kan B – kanamycin B; gnt – gentamicin C complex; neoB – neomycin; prm – paromomycin; amk – amikacin; Isep – isepamicin; ribo – ribostamycin; but – butirosin; liv – lividomycin; ntl – netilmicin; 6'-ntl – 6'-N-ethylnetilmicin; tob – tobramycin; siso - sisomicin

^bAPH(3')-IIIa follows a Theorell-Chance kinetic mechanism with an ordered substrate binding and product release. ATP is first bound to the enzyme followed by the aminoglycoside. The phosphorylated aminoglycoside is then released prior to the slow release of ADP, which has been determined as the rate-limiting step in the reaction. The kinetic mechanisms for AAC(3)-IIIb and AAC(3)-VIa has yet to be determined.

CHAPTER II: GENTAMICIN C COMPLEX

A. Background

I. Origin and Structure

Gentamicin C complex is synthesized from *Micromonospora purpurea*, a Gram-positive organism, and is produced as a mixture of components used to treat Gram-negative bacterial infections. The main active components are gentamicin C_{1a}, C₁, and C₂, which make up about 80% of the complex while the remaining 20% are composed of minor components. These three active components only differ in the degree of methylation on the 2,6-diaminosugar on the purpurosamine ring (figure 10). The degree of methylation increases by one methyl group: gentamicin C₁ > gentamicin C₂ > gentamicin C_{1a}.^{5,33,34}

Individual components of gentamicin C are not commercially distributed; rather the antibiotic is marketed as a complex with sulfate. In order to study the differences in activation energy affected by the degree of methylation of each component, gentamicin C complex must first be separated into its respective components. Separation of gentamicin C complex can be achieved by various chromatography techniques, particularly ion exchange and thin-layer chromatography due to the antibiotic being highly polar, negatively charged, and hydrophobic.

II. CHSQC-TOSCY Experiments

Homonuclear Total Correlation Spectroscopy (TOCSY) in combination with Carbon Heteronuclear Decoupled Single Quantum Coherence (CHSQC) is commonly used to analyze the structure of polysaccharides. In a simple TOSCY experiment, each monosaccharide unit is associated with a single ¹H spin system allowing differentiation between resonances belonging to a single monosaccharide unit. The addition of CHSQC pulse sequence identifies

the correlation of nuclear spins between α -carbons. CHSQC – spectrum shows cross-peaks correlating carbon resonances with their respective hydrogen atoms. Proton-proton connectivity's determined from TOCSY experiments were then used to make assignments of carbon atoms^{35,36} (figure 11).

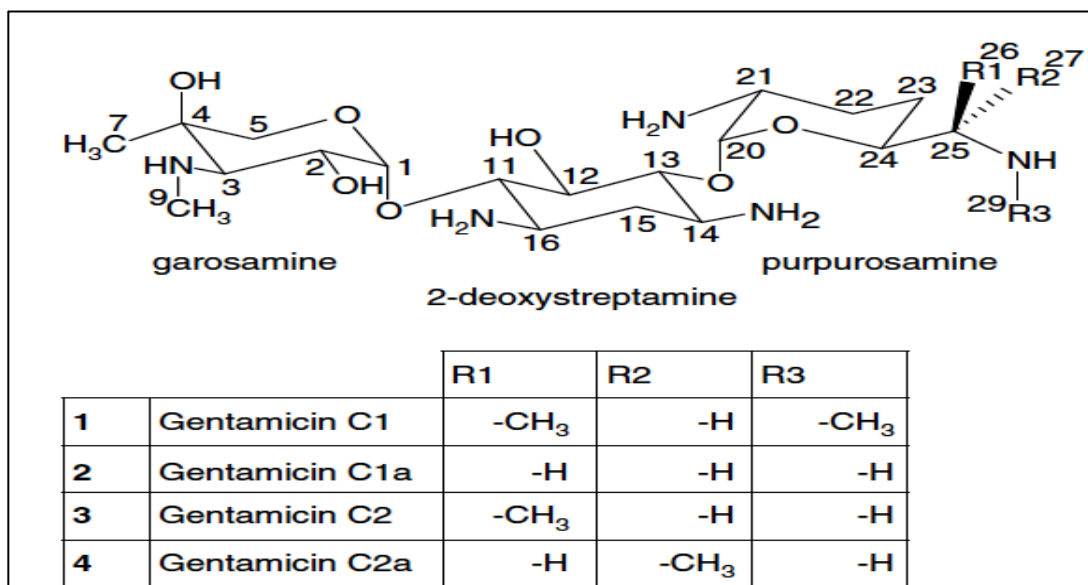


Figure 10: Structure of gentamicin C complex⁴². Gentamicin complex is composed of a mixture of components that are differentiated based on the degrees of methylation at the R1, R2, and R3 positions.

B. Experimental Procedure

I. Separation of Gentamicin C Complex by Thin Layer Chromatography

Gentamicin C complex (30ng) was placed on 20x20 silica gel plates purchased from Sigma and placed in a chamber pre-saturated with the mobile phase (3:2:1 methanol: propanol: ammonium hydroxide). Plates were removed after the mobile phase saturated $\frac{3}{4}$ of the silica gel plate. TLC plates were dried and samples were analyzed by ninhydrin spray (2.0g ninhydrin, 100ml dH₂O, 100ml 1-butanol, 100mL Acetic Acid) and heated at 100°C for 10 minutes.

II. Separation of Gentamicin C Components, C₁, C₂, C_{1a}

Gentamicin C complex separation protocol was modified from Thomas et al³⁷. Gentamicin C complex (80mg) was dissolved in 1.0mL of 2.0M NaCl solution and loaded onto an ion exchange Whatmann Cellulose Phosphate P-11 column (1.0cm x 15cm). A linear gradient composed of 60mL of 2.0M NaCl and 60mL of 2.2M NaCl was applied to the column. Fractions (2ml) were collected and analyzed by thin layer chromatography on silica gel G plates and ninhydrin spray³⁷. Fractions with respective purified components were lyophilized, dissolved in 1mL of dH₂O, and loaded onto a Sephadex G-10 column (30ml of resin slurry on a 1.0cm by 50cm column) for desalting. The column was washed with 40ml of dH₂O and 1mL fractions were collected and analyzed by thin layer chromatography with ninhydrin spray. Desalting of purified gentamicin components on a Sephadex G-10 column was performed twice to ensure the removal of all salt. Following the second desalting of purified gentamicin components, compounds were lyophilized and stored at -20°C.

III. NMR Verification of Purified Gentamicin C Components by ¹H 1D NMR, Total Correlation Spectroscopy (TOCSY), and Carbon Heteronuclear Single Quantum Coherence (CHSQC)

Purified gentamicin components were dissolved, lyophilized, and redissolved in D₂O. ¹H 1D NMR, 2D CHSQC, and TOCSY NMR experiments were used to analyze the purified gentamicin components. Coupled and decoupled CHSQC experiments were run at 25°C. TOCSY experiments were run at 25°C with 15ms, 30ms, 50ms, and 80ms mixing times. ¹H 1D NMR spectra were analyzed by Mestrenova NMR analysis and CHSQC and TOCSY experiments were analyzed by Sparky³⁵.

C. Results and Discussion

I. Gentamicin C Complex Separation by Thin Layer Chromatography

Gentamicin C complex separation can be shown by thin-layer chromatography on silica gel plates^{38,39}. Various mobile phase mixture systems consisting of methanol, propanol, and ammonium hydroxide were analyzed to determine the best ratio of components. For all mixture systems, diluting ammonium hydroxide yielded a less pronounced separation. The mobile phase consisting of 2:3:1 ratio of chloroform: methanol: ammonia hydroxide exhibited the best separation for gentamicin and darkest spots on the silica gel plates (figure 12).

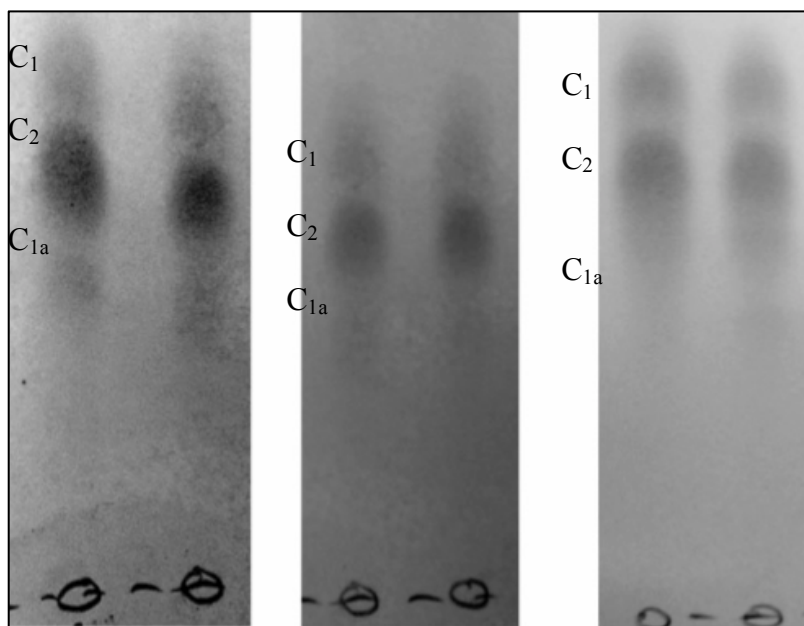


Figure 11: Determination of mobile phase components for thin layer chromatography on silica gel plates. Gentamicin C complex (30ng) was placed on silica gel plates 1-2inches apart. Various mobile phases were studied composed of 3:2:1 methanol:propanol: ammonium hydroxide (left), 3:2:1 methanol:propanol:25%ammonium hydroxide (middle), and 1:1:1 methanol:propanol:16% ammonium hydroxide (right). The mobile phase consisting of 3:2:1 methanol:propanol:ammonium hydroxide (left) exhibited the best separation of gentamicin components: gentamicin C₁ (top), gentamicin C₂ (middle), and gentamicin C_{1a} (bottom).

II. Separation of Gentamicin Components by Ion Exchange Chromatography

A majority of fractions contained a mixture of C₁ and C₂ components with the exception of the first and last few fractions (figure 13). Gentamicin C₁ was eluted in the first four fractions followed by the elution of gentamicin C₂, which was present in fractions 20-24. Gentamicin C_{1a} was not purified and was only eluted as a mixture containing gentamicin C₂. Additional purification attempts with fractions containing a mixture of components did not yield significant separation. We observed that increasing the size of the ion exchange column to purify more gentamicin did not provide significant separation of components. Also, minimizing the molarity of the salt gradient did not improve the separation.

Once separated by ion exchange, a Sephadex G-10 column desalted purified gentamicin components. The initial desalting of gentamicin components still contained a high level of salt, indicated by ¹H 1D NMR, which showed a high level of interference in the spectrum. After the second round of desalting, the ¹H 1D NMR showed sharper signals in the spectrum. Separation of gentamicin C₁ and C₂ yielded small quantities of purified components, 1.5mg and 0.65mg, respectively. The small quantities of gentamicin are most likely due to the numerous purification steps required to separate and desalt purified fractions. In addition to the several purification steps, samples were lyophilized after each round of purification, also leading to the loss of product. There have been several previously published methods of separation of gentamicin components. In particular, separations by a silica gel column and High Performance Liquid Chromatography (HPLC) have been the most recent methods described^{40,41}. Both of these methods were tested to improve the separation and increase the quantity of product but neither yielded sufficient separation of gentamicin components. Other methods described for separation include derivatized gentamicin. These methods were not studied due to the potential structural changes that may be induced by derivatization^{40,41}.

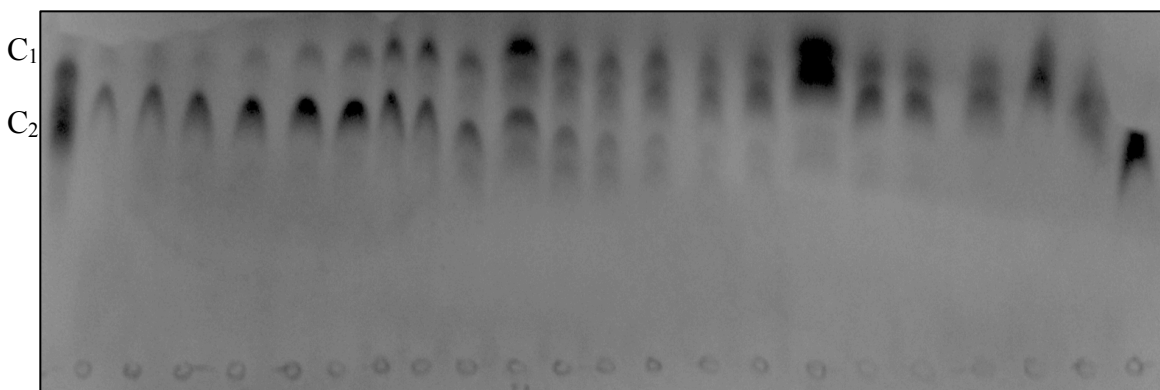


Figure 12: Gentamicin Separation by ion exchange chromatography with an 2.0-2.2M NaCl gradient.

III. Verification of Purified Gentamicin C₁ and C₂ Components by ¹H 1D NMR, TOCSY, and CHSQC 2D NMR Experiments

The structure of gentamicin C₁ and C₂ were analyzed by ¹H 1D NMR to verify the purity of each component (figures 14 and 15, respectively). Peak assignments in both spectrums were assigned by the reference spectra determined by Deubner et al. and desulfated gentamicin complex (figure 16)^{42,43,44}. On the NMR spectra, the labeled peaks correspond with the numbering of carbons shown in figure 10. There are two main differences in the gentamicin C₁ and C₂ spectra correlating to the number of methyl groups present on the 6'-carbon and 6'-amine group. Both components contain a methyl group on the 6'-carbon shown at 1.2 ppm (H-26 and H-27). The ¹H peak associated with the additional methyl group on the 6'-amine group for gentamicin C₁ is present at 2.6ppm labeled as H-29 (figure 14). This peak is not present in the gentamicin C₂ spectrum, indicating the differences in the two compounds. The additional methyl groups labeled as H-7 and H-9, are the present for all the components in the gentamicin C complex. It is to be noted that the chemical shifts are not identical for the gentamicin spectra.

Gentamicin C₂ is epimeric at the 6'-carbon position. The two epimers are denoted as gentamicin C₂ and gentamicin C_{2a} with R and S configurations, respectively. These two epimers are purified as a single component and exhibit slight variations in the ¹H 1D NMR

spectrum (figure 15). The additional doublets of peaks at 1.2 ppm (H-26 and H-27) are consistent with the reference spectra and correlate with the epimer structures⁴².

Total Correlation Spectroscopy (TOCSY) and ^1H ^{13}C HSQC coupled and uncoupled spectra were analyzed to verify the results found from the ^1H 1D NMR spectra. Peak assignments for TOCSY and CHSQC spectra were identified similar to Cox et al.³⁵. Both spectra indicated the presence of two methyl groups associated with gentamicin C_1 whereas gentamicin C_2 only contained the presence of one methyl group on the 6'-carbon. For gentamicin C_2 , the TOSCY and CHSQC spectra confirmed that the additional peaks shown in the ^1H 1D NMR spectrum were due to epimeric carbon at the 6'-position and not from impurities.

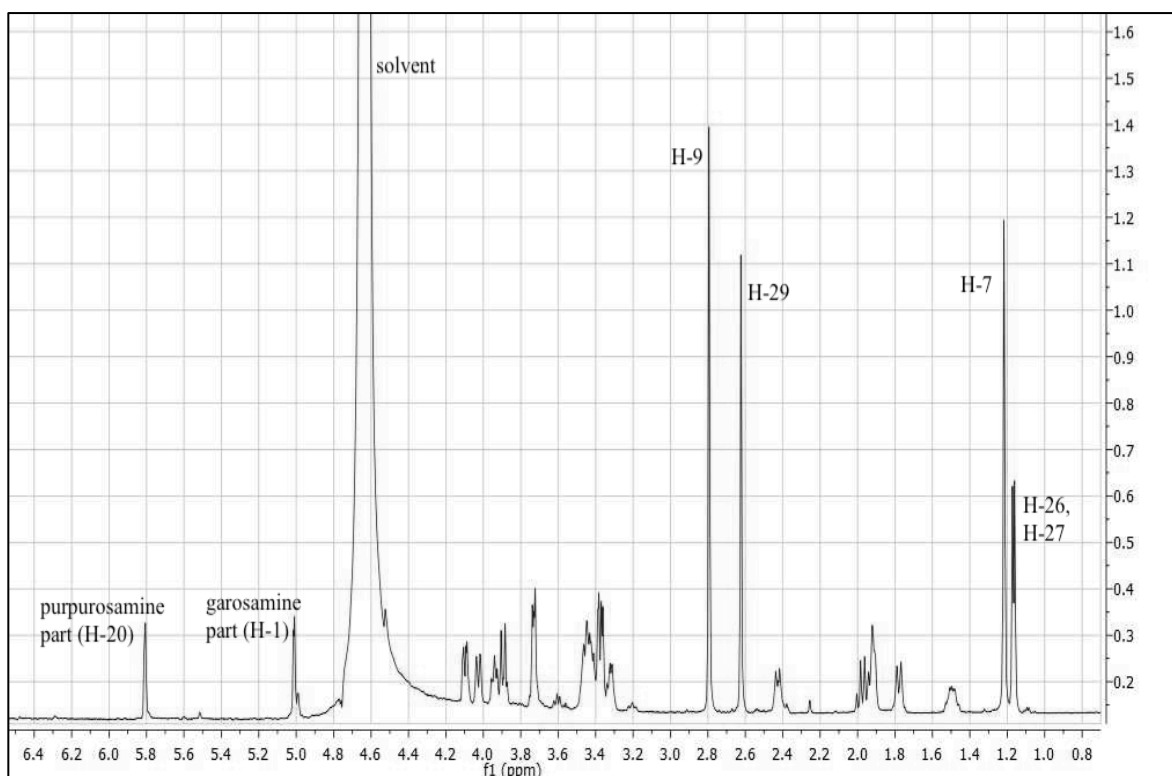


Figure 13: ^1H 1D NMR spectrum of gentamicin C_1 component in D_2O at 25°C .

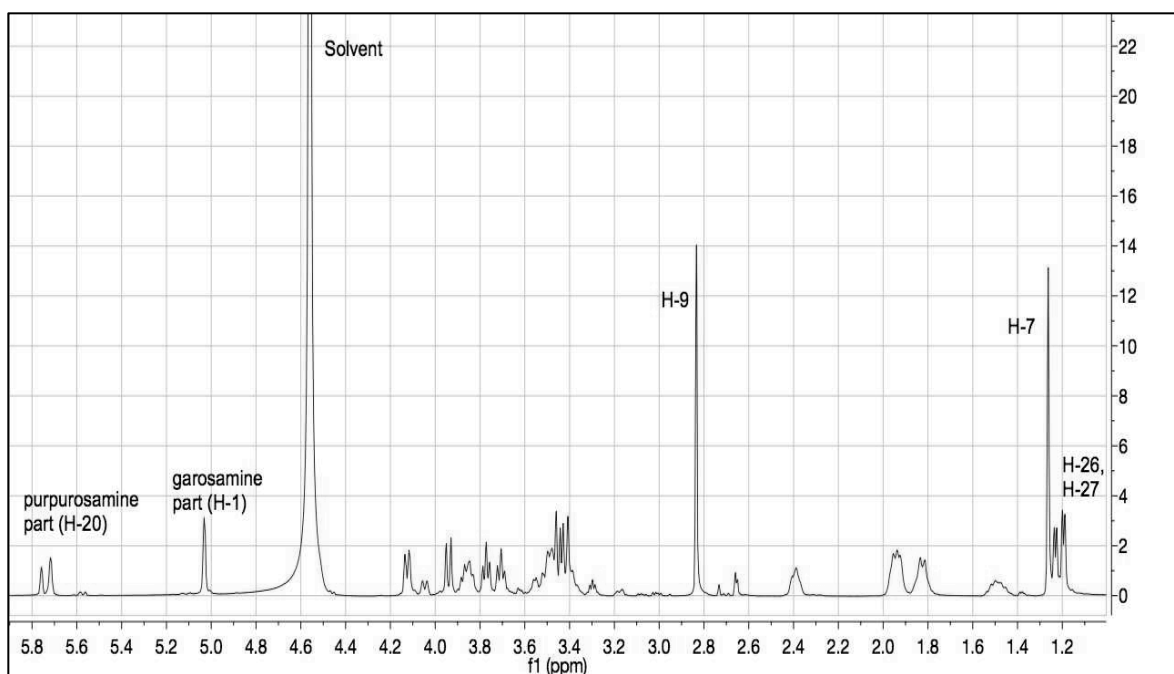


Figure 14: ^1H 1D NMR spectrum of gentamicin C_2 component in D_2O at 25°C .

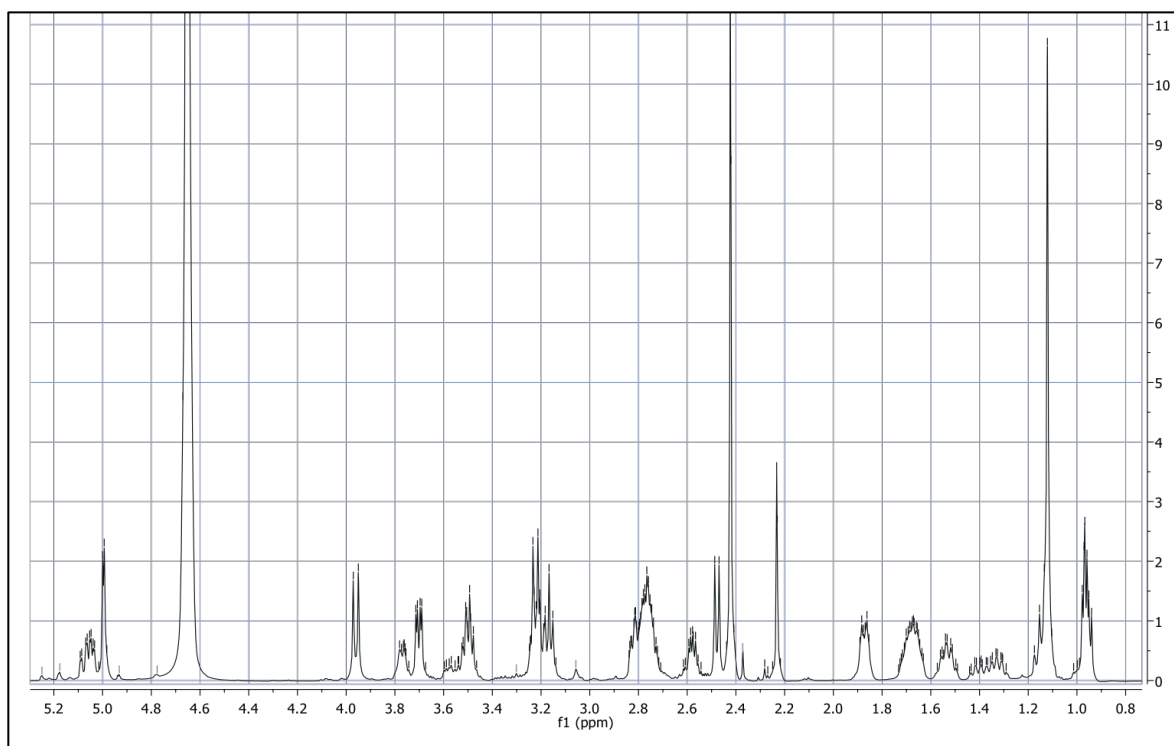


Figure 15: ^1H 1D NMR spectrum for desulfated gentamicin C complex at 25°C .

CHAPTER III: TEMPERATURE-DEPENDENT KINETIC ASSAYS FOR APH(3')-IIIa, AAC(3)-IIIb, AAC(3)-VIa

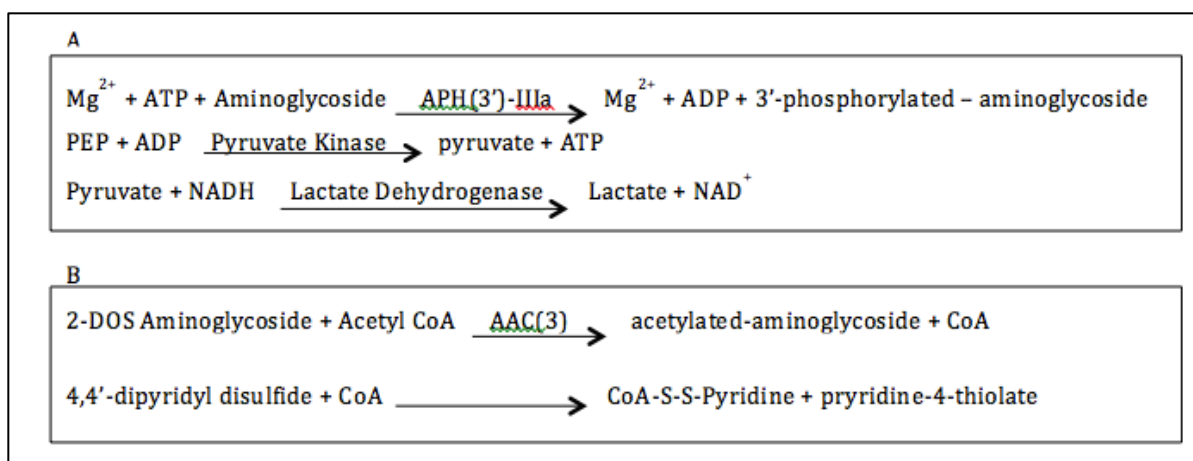
A. Background

I. Coupled Catalyzed Reactions

Modification reactions of aminoglycosides catalyzed by APH and AAC family of enzymes are coupled with pyruvate kinase/lactate dehydrogenase (PK/LDH) and 4,4'-dipyridyl disulfide, respectively (scheme 1). In general, coupled assays involve the addition of one or more enzymes to catalyze a reaction with one of the products produced by the enzyme being observed to produce another product that can be directly detected⁴⁵.

For APH, ATP is regenerated from ADP, one product produced by the catalyzed reaction of APH, by pyruvate kinase, which produces pyruvate. The production of pyruvate is used as a reactant to oxidize NADH by lactate dehydrogenase. The disappearance of NADH is directly measured at 340nm and is proportional to the production of the phosphorylated-aminoglycoside. In theory, the regeneration of ATP by the coupled reaction with pyruvate kinase will prevent the depletion of this substrate, thereby eliminating the potential reduction in initial velocity of the catalyzed reaction by APH¹³.

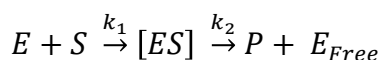
The AAC assay does not involve an additional coupled enzyme but contains the presence of 4,4'-dipyridyl disulfide, which is reduced by CoA to produce pyridine-4-thiolate. The production of pyridine-4-thiolate and disappearance of the disulfide can both be quantitatively measured at 324nm by UV-Vis spectrometry and are proportional to the production of the acetylated-aminoglycoside^{45,46}.



Scheme 1: Coupled catalyzed reactions for the APH(3')-IIIa (a) and AAC(3) families of enzymes (b). APH(3')-IIIa utilizes a PK/LDH coupled enzyme assay to measure the oxidation of NADH at 340nm, which is proportional to the production of phosphorylated aminoglycoside. The coupled reaction for the AAC(3) family of enzymes measures the production of pyridine-4-thiolate at 324nm, which is proportional to the acetylated-aminoglycoside product.

II. Michaelis-Menten Plots

Michaelis-Menten kinetics describes the rate enhancement of the chemical reactions catalyzed by an enzyme. In general, an enzyme in the presence of a substrate will form an enzyme-substrate complex, which is followed by the decomposition of this complex to form free enzyme and product (equation 1). Under rapid equilibrium conditions, initial velocity rates increase with an increase in substrate and enzyme concentrations. At saturating substrate concentrations, the maximum velocity (V_{max}) for a catalyzed reaction is achieved (equation 2). The K_m of a reaction is determined by, $K_m = \frac{V_{max}}{2}$, where half of the active sites on the enzyme are filled by a substrate. Catalytic turnover rates, the maximum number of substrate molecules converted to product per second, can be determined by the relationship of $k_{cat} = V_{max}/[E_{total}]$ ^{1,2,3,4,47,48}.



Equation 1

$$v = \frac{v_{max} * S}{(K_m + S)}$$

Equation 2

III. Determination of Gibbs Free Energy, Enthalpy, and Entropy of Activation

In 1889, Svente Arrhenius directly observed a relationship between rate constants and temperature. Arrhenius plots are based on the collision theory that states that molecules must possess enough kinetic energy to collide with one another to provoke product formation. Arrhenius plots are obtained by plotting the natural log of the catalytic turnover rates as a function of inverse temperature (K^{-1}) yielding a linear relationship in the form of $y = mx + b$. The slope of the Arrhenius plot directly measures the activation energy (average kinetic energy) required for a reaction to proceed to product; whereas the y-intercept of the Arrhenius plot provides the pre-exponential factor ($\ln A$), which can be used to determine the rate constant from the Arrhenius equation, $k_{cat} = Ae^{-\frac{E_a}{RT}}$, where R is the gas constant. The Gibbs free energy of activation can be described by: $\Delta G^\ddagger = -RT \ln(k_{cat}^\ddagger h / k_B T)$, where h is Planck's constant and k_B is the Boltzmann constant. The activation energy of a reaction is analogous to the enthalpy of activation by the Eyring equation: $E_a = \Delta H^\ddagger + RT$. The activation energy can be obtained from the slope (equation 3). From the thermodynamic relationship, $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$, the entropy of activation for the reaction to the transition state can be determined^{1,2,3,4,47,49,50}.

$$\text{Slope} = \frac{-E_a}{R}$$

Equation 3

B. Experimental Procedure

I. Chemicals and Reagents

All chemicals and reagents used were of the highest purity. Aminoglycoside antibiotics, adenosine 5'-triphosphate (ATP), and other chemicals required for kinetic activity assays and protein were obtained from Sigma (St. Louis, MO). Sepharose Ni²⁺ NTA resin was obtained from GE Healthcare and High MacQ anion exchange column resin was obtained from BioRad. Kinetic assays were conducted using a Varian Carey 50Bio UV-Vis spectrophotometer and temperature was controlled using a Brinkmann Lauda RM6 circulating water bath system.

II. Purification of APH(3')-IIIa

APH (3')-IIIa was expressed in *Escherichia coli* BL21 (DE3) containing the overexpression plasmid pETPCR6 provided by Dr. Gerard D. Wright, and was recloned to include an N-terminal 6-his-tag. APH (3')-IIIa was purified using a modified protocol described by McKay et al¹³. Colonies were grown at 37°C overnight on Luria broth (LB) agar from a 20% glycerol stock. A 10ml subculture was inoculated from one colony in LB broth containing 0.1mg/ml ampicillin and grown overnight at 37°C with shaking. Two liters of (LB) media containing 0.1mg/ml of ampicillin were inoculated with 20ml from the overnight subculture and grown at 37°C with shaking. Growth of cells was monitored by OD₆₀₀ every hour until mid log phase was reached (0.6-0.8 absorbance). Protein expression was induced with 1mM IPTG for four hours and harvested by centrifugation at 6,000xg for 15 minutes at 4°C. Harvested cells were washed with 0.85% NaCl solution, centrifuged at 6,000xg at 4°C, and prepped for lysis. Cell pellet was solubilized in 20mL of lysis buffer (50mM Tris-HCL pH 7.6, 100mM NaCl, 20mM Imidazole, 100mM phenylmethylsulfonyl fluoride (PMSF)) and lysed with 3 passes of the French Press at 15,000 psi. The lysate was centrifuged at 35,000xg for one hour to remove cell debris. Lysate was loaded onto a Sepharose Ni²⁺ NTA column (1.5mL slurry resin in 1.5x5 column) and washed with 150mL of wash buffer (50mM Tris-

HCL pH 7.6, 100mM NaCl, 20mM Imidazole). Purified protein was eluted using a step gradient consisting of 15ml wash buffer (50mM Tris-HCL pH 7.6, 100mM NaCl, 100mM Imidazole) and 10mL of elution buffer (50mM Tris-HCL pH 7.6, 100mM NaCl, 500mM Imidazole) and dialyzed 3 times against 350ml of dialysis buffer (50mM Tris-HCL pH 7.6, 100mM NaCl) for 1.5 hours. The N-terminal 6-His tag was removed by thrombin, 1:750 ratio of thrombin to protein (w/w) for one hour at room temperature with light shaking and reloaded onto a Sepharose Ni²⁺ column. Flow through containing purified protein was measured at 280/260nm ratio. Purified protein was loaded onto a high MacQ anion exchange column using Fast Protein Liquid Chromatography (FPLC). Protein fractions (2ml) were eluted using a linear gradient (0.1M to 2M NaCl, 50mM Tris-HCL pH 7.6) with a flow rate of 2.0ml/min and collected with a fraction collector. Purified protein fractions were verified by SDS-PAGE electrophoresis. Protein was dialyzed against 350ml of dialysis buffer 3 times for 1.5hours and stored at -80°C in 1ml aliquots.

II. Purification of AAC(3)-IIIb

Purified AAC(3)-IIIb (46.8µM) was kindly provided by Sherin Raval, a recent member of the lab. Before conducting kinetic assays, protein activity was verified to be equal to the original activity (63.5µmol/min*mg). The protein purification procedure used by Raval was adopted from the original procedure written by Owston et al. and Norris et al^{27,31}. AAC(3)-IIIb was originally isolated from *Pseudomonas aeruginosa* [provided by Dr. George Miller and Karen Shaw of Schering Plough] and transformed into a pET22b plasmid vector containing a N-terminal 6-his-tag and was expressed by *Escherichia coli* BL21 (DE3) cells. Transformed cells were first overexpressed by inoculating 1ml of LB broth with a single colony of cells grown on agar plates containing 0.1mg/ml ampicillin for 6-8hours at 37°C with shaking. From the initial inoculation, an overnight culture containing the initial 1ml inoculation and 0.1mg/ml ampicillin was grown overnight for 18-24hours at 37°C with shaking. The overnight culture was added to fresh LB media containing 01.mg/ml ampicillin and grown at 37°C with shaking. Cell growth was monitored every hour by OD₆₀₀ until mid log phase was reached at an absorbance between 0.6-0.8. At mid log phase, 1mM of IPTG was added to protein overexpression and grown for an additional 4 hours. Cells were

harvested by centrifugation at 6,000xg for 13 minutes at 4°C. The harvested cell pellet was resuspended and washed with 150ml of 0.85% NaCl, centrifuged at 6,000xg at 4°C, and prepped for cell lysis. Cells were resuspended in 15ml of lysis buffer (100mM PMSF, 50mM Tris-HCL pH 7.6, 100mM NaCl, 20mM Imidazole) and homogenized. Cells were lysed with 3 passes of the French press at 15,000 PSI and centrifuged at 35,000xg for 1 hour. Crude lysate was loaded onto a Sepharose Ni²⁺ NTA column (1.5ml resin slurry onto a 5x5 column). Column was initially washed with 150ml of wash buffer (50mM Tris-HCL pH 7.6, 100mM NaCl, 20mM Imidazole). Protein was eluted with 40ml of 50mM Tris-HCL, pH 7.6, 100mM NaCl, and an Imidazole gradient from 100mM to 350mM by a 100ml gradient maker. Protein fractions (2mL) were collected and measured for purity (1:40 dilution) at 280/260 on a CaryWin UV-Vis Spectrophotometer. Purified protein fractions (1.4 or higher absorbance ratio) were pooled and dialyzed against 350ml of dialysis buffer #1 (50mM Tris-HCL pH 7.6, 100mM NaCl) three times for 1.5hours. Thrombin, 1:100 ratio of thrombin to protein (w/w), was added and incubated for 3 hours at room temperature with gentle shaking. Protein containing thrombin was reloaded onto the Sepharose Ni²⁺ column and washed with 10ml of wash buffer. Flow through was collected and analyzed on CaryWin UV-Vis spectrophotometer to determine the 280/260nm ratio. Purified protein was dialyzed against 3 changes of 350ml of dialysis buffer #2 (50mM Tris-HCL, pH 8.0) for 1.5hours. To remove the remaining salt, protein was loaded onto a MacroPrep Q anion exchange column (25ml resin slurry in a 1.5x25 column). Protein was loaded onto the anion exchange column and washed with 50ml of ion exchange wash buffer (50mM Tris-HCL, pH 8.0). Purified protein was eluted by 50ml salt gradient containing 50mM-Tris-HCL pH 8.0 and increasing salt concentration from 0-700mM NaCl. Protein fractions were collected in 3-4ml fractions and verified by 280/260nm ratio and SDS-PAGE electrophoresis. Purified protein was dialyzed 3 times with 500ml dialysis buffer #2 for 1.5hours. Aliquots containing 1ml of protein were stored at -80°C.

III. Purification of AAC(3)-VIa

Purified AAC(3)-IIIb (49.0μM) was kindly provided by Prashasti Kumar, a current member of the lab. Before conducting kinetic assays, protein activity was verified to be equal

to the original activity. AAC(3)-VIa was transformed into a pET15b plasmid vector containing a N-terminal 6-his tag by GeneScript. Plasmid was transformed and overexpressed into *E. coli* BL21 (DE3) cells. Colonies were grown on agar plates overnight at 37°C. A subculture grown in LB media containing 0.1mg/ml ampicillin was inoculated with a single colony and incubated at 37°C with shaking at 200rpm overnight. The overnight culture was added to fresh LB media containing 0.1mg/ml ampicillin and grown at 37°C with shaking at 200rpm until mid log phase was reached at OD₆₀₀ 0.6-0.8 absorbance. Protein overexpression was induced with 0.5mM of IPTG for 3.5 hours at 37 °C with shaking at 200rpm. Cells were harvested by centrifugation at 6,000xg for 15 minutes at 4°C. Cell pellet was resuspended with 0.85% NaCl and centrifuged at 4°C for an additional 15 minutes at 6,000xg. Cell pellet was resuspended in 25ml of lysis buffer (100mM PMSF, 50mM Tris-HCL pH 7.6, 100mM NaCl, 20mM Imidazole) and lysed by 3 passes of the French Press at 15,000 PSI. Lysate was centrifuged at 4°C for 1 hour at 35,000xg. Lysate was loaded onto a Sepharose Ni²⁺ column (1.5ml resin slurry in 1.5x5 column) and washed with 150ml of wash buffer (50mM Tris-HCL pH 7.6, 100mM NaCl, 20mM Imidazole). Protein was eluted with 15ml of elution buffer (50mM Tris-HCL, pH 7.6, 100mM NaCl, 300mM Imidazole) with 5-6ml fractions collected. Fractions were analyzed on a CaryWin UV-Vis spectrophotometer at 280/260nm ratio. Purified protein was dialyzed against 1L of dialysis buffer (50mM Tris-HCL pH 8.0, 100mM NaCl) 3 times for 1.5 hours at 4°C. The N-terminal 6-His-tag was cleaved by thrombin, 1:750 ratio of thrombin to protein (w/w), with 50mM MOPS for 4 hours at room temperature with light shaking. Protein was reloaded onto a Sepharose Ni²⁺ column and washed with 10ml of dialysis buffer. Flow through was collected in 2-3ml fractions and absorbance was monitored at 280/260nm. Protein was dialyzed against 1L of 50mM Tris-HCL pH 8.0 3 times for 1.5 hours. Dialyzed protein was loaded onto a MacroQ anion exchange column (1.5x25 column) and washed with 2 column volumes of ion exchange buffer A (50mM Tris-HCL pH 8.0). Protein was eluted by a 0-1M salt gradient and 2ml fractions were collected. Purified protein fractions were determined by SDS-PAGE electrophoresis, dialyzed again with 1L of 50mM Tris-HCL pH 8.0 buffer 3 times for 1.5hours, and stored at 4°C.

IV. Kinetic Assays for APH(3')-IIIa

APH(3')-IIIa activity assay protocol was adapted from McKay et al¹³. Phosphorylation of aminoglycoside antibiotics was monitored by the coupled enzymatic assay by measuring the production of NAD⁺ converted by Kinase/lactate dehydrogenase (PK/LDH) reaction (scheme 1A). Oxidation of NADH was monitored at 340nm absorbance, which is proportional to the production of phosphorylated aminoglycoside. Assay mixture was composed of assay buffer (50mM Tris pH 7.6, 100mM KCl, 1.5mM MgCl₂, 150μM NADH, 2mM phosphoenolpyruvate, 1mM ATP), various concentrations of aminoglycoside antibiotics, and 5μL of PK/LDH (0.02mg/ml) enzyme solution for a total volume of 1ml. The mixture was pre-incubated at various temperatures for 15 minutes and initiated by a rapid addition of purified APH(3')-IIIa (32.5μM) stock solution. Initial rates were obtained from enzymatic reaction curves and analyzed by Michaelis-Menten fitting (equation 2) in Prism Graphpad 6.0 software.

V. Kinetic Assays for AAC(3)-IIIb and AAC(3)-VIa

AAC(3)-IIIb and AAC(3)-VIa catalyzed modification reaction was measured using a coupled assay (scheme 1B)³¹. The product formation of pyridine-4-thiolate was measured at 324nm absorbance, which was proportional to the production of acetylated aminoglycoside. Assay mixture was composed of assay buffer (50mM Tris-HCL pH 7.6, 100mM NaCl, 750μM Aldiothiol, 100μM Acetyl CoA, 100mM EDTA) and various concentrations of aminoglycoside antibiotics for a total volume of 1ml. The mixture was pre-incubated at various temperatures for 15 minutes and initiated by a rapid addition of purified AAC(3)-IIIb or AAC(3)-VIa (1 μM) stock solution. Initial rates were obtained from enzymatic reaction curves and analyzed by Michaelis-Menten fitting (equation 2) in Prism Graphpad 6.0 software.

C. Results and Discussion

I. Kinetic Assays for APH(3')-IIIa, AAC(3)-IIIb, and AAC(3)-VIa

Michaelis-Menten plots were used to determine the kinetic parameters for APH(3')-IIIa, AAC(3)-IIIb, and AAC(3)-VIa with various aminoglycosides at a temperature range between 10°C to 37°C (table 2). For all three enzymes, there was no problem with the coupled reaction. For the coupled reaction with APH(3')-IIIa, the concentration of APH(3')-IIIa was doubled with 100µM aminoglycoside concentration. When the concentration of APH(3')-IIIa was doubled, the initial rates were remained the same. In theory, the coupled reaction with PK/LDH is a 1:1:1 ratio. Therefore, doubling the concentration of APH(3')-IIIa should not affect the initial velocity rates when measuring the amount of phosphorylated aminoglycoside. For the coupled reaction with AAC(3)-IIIb and AAC(3)-VIa, the concentration of AAC(3)-IIIb or AAC(3)-VIa was doubled with 100µM aminoglycoside. When the initial rates were doubled for either enzyme, the initial velocity rates were proportional to the rates at standard conditions.

The initial rates for the catalyzed reactions with all three enzymes were observed to decrease at lower temperatures. To compensate for the decrease in rate, the concentration for all three enzymes was increased by at least 1µM. However, the increase in enzyme concentration was not consistent for all three enzymes and depended on which substrate was being studied.

The chemical and rate-limiting step for APH(3')-IIIa has been previous determined to be the binding of ATP and the release of the second product, ADP, respectively. The rate-limiting step for both AAC(3) enzymes has yet to be determined. These enzymes may exhibit similar kinetic mechanisms to those been previously determined for two other aminoglycoside – 3 – N – acteyltransferases, AAC(3)-IVa and AAC(3)-I^{51,52}. AAC(3)-IVa and AAC(3)-I were shown to exhibit a random sequential kinetic mechanism, where both substrates must first bind to the enzyme in order for catalysis to occur. For both enzymes, AAC(3)-IVa and

AAC(3)-I, a preferred order of substrate binding was proposed, depending on the affinity for the aminoglycoside and acetyl coenzyme A concentration, where acetyl coenzyme A is bound first followed by the binding of the aminoglycoside^{51,52}.

The kinetic parameters for all three enzymes exhibited a general trend with a decrease in $V_{\max}$ as temperature decreased. The K_m values were expected to follow this similar trend but this behavior was not observed. However, the determined K_m values did fall within the previously reported range of K_m values^{13,27}. The inconsistent decrease in K_m values may be due to variability within the assay and few substrate concentrations below the measured K_m values. The kinetic assays with APH(3')-IIIa and paromomycin showed the largest discrepancies between K_m values with little variation with the temperature. Assays at 30°C were repeated and additional temperatures close in range were performed for better fitting of the Arrhenius plot.

Table 2: Temperature-dependent kinetic parameters for APH(3')-IIIa, AAC(3)-IIIb, and AAC(3)-VIa with numerous aminoglycoside antibiotics at various substrate concentrations and temperatures ranging from 10°C -37°C.

Aminoglycoside	°C	^a K _m (μM)	^a k _{cat} (s ⁻¹)	k _{cat} /K _m (M ⁻¹ S ⁻¹)
APH(3')-IIIa				
<i>Kanamycin A</i>	37	12±2	1.9±0.1	1.50E+05
<i>Kanamycin A</i>	30	21±4	1.0±0.2	4.86E+04
<i>Kanamycin A</i>	25	6.0±1	0.5±0.04	8.64E+04
<i>Kanamycin A</i>	21	8.9±1	0.3±0.03	3.74E+04
<i>Kanamycin A</i>	15	14±4	0.3±0.04	1.86E+04
<i>Kanamycin A</i>	12	9.0±3	0.1±0.02	1.31E+04
<i>Paromomycin</i>	37	14±3	1.2±0.2	8.80E+04
<i>Paromomycin</i>	36	32±15	2.1±1	6.63E+04
<i>Paromomycin</i>	30	17±7	1.0±0.4	5.90E+04
<i>Paromomycin</i>	30	16±4	0.9±0.2	6.08E+04
<i>Paromomycin</i>	26	14±7	0.8±0.4	5.40E+04
<i>Paromomycin</i>	25	6.3±2	0.5±0.1	7.36E+04
<i>Paromomycin</i>	21	5.8±1	0.2±0.02	3.30E+04
<i>Paromomycin</i>	20	17±6	0.4±0.2	2.61E+04
<i>Paromomycin</i>	15	16±2	0.3±0.04	1.71E+04
<i>Paromomycin</i>	10	8±4	0.1±0.03	1.07E+04
<i>Ribostamycin</i>	37	48±23	3.0±2	6.30E+04
<i>Ribostamycin</i>	30	31±10	1.6±0.6	5.24E+04
<i>Ribostamycin</i>	25	9.2±5	0.7±0.3	7.81E+04
<i>Ribostamycin</i>	21	5.5±1	0.4±0.04	7.98E+04
<i>Ribostamycin</i>	15	5.9±2	0.2±0.04	3.18E+04
<i>Ribostamycin</i>	12	14±5	0.2±0.05	1.15E+04
<i>Neomycin B</i>	37	2.9±1	1.1±0.1	3.72E+05
<i>Neomycin B</i>	30	4.7±1	0.5±0.1	1.16E+05
<i>Neomycin B</i>	25	3.2±1	0.4±0.1	1.25E+05
<i>Neomycin B</i>	18	2.6±1	0.2±0.03	7.27E+04
<i>Neomycin B</i>	10	3.8±1	0.1±0.02	2.45E+04
AAC(3)-IIIb				
<i>Neomycin B</i>	35	2.3±1	6.6±0.7	2.94E+06
<i>Neomycin B</i>	30	7.7±2	7.2±1	9.36E+05
<i>Neomycin B</i>	25	3.0±1	5.0±0.9	1.68E+06
<i>Neomycin B</i>	20	1.7±0.3	4.0±0.3	2.38E+06
<i>Neomycin B</i>	15	3.6±1	2.0±0.3	5.63E+05
<i>Neomycin B</i>	12	0.6±0.1	1.1±0.04	2.07E+06

Table 2: Continued.

Aminoglycoside	°C	^a K _m (μM)	^a k _{cat} (s ⁻¹)	k _{cat} /K _m (M ⁻¹ S ⁻¹)
<i>Kanamycin A</i>	37	46±8	125±21	2.72E+06
<i>Kanamycin A</i>	30	20±3	78±8	3.93E+06
<i>Kanamycin A</i>	25	7.6±1	46±4	6.04E+06
<i>Kanamycin A</i>	19	4.3±1	21±1	4.86E+06
<i>Kanamycin A</i>	10	2.5±1	9.0±1	3.64E+06
<i>Paromomycin</i>	37	6.5±2	32±4	4.91E+06
<i>Paromomycin</i>	30	6.3±2	24±4	3.88E+06
<i>Paromomycin</i>	25	5.3±1	20±2	3.66E+06
<i>Paromomycin</i>	18	1.9±1	11±1	5.88E+06
<i>Paromomycin</i>	12	1.8±1	8.6±2	4.87E+06
<i>Ribostamycin</i>	37	24±5	33±5	1.40E+06
<i>Ribostamycin</i>	30	12±3	23±4	2.02E+06
<i>Ribostamycin</i>	25	16±1	20±2	1.24E+06
<i>Ribostamycin</i>	18	5.7±1	10±0.9	1.69E+06
<i>Ribostamycin</i>	12	4.6±0.3	7.3±0.3	1.59E+06
<i>Gentamicin C₂</i>	37	5.7±1	50±6	8.74E+06
<i>Gentamicin C₂</i>	30	2.8±0.4	41±2	1.47E+07
<i>Gentamicin C₂</i>	25	2.7±1	35±3	1.26E+07
<i>Gentamicin C₂</i>	18	3.1±0.3	24±1	7.53E+06
<i>Gentamicin C₂</i>	12	2.0±0.3	19±0.9	9.32E+06
<i>Gentamicin C₁</i>	30	3.7±0.4	24±1	6.55E+06
<i>Gentamicin C₁</i>	25	3.9±2	25±7	6.41E+06
<i>Gentamicin C₁</i>	18	2.9±1	21±3	7.53E+06
<i>Gentamicin C₁</i>	12	3.1±1	17±4	5.46E+06
AAC(3)-VIa				
<i>Gentamicin C₂</i>	37	1.6±0.2	13±1	8.45E+06
<i>Gentamicin C₂</i>	30	2.6±1	12±3	4.71E+06
<i>Gentamicin C₂</i>	25	0.6±0.1	11±0.4	1.89E+07
<i>Gentamicin C₂</i>	18	1.1±0.2	8.3±0.7	7.69E+06
<i>Gentamicin C₂</i>	12	1.7±1	5.1±1	3.07E+06
<i>Gentamicin C₁</i>	30	3.8±1	17±1	4.47E+06
<i>Gentamicin C₁</i>	25	2.2±0.5	11±1	5.20E+06
<i>Gentamicin C₁</i>	18	2.0±0.4	10±0.8	5.13E+06
<i>Gentamicin C₁</i>	12	2.4±1	6.5±1	2.74E+06

^aError calculations for K_m and k_{cat} values were determined by Prism GraphPad 6.0.

II. Activation Energies for APH(3')-IIIa with various 4,5- and 4,6-disubstituted Aminoglycosides

Table 3: Thermodynamic activation parameters for APH(3')-IIIa, AAC(3)-IIIb, and AAC(3)-VIa. Enthalpy, entropy, and free energy of activation were determined at 25°C. The activation energy was determined by the slope of the Arrhenius plot (equation 3).

	^a E_a (kcal /mol)	^a k_{cat} (s ⁻¹) _{25°C}	$\Delta G_{25^\circ\text{C}}^\ddagger$ (kcal/mol)	$\Delta H_{25^\circ\text{C}}^\ddagger$ (kcal/mol)	$T\Delta S_{25^\circ\text{C}}^\ddagger$ (kcal/mol)
APH(3')-IIIa					
<i>kanamycin A</i>	18±1	0.6±1	18±1	17±1	-0.5±1
<i>neomycin B</i>	16±0.3	0.4±1	18±0.3	15±0.3	-2.9±0.4
<i>paromomycin</i>	18±1	0.6±1.2	18±1	17±1	-0.4±2
<i>ribostamycin</i>	22±0.6	0.8±0.6	17±0.6	21±0.6	3.3±1
AAC(3)-IIIb					
<i>kanamycin A</i>	18±0.5	41±0.5	15±0.5	15±0.5	0.1±2
<i>gentamicin C1</i>	3.5±0.5	24±0.5	16±0.5	17±0.5	1.2±0.7
<i>gentamicin C2</i>	7.1±0.2	33 ±0.2	16±0.2	16±0.2	1.0±0.3
<i>neomycin B</i>	17±1	5.1±0.3	17±1	15±1	-1.1±2
<i>paromomycin</i>	9.6±0.4	17±0.4	16±0.4	16±0.4	-0.1±0.6
<i>ribostamycin</i>	11±0.5	17±0.5	16±0.5	16±0.5	-0.1±0.7
AAC(3)-VIa					
<i>gentamicin C1</i>	8.2±1	13±1	16±1	17±1	0.9±1
<i>gentamicin C2</i>	6.4±0.6	9.6±1	16±0.6	17±0.6	0.8±1

^a Calculated errors for the activation energy (E_a) and k_{cat} values were determined by Prism Graphpad 6.0.

The activation energies for APH(3')-IIIa were determined for kanamycin A, neomycin B, ribostamycin, and paromomycin (table 3). There were no significant trends differing between the 4,6- and 4,5-di-substituted aminoglycosides. These data also agree with previous findings where the turnover rates and thermodynamic parameters did not distinguish between the kanamycin-like and neomycin-like classes of aminoglycosides^{13,14}. The slopes of the Arrhenius plots were extrapolated and shown to intersect at similar temperatures ranging from -5°C to 12°C, at which APH(3')-IIIa was able to modify all substrates equally fast (figure 17). The order of decreasing activation energy and catalytic turnover rates at 25°C for substrates was ribostamycin > paromomycin ≥ kanamycin A > neomycin B. Kanamycin A (three modified amino-sugar rings) and paromomycin (four modified amino-sugar rings) exhibited similar activation energies and identical turnover rates. Due to the similar activation energies for kanamycin A and paromomycin, it may be suggested that APH(3')-IIIa does not prefer a particular size of substrate in order to reduce the activation energy. This differs from previous thermodynamic data where the size of aminoglycosides was proposed to influence the thermodynamic parameters for the formation of the ternary complex¹⁴.

Ribostamycin was observed to have the least favorable enthalpy of activation accompanied by a more favorable entropy of activation. These observations correlate with the thermodynamic parameters in the formation of the ternary complex where ribostamycin exhibited a less favorable intrinsic enthalpy compared to other 4,5-disubstituted 2-DOS aminoglycosides. It was suggested that the fourth rings of 4,5-disubstituted aminoglycosides (neomycin B and paromomycin) contribute to the binding within the active site^{14,15}. These observations suggest that the more positive $\Delta H^\ddagger$ associated with ribostamycin may be due to additional breaking of bonds at the transition state to adopt a certain conformation. In doing so, a more positive $T\Delta S^\ddagger$ may be due to the rearrangement of solvent to yield similar $\Delta G^\ddagger$ values for the substrates studied. On the other hand, neomycin B was observed to exhibit a less positive $\Delta H^\ddagger$ and a negative $T\Delta S^\ddagger$ value. This may be due to a more structured transition state when bound to neomycin B^{53,54,55}.

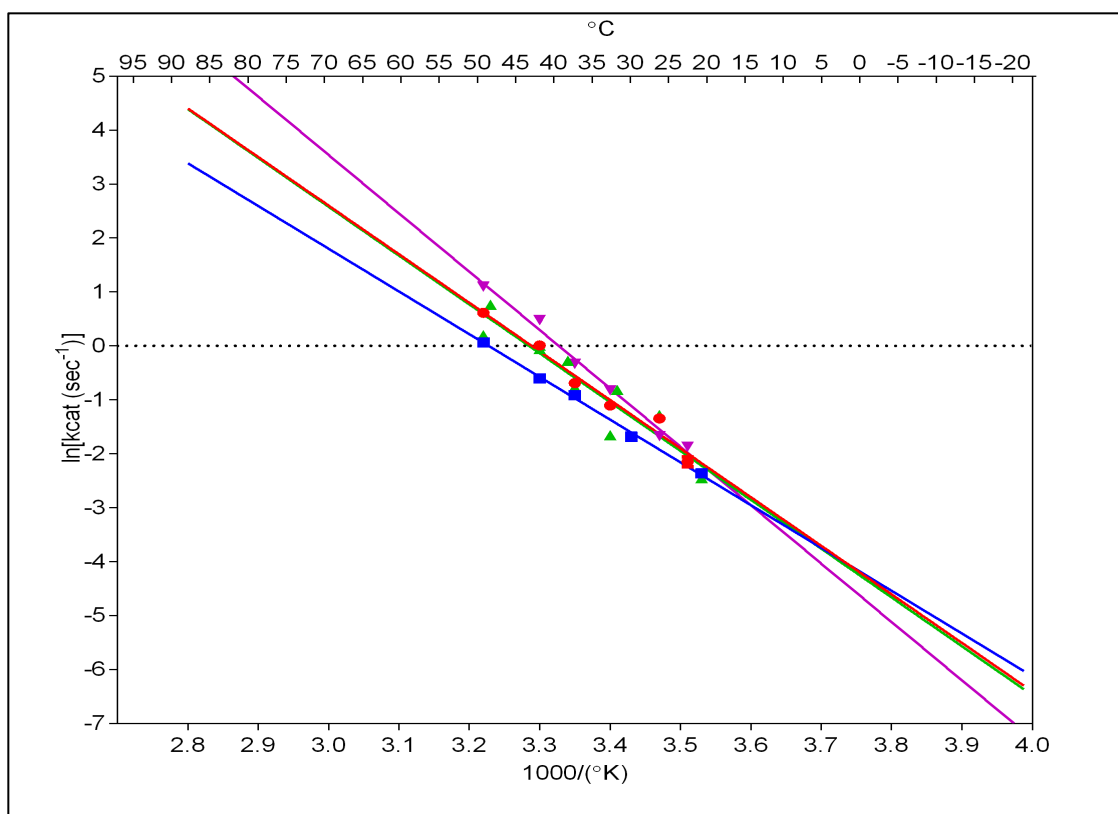


Figure 16: Arrhenius plots for APH(3')-IIIa with kanamycin A(red), neomycin B(blue), parmomycin(green), and ribostamycin(magenta). There were no significant trends in activation energy distinguishing the 4,5-disubstituted and 4,6-disubstituted aminoglycosides: ribostamycin>parmomycin≥kanamycin A>neomycin B.

III. Activation Energy for AAC(3)-IIIb with Various Aminoglycosides

The activation energies for AAC(3)-IIIb were determined for kanamycin A, gentamicin C₁, gentamicin C₂, neomycin B, paromomycin, and ribostamycin (table 3). Similar to APH(3')-IIIa, there were no significant trends in the activation energies that distinguished between the 4,5- and 4,6-disubstituted aminoglycosides: gentamicin C₁ > gentamicin C₂ > paromomycin > ribostamycin > neomycin B > kanamycin A. This agrees with the previously reported data, which illustrated that there were no significant trends distinguishing between the two classes of aminoglycosides based on the thermodynamic parameters but did show a difference between the two classes in the overall catalytic turnover rates, which was lower for the 4,5-disubstituted aminoglycosides²⁷.

In general, both gentamicin components had the shallowest slopes, whereas kanamycin A had the steepest slope (figure 18). Both gentamicins carry hydrophobic methyl groups, which are absent in kanamycin A, neomycin B, paromomycin, and ribostamycin. It may be suggested that an increase in hydrophobicity may contribute to a decrease in activation energy for AAC(3)-IIIb. From homology modeling, AAC(3)-IIIb contains several nonpolar and hydrophobic residues that line the antibiotic binding site (figure 19). These residues may increase the number of interactions with the primed ring of gentamicin, which makes the most important contacts within the active site, allowing the orientation of the antibiotic to be better optimized for catalysis^{27,28,29}.

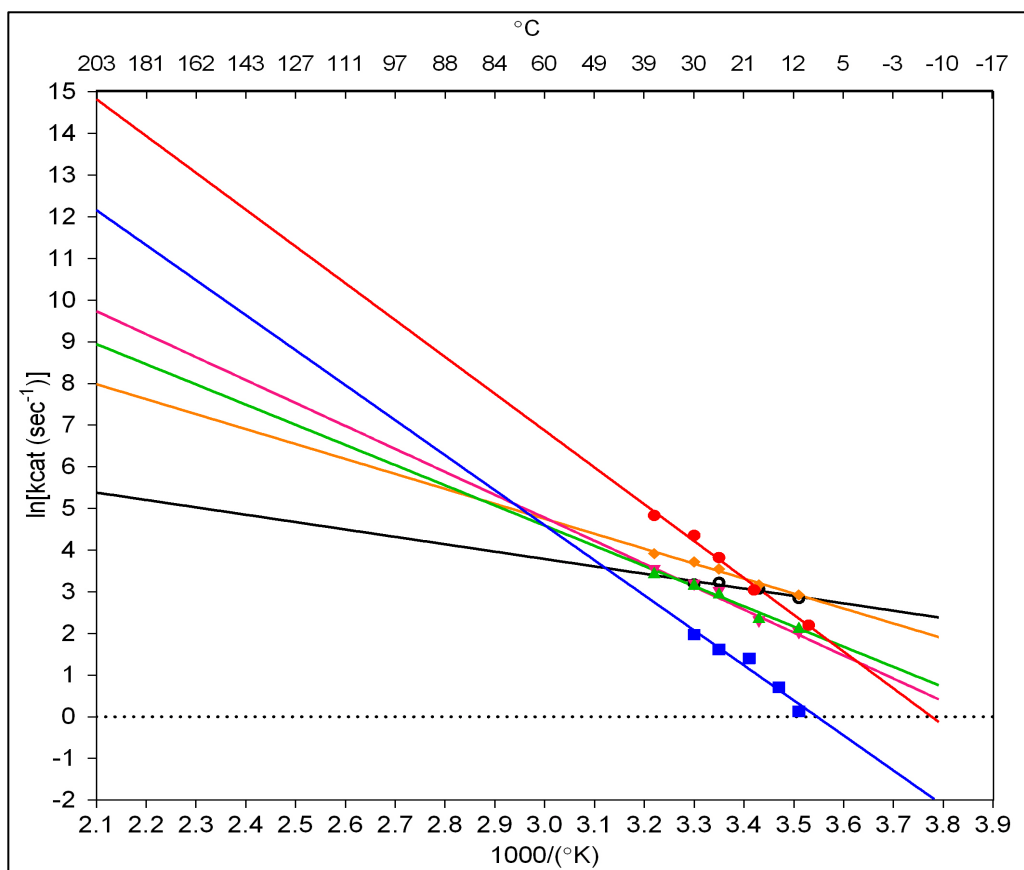


Figure 17: Arrhenius plots for AAC(3)-IIIb with kanamycin A (red), gentamicin C₁ (black), gentamicin C₂ (orange), neomycin B (blue), paromomycin (green), and ribostamycin (magenta). There were no significant trends in activation energy distinguishing the 4,5-disubstituted and 4,6-disubstituted aminoglycosides with the Arrhenius slopes each substrate interacting at a wide range of temperatures.

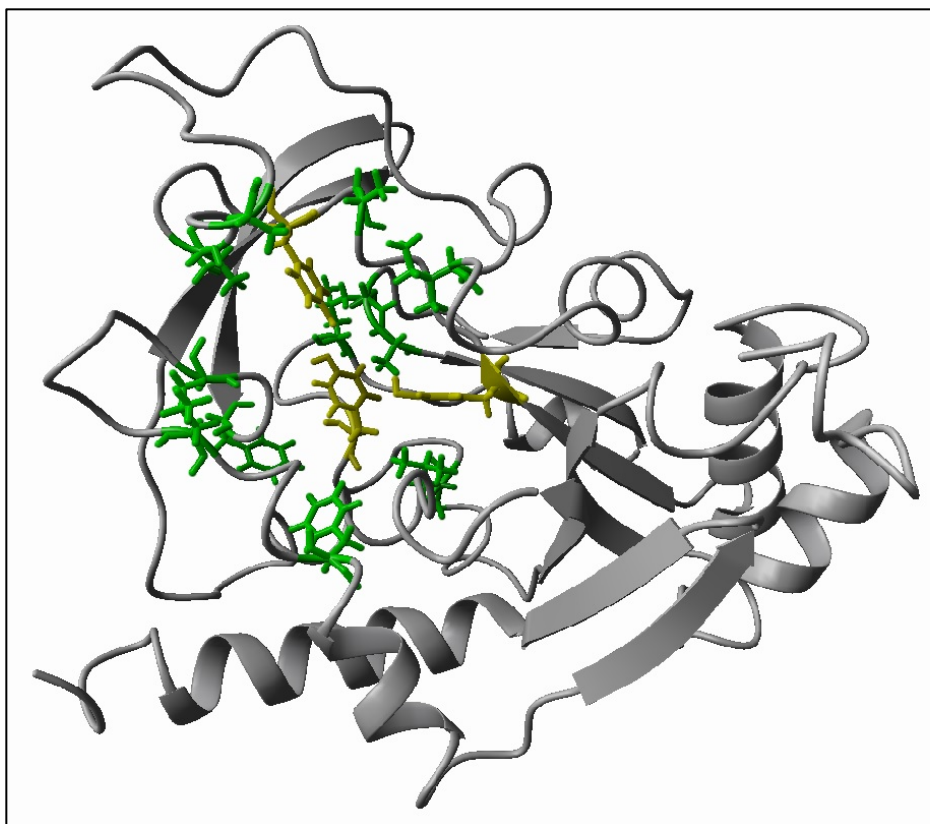


Figure 18: A cartoon representation of the homology model of AAC(3)-IIIb from AAC(3) in complex with CoASH (PDB: 3IJW) from *Bacillus*. The lining of the antibiotic binding loop has been proposed to contain hydrophobic residues (green) and three tyrosine residues (yellow)²⁷.

The thermodynamic activation parameters were fairly consistent for all aminoglycosides. The gentamicin components had the least favorable free energy of activation due to a slightly more positive enthalpy of activation, which was compensated with a slightly positive entropy of activation. Although there have been no structural or thermodynamic studies conducted with AAC(3)-IIIb and the gentamicin components, the slightly positive entropy of activation may suggest an increase in the degrees of freedom of the ternary complex at the transition state if the k_{cat} is shown to monitor the chemical step^{53,54,55}. This may be attributed to the potential increase in hydrophobic and nonpolar residues lining the active site on the antibiotic binding loop, which would promote more interactions with the gentamicin primed ring and an increase in the rearrangement of solvent^{27,28,29}.

The 4,5-disubstituted aminoglycosides were observed to all have a slightly negative entropy of activation while the 4,6-disubstituted aminoglycosides all exhibited a slightly positive entropy of activation. When comparing neomycin B to kanamycin A, the enthalpies of activation are nearly the same with a ~ 1 kcal/mol difference in the free energy of activation.

When comparing the 4,6-disubstituted aminoglycosides, the highest activation energy correlated with the fastest turnover rates: kanamycin A > gentamicin C₂ > gentamicin C₁. When the slopes on the Arrhenius plots are extrapolated, kanamycin A was observed to be modified equally as fast as gentamicin C₁ and C₂ at temperatures 18°C and 21°C, respectively; whereas gentamicin C₂ and gentamicin C₁ were observed to be modified at equivalent rates at 10°C (figure 20). The enthalpy and entropy of activation for all three 4,6-disubstituted aminoglycosides were fairly similar while the catalytic turnover rates measured at 25°C varied. Therefore, the differences in activation energies for 4,6-disubstituted aminoglycosides may be due to the orientation of the aminoglycoside in the active site, which allows AAC(3)-IIIb to catalyze the reaction more quickly for gentamicin components than for kanamycin A.

When comparing the 4,5-disubstituted aminoglycosides, these substrates all had similar activation energies, neomycin B > ribostamycin > paromomycin, which was proportional to the catalytic turnover rates measured at 25°C. The correlation between lowest activation energy and highest catalytic turnover rate was only observed for AAC(3)-IIIb bound with 4,5-disubstituted aminoglycosides. When extrapolating the linear regression line, neomycin B was modified equally as fast as paromomycin and ribostamycin at higher temperatures, 60°C and 68°C, respectively; whereas ribostamycin and paromomycin were modified equally as fast at cooler temperatures around 30°C (figure 21). All three substrates exhibited similar enthalpies of activation and slight negative entropies of activation. Neomycin B was observed to have ~ 1 kcal/mol more positive free energy of activation compared to paromomycin and ribostamycin. Previous data suggested that the size of the aminoglycoside and presence of a 6'-amine group influences the affinity of the substrate to AAC(3)-IIIb. The activation energies do not appear to correlate with these findings, where ribostamycin, the smallest of the 4,5-aminoglycosides studied, and paromomycin, containing a 6'-hydroxyl group, exhibited a lower activation energy compared to neomycin B^{27,28}.

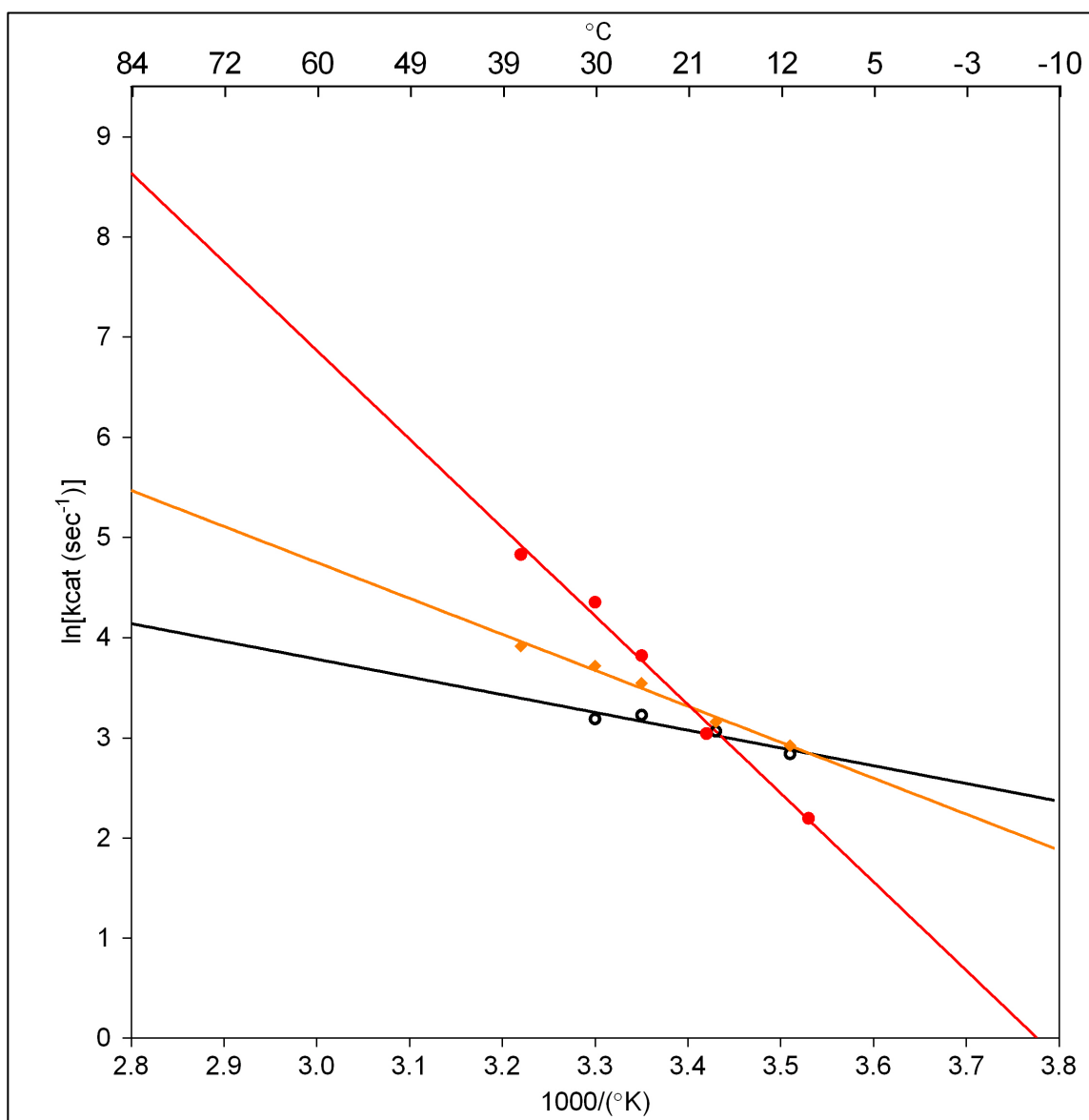


Figure 19: Arrhenius plots for AAC(3)-IIIb with kanamycin A(red), gentamicin C₁ (black), and gentamicin C₂ (orange). AAC(3)-IIIb modified both genamicin components at 10°C while kanamycin A was modified as quickly as gentamicin C₁ and C₂ at 18°C and 21°C, respectively.

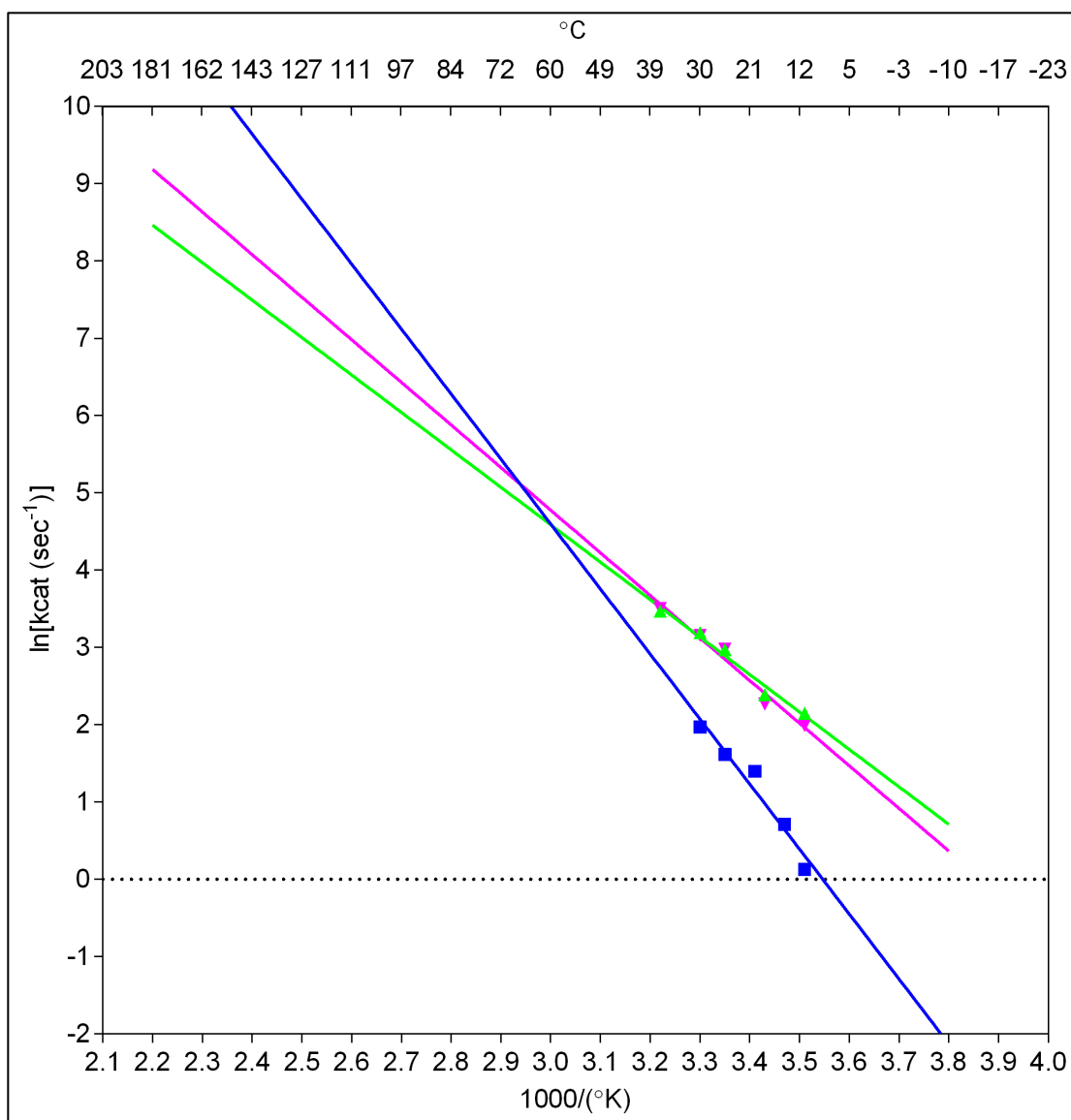


Figure 20: Arrhenius plots for AAC(3)-IIIb with neomycin B (blue), paromomycin (green), and ribostamycin (magenta). The Arrhenius slopes intersected at much greater temperature ranges compared to the 4,6-disubstituted aminoglycosides. AAC(3)-IIIb modifies neomycin B equally as fast as paromomycin and ribostamycin at temperatures 60°C and 68°C, respectively, whereas paromomycin and ribostamycin are modified equally as fast at 30°C.

III. Activation Energy for AAC(3)-VIa with Gentamicin C₁ and C₂

The activation energy for AAC(3)-VIa was determined for gentamicin C₁ and gentamicin C₂ (table 3). Gentamicin C₂ had a lower activation energy and catalytic turnover rate than gentamicin C₁. The enthalpy, entropy and free energy of activation for both gentamicin components were almost identical. When extrapolating the slopes on the Arrhenius plot, gentamicin C₁ and gentamicin C₂ were modified equally as fast at -1°C (figure 22).

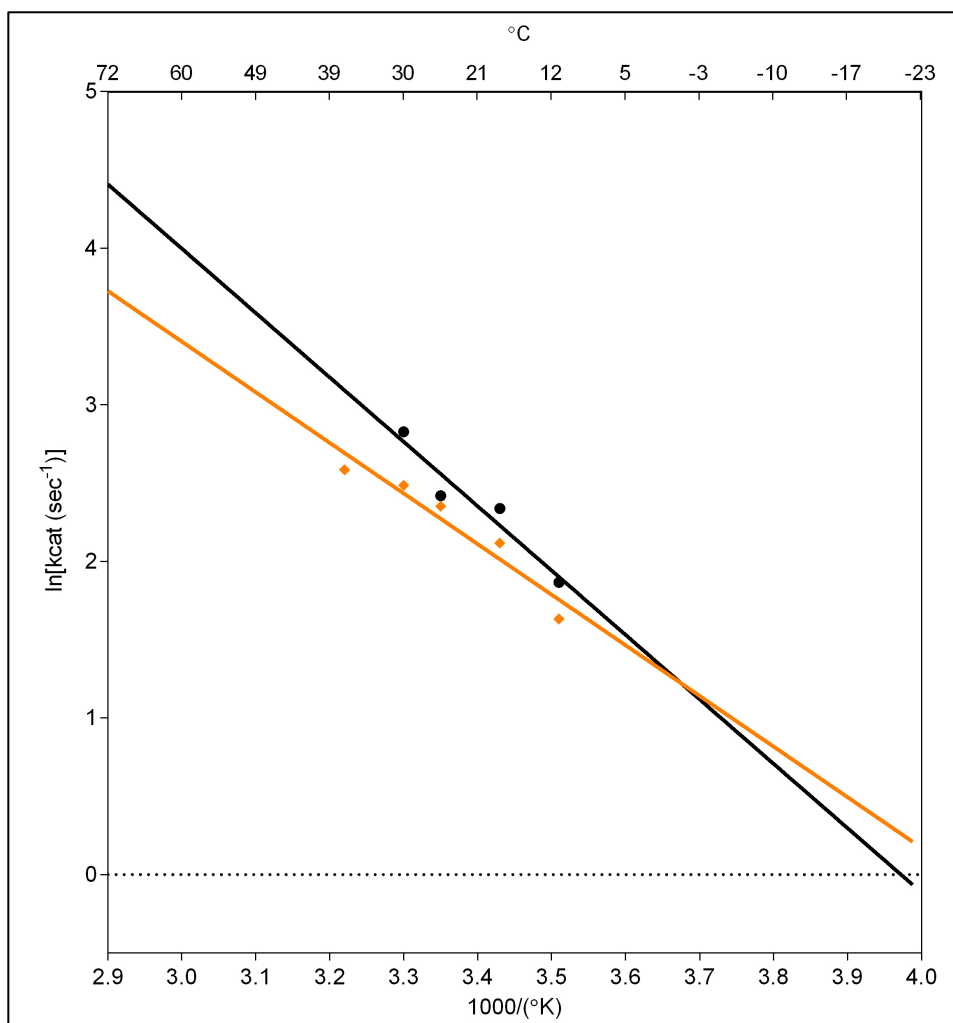


Figure 21: Arrhenius plots for AAC(3)-VIa with gentamicin C₁ (black) and gentamicin C₂ (orange). Gentamicin components were modified equally as fast at -1°C.

CHAPTER IV: FUTURE RESEARCH

It is well known that the addition of heat increases the rate of most reactions. Many spontaneous reactions occur at extremely high temperatures that are not biologically feasible. To better understand the release of enthalpy and rate enhancement produced by a catalyst, spontaneous, uncatalyzed reaction rates need to be observed. Several complications arise when measuring the spontaneous reactions catalyzed by aminoglycoside modifying enzymes. For the APH family of enzymes, a coupled reaction measuring the disappearance of NADH is used to measure the initial velocity rates. NADH becomes inactive at temperatures above 50°C. For the AAC family of enzymes, Acetyl coenzyme A is used as the acetyl donor in the modification reaction. Acetyl CoA becomes unstable at temperatures higher than 37°C. In order to measure the spontaneous reactions, variations of these reactions will need to be developed. The difference in the rate enhancement produced by APH(3')-IIIa and AAC(3)-IIIb may provide a better understanding for their promiscuous nature while exhibiting very different protein dynamics in the apo- and enzyme bound-form. Comparing AAC(3)-IIIb and AAC(3)-VIa may also provide further insight to the selectivity of substrates between two enzymes within the same family of aminoglycoside modifying enzymes that catalyze the modification reaction by the same mechanism.

In addition to measuring the uncatalyzed reactions, future studies should consist of determining the kinetic mechanism for AAC(3)-IIIb and AAC(3)-VIa, thermodynamic properties with gentamicin components, and protein dynamics for AAC(3)-VIa. Viscosity experiments should be conducted to determine the rate-limiting step for both of these enzymes. Understanding the kinetic mechanism will provide further support on the stability of the transition state. The thermodynamic parameters and protein dynamics when bound to gentamicin C₁ and C₂ will provide further understanding for the potential hydrophobic contributions that effect the activation energy.

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APPENDIX

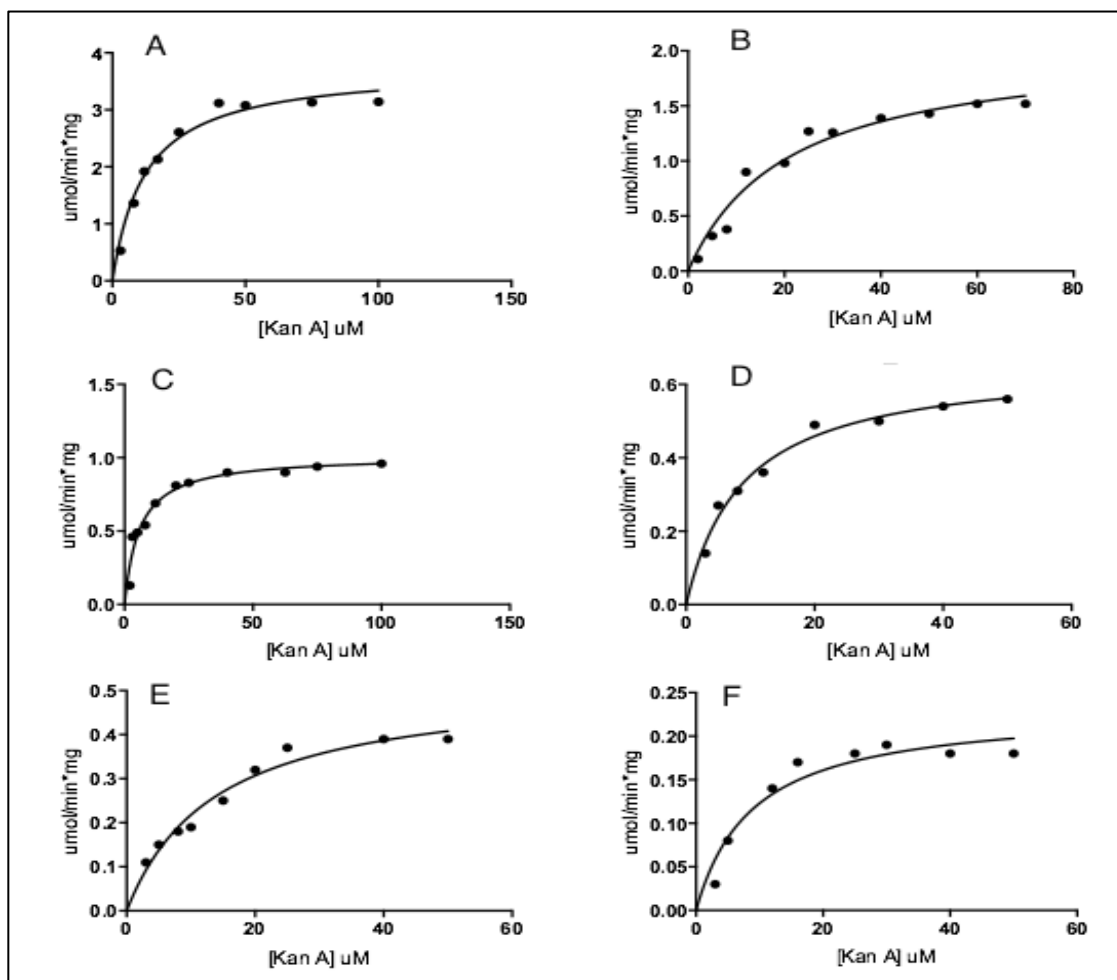


Figure 22: Temperature-dependent Michaelis-Menten curves for APH(3')-IIIa with kanamycin A. Initial velocity rates were measured by a coupled-enzyme assay (scheme 1A) with various aminoglycoside concentrations at temperatures 37°C (A), 30°C (B), 25°C (C), 21°C (D), 15°C (E), 12°C (F). Steady-state kinetic parameters are shown in table 2.

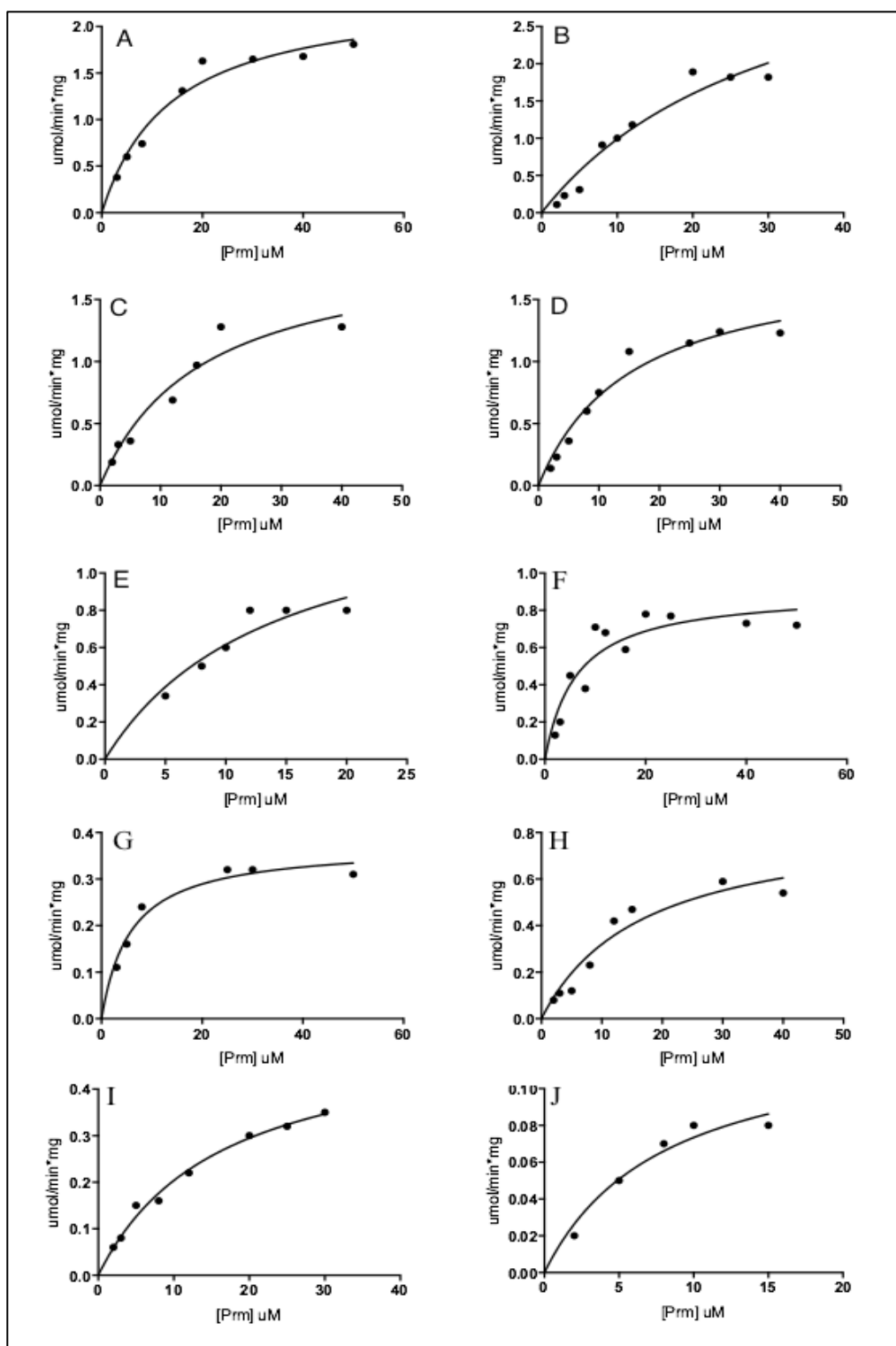


Figure 23: Temperature-dependent Michaelis-Menten curves for APH(3')-IIIa with paromomycin. Initial velocity rates were measured by a coupled-enzyme assay (scheme 1A) with various aminoglycoside concentrations at temperatures 37°C (A), 36°C (B), 30°C (C and D), 26°C (E), 25°C (F), 21°C (G), 20°C (H), 15°C (I), 10°C (J). Steady-state kinetic parameters are shown in table 2.

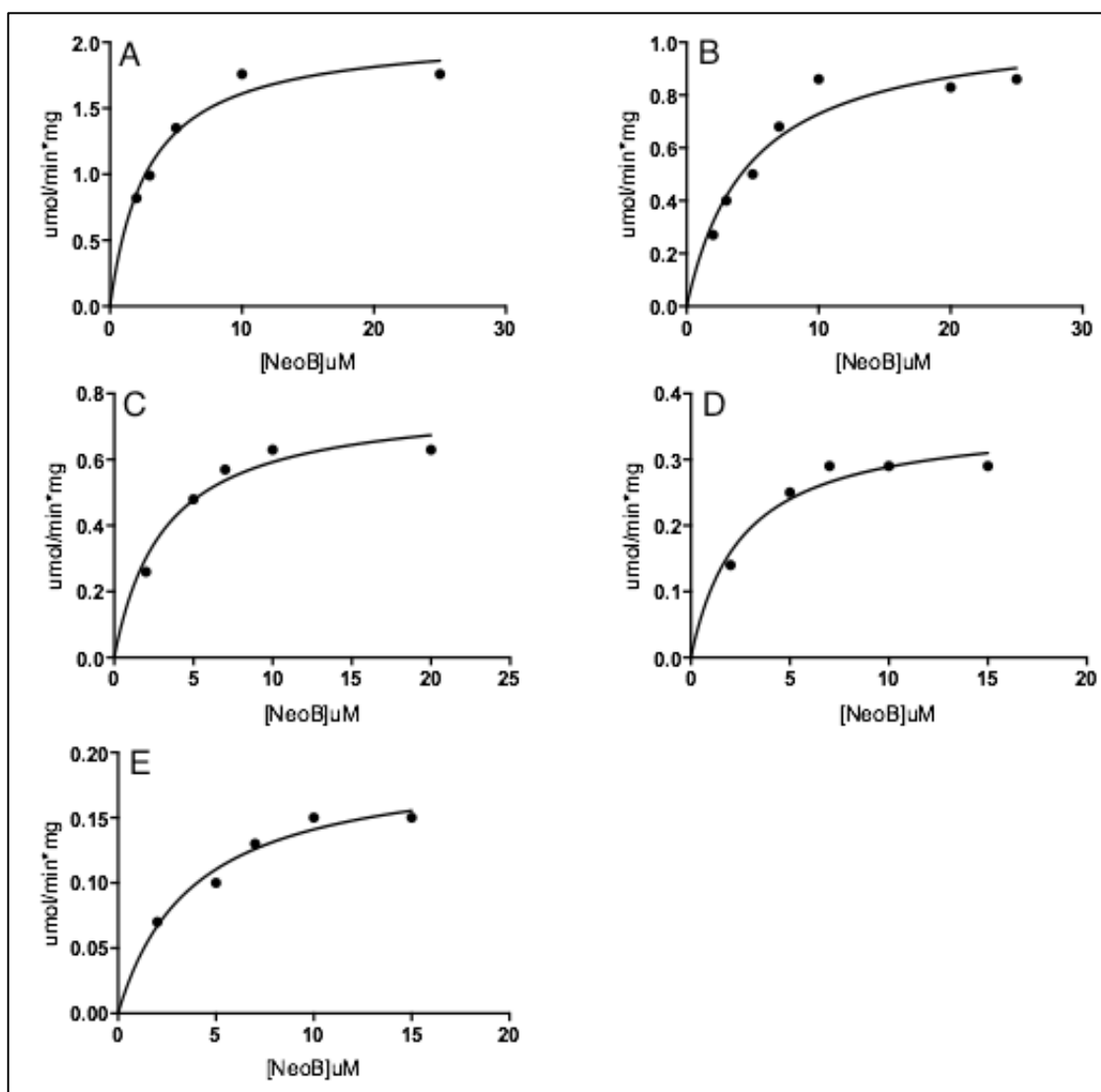


Figure 24: Temperature-dependent Michaelis-Menten curves for APH(3')-IIIa with neomycin B. Initial velocity rates were measured by a coupled-enzyme assay (scheme 1A) with various aminoglycoside concentrations at temperatures 37°C (A), 30°C (B), 25°C (C), 18°C (D), 10°C (E). Steady-state kinetic parameters are shown in table 2.

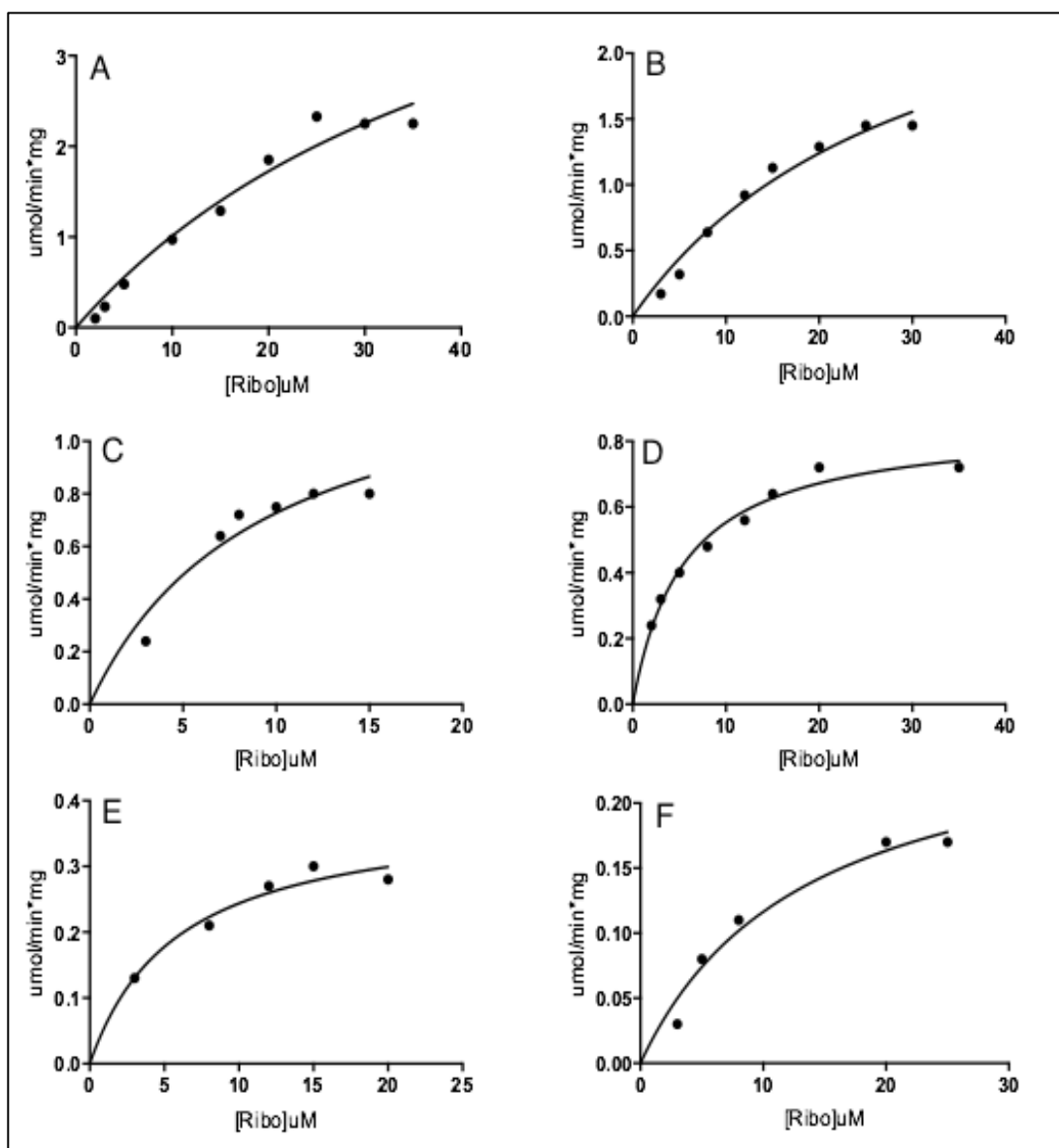


Figure 25: Temperature-dependent Michaelis-Menten curves for APH(3')-IIIa with ribostamycin. Initial velocity rates were measured by a coupled-enzyme assay (scheme 1A) with various aminoglycoside concentrations at temperatures 37°C (A), 30°C (B), 25°C (C), 21°C (D), 15°C (E), 12°C (F). Steady-state kinetic parameters are shown in table 2.

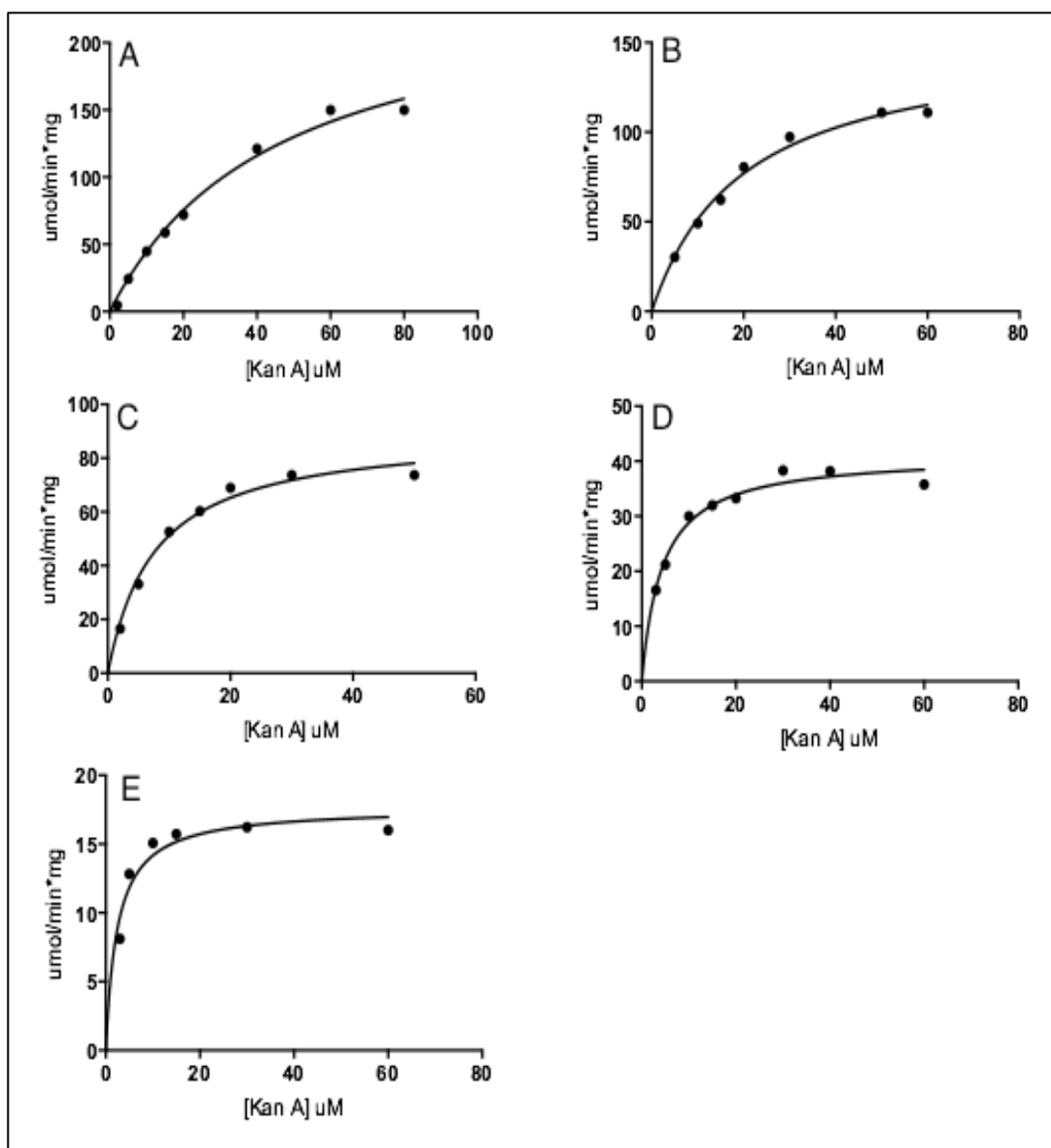


Figure 26: Temperature-dependent Michaelis-Menten curves for AAC(3)-IIIb with kanamycin A. Initial velocity rates were measured by a coupled reaction assay (scheme 1B) with various aminoglycoside concentrations at temperatures 37°C (A), 30°C (B), 25°C (C), 19°C (D), 10°C (E). Steady-state kinetic parameters are shown in table 2.

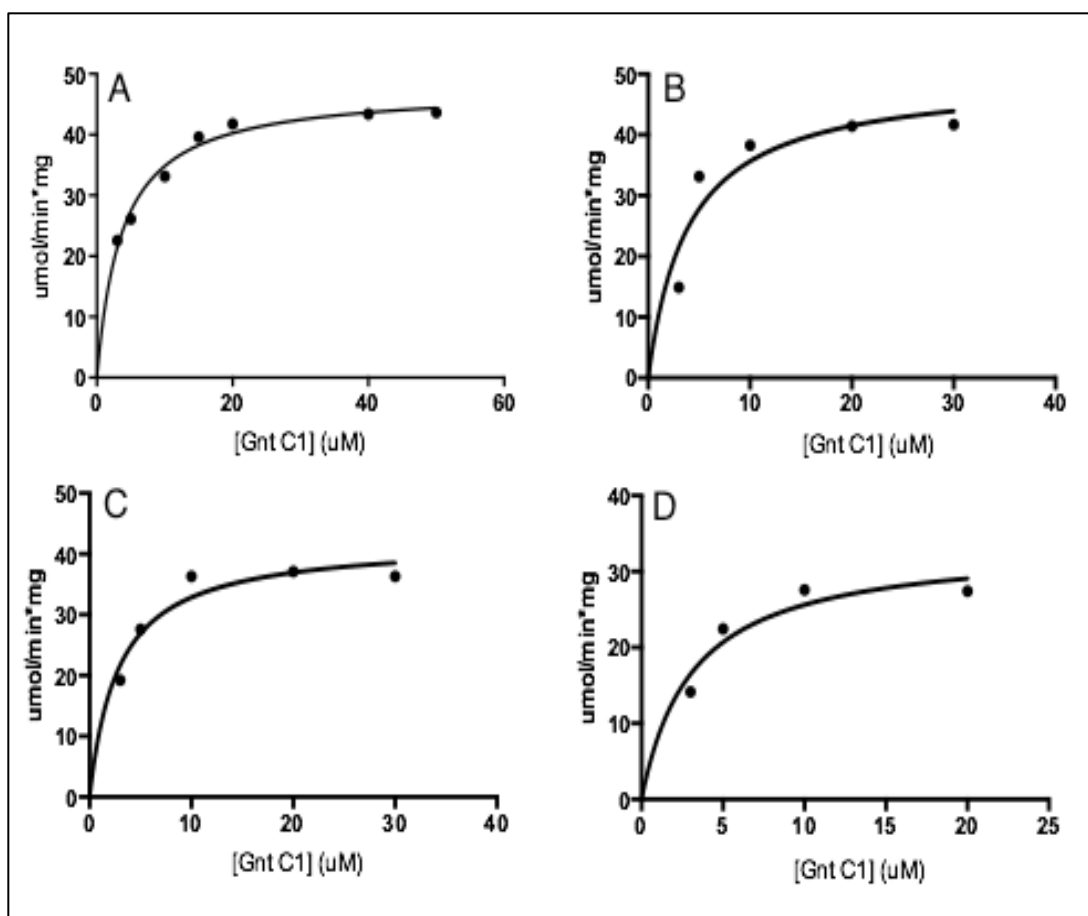


Figure 27: Temperature-dependent Michaelis-Menten curves for AAC(3)-IIIb with gentamicin C₁. Initial velocity rates were measured by a coupled reaction assay (scheme 1B) with various aminoglycoside concentrations at temperatures 30°C (A), 25°C (B), 18°C (C), 12°C (D). Steady-state kinetic parameters are shown in table 2.

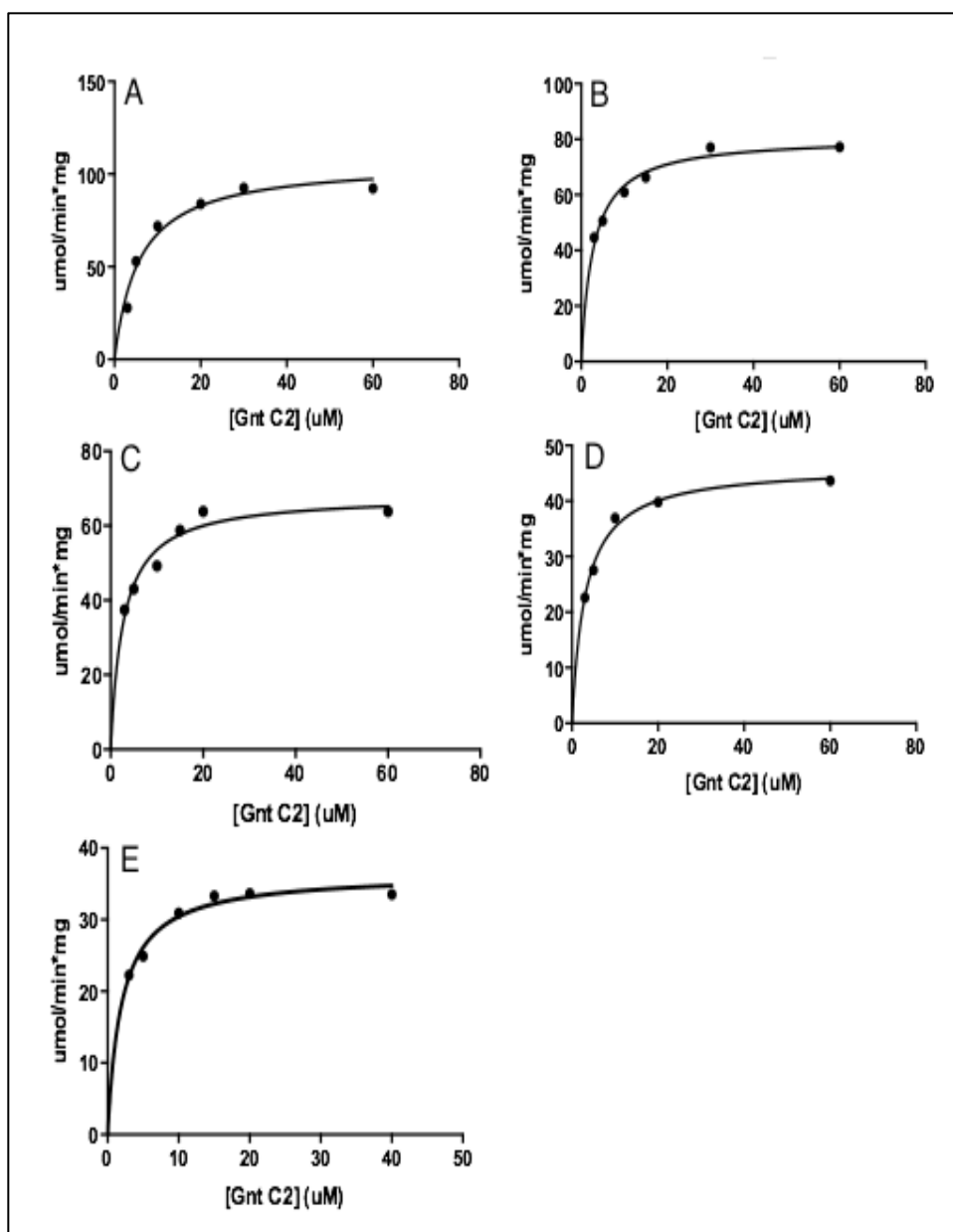


Figure 28: Temperature-dependent Michaelis-Menten curves for AAC(3)-IIIb with gentamicin C₂. Initial velocity rates were measured by a coupled reaction assay (scheme 1B) with various aminoglycoside concentrations at temperatures 37°C (A), 30°C (B), 25°C (C), 18°C (D), 12°C (E). Steady-state kinetic parameters are shown in table 2.

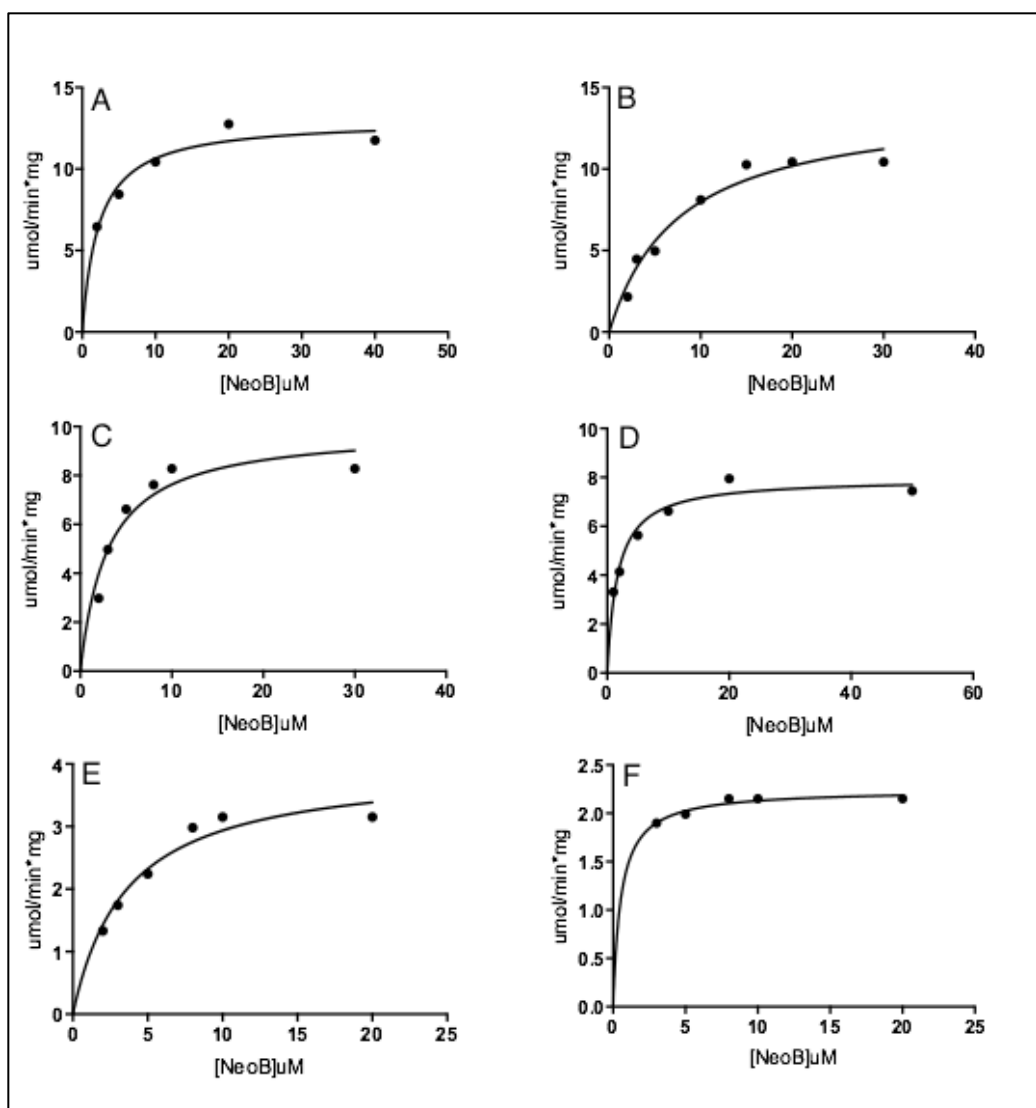


Figure 29: Temperature-dependent Michaelis-Menten curves for AAC(3)-IIIb with neomycin B. Initial velocity rates were measured by a coupled reaction assay (scheme 1B) with various aminoglycoside concentrations at temperatures 35°C (A), 30°C (B), 25°C (C), 20°C (D), 15°C (E), 10°C (F). Steady-state kinetic parameters are shown in table 2.

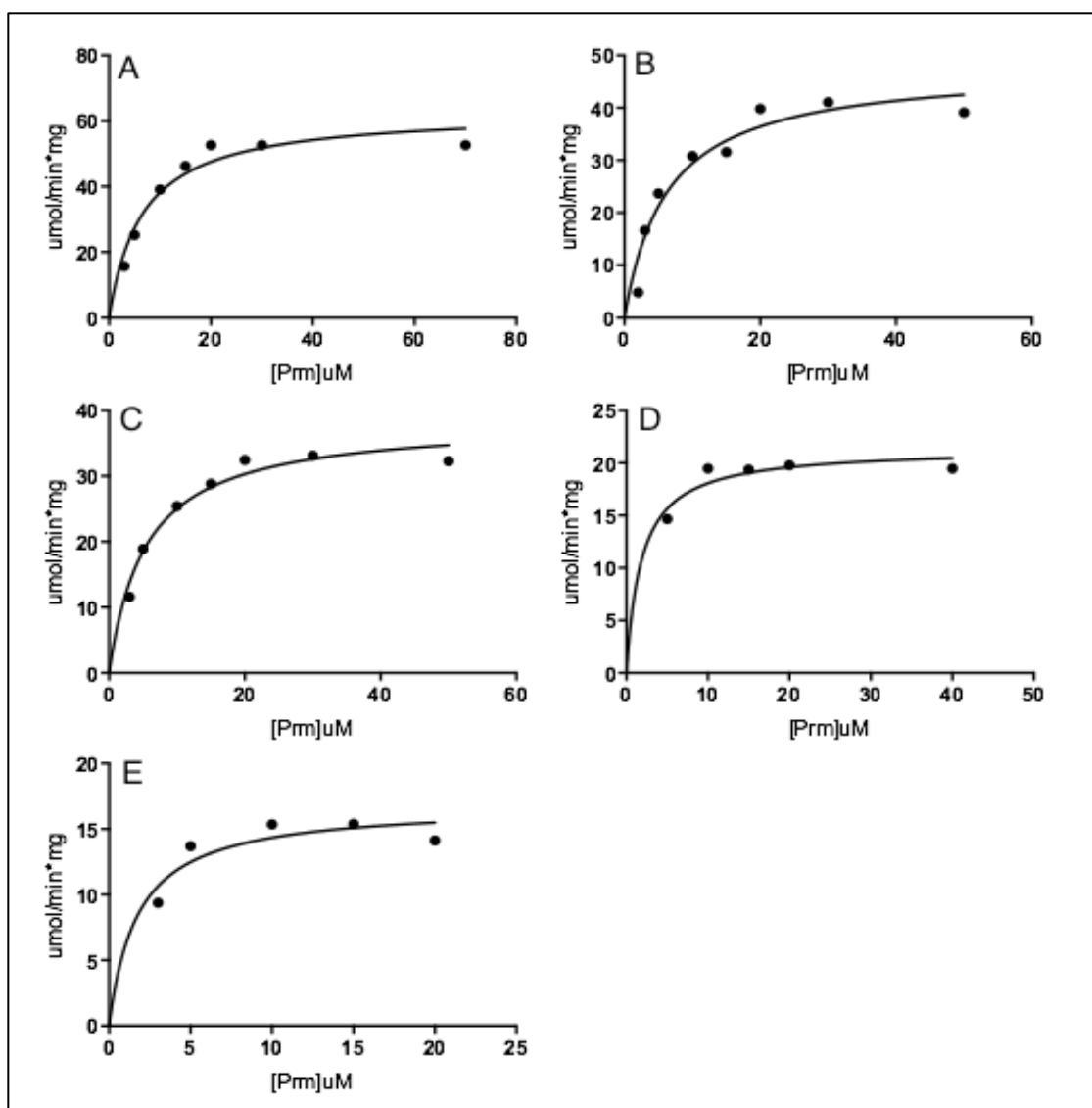


Figure 30: Temperature-dependent Michaelis-Menten curves for AAC(3)-IIIb with paromomycin. Initial velocity rates were measured by a coupled reaction assay (scheme 1B) with various aminoglycoside concentrations at temperatures 37°C (A), 30°C (B), 25°C (C), 18°C (D), 12°C (E). Steady-state kinetic parameters are shown in table 2.

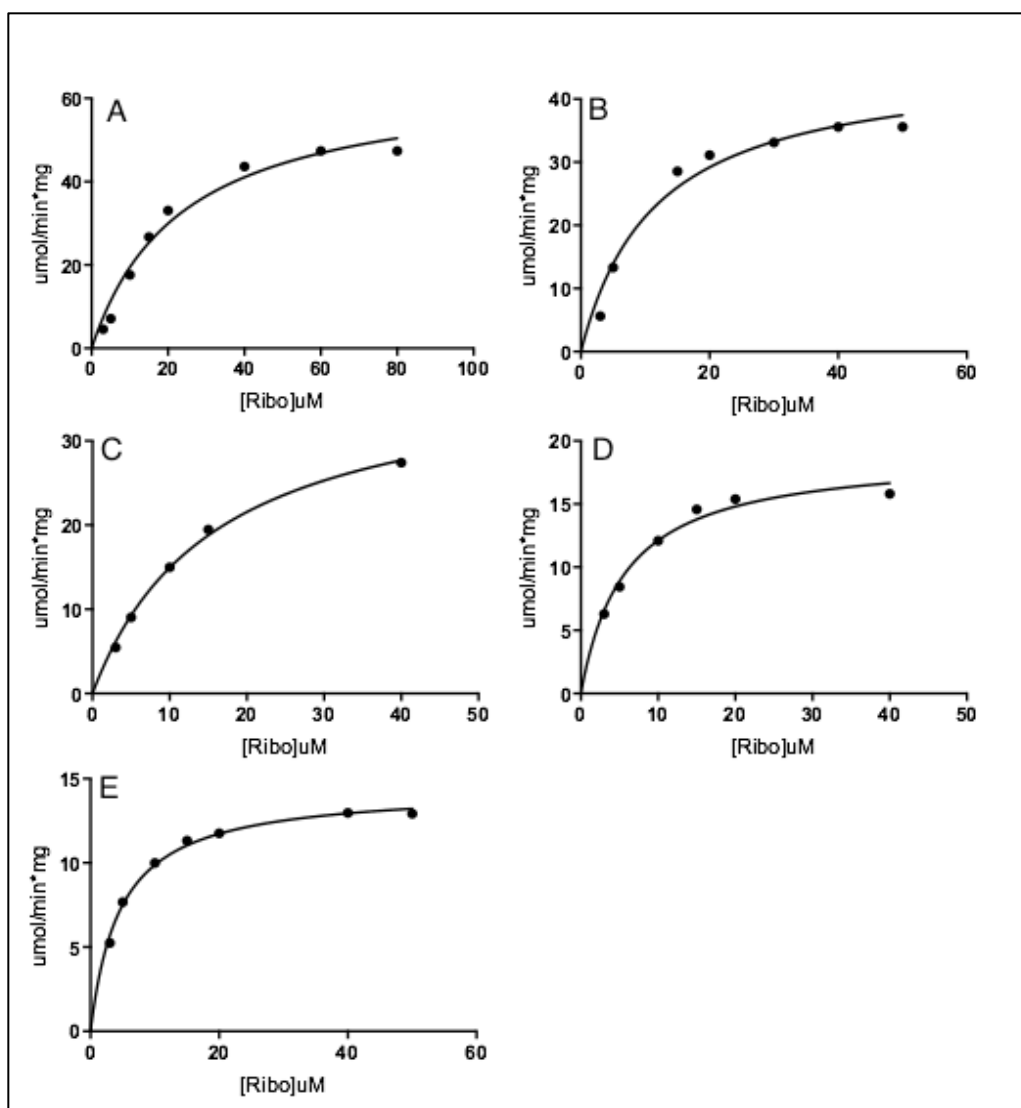


Figure 31: Temperature-dependent Michaelis-Menten curves for AAC(3)-IIIb with ribostamycin. Initial velocity rates were measured by a coupled reaction assay (scheme 1B) with various aminoglycoside concentrations at temperatures 37°C (A), 30°C (B), 25°C (C), 18°C (D), 12°C (E). Steady-state kinetic parameters are shown in table 2.

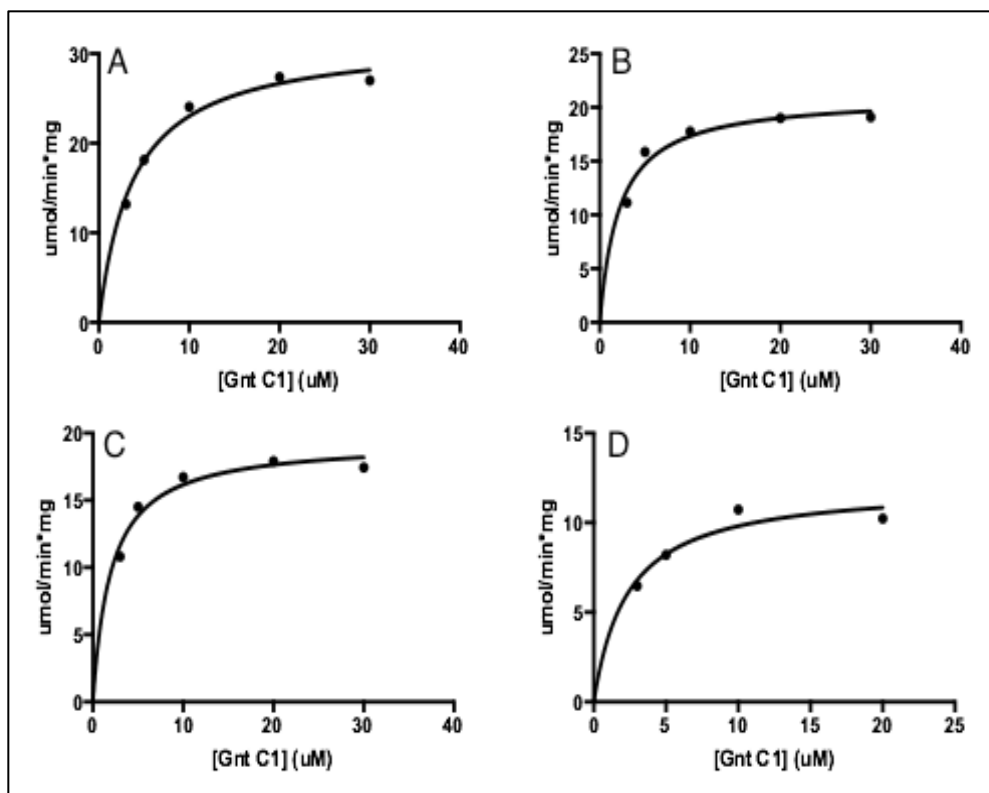


Figure 32: Temperature-dependent Michaelis-Menten curves for AAC(3)-VIa with gentamicin C₁. Initial velocity rates were measured by a coupled reaction assay (scheme 1B) with various aminoglycoside concentrations at temperatures 30°C (A), 25°C (B), 18°C (C), 12°C (D). Steady-state kinetic parameters are shown in table 2.

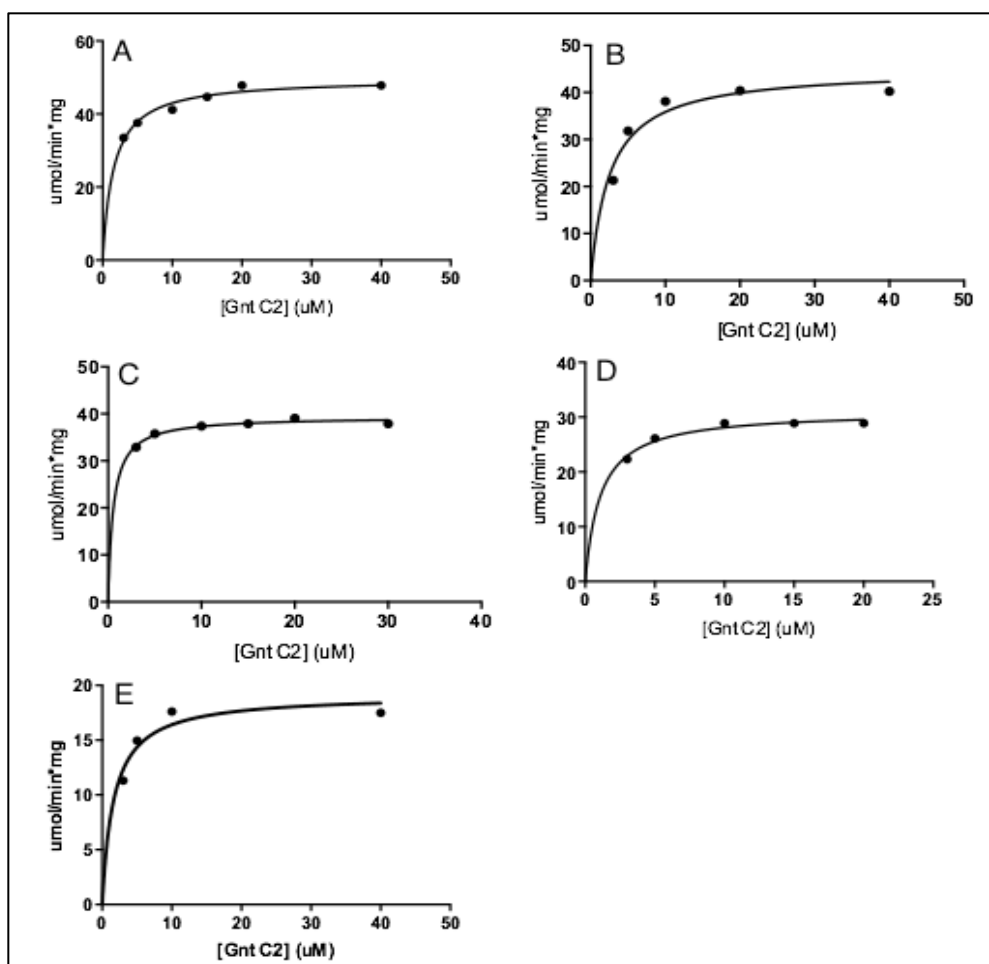


Figure 33: Temperature-dependent Michaelis-Menten curves for AAC(3)-VIa with gentamicin C₂. Initial velocity rates were measured by a coupled reaction assay (scheme 1B) with various aminoglycoside concentrations at temperatures 37°C (A), 30°C (B), 25°C (C), 18°C (D), 12°C (E). Steady-state kinetic parameters are shown in table 2.

VITA

On May 2, 1989, Brittany S. Soto was born to Andrea-Lee Friedman and Ron Soto in Sarasota, FL. Since the age of 4, Brittany has resided in Georgia, where she graduated high school in May 2007 from South Forsyth High School in Cumming, GA. After graduating high school, Brittany attended Winthrop University in Rock Hill, South Carolina on a full athletic scholarship for soccer. At Winthrop, she pursued her Bachelor of Science degree in Human Nutrition of Science. In May 2009, Brittany transferred from Winthrop University to Birmingham-Southern College located in Birmingham, Alabama. At Birmingham-Southern College, Brittany finished her athletic career as a two-sport athlete in soccer and softball and graduated with her Bachelor of Science degree in chemistry in May 2012. While attending Birmingham-Southern College, Brittany conducted undergraduate research in Dr. William Kettleman's lab. To further her education, Brittany received the Program of Excellence and Equity in Research (PEER) fellowship to attend graduate school at The University of Tennessee, Knoxville in the Biochemistry, Cellular and Molecular Biology Department. Prior to attending graduate school (Summer 2012), Brittany continued her soccer career in Pensacola, Florida, playing for the Pensacola Gulf Coast Texans, a Women's Professional – Amateur team participating in the W-league. During her time at the University of Tennessee, Knoxville, Brittany also played for the Knoxville Lady Force, a Women's semi-professional amateur soccer team participating in the Women's Premier Soccer League (WPSL). She completed her Thesis in the summer of 2015 from the University of Tennessee, Knoxville with her Master of Science degree in Biochemistry, Cellular and Molecular Biology. After completing her Thesis in the summer of 2015, Brittany went on to pursue a second Master of Science degree in Actuarial Science from Georgia State University located in Atlanta, GA. She plans to become a certified actuary working with pharmaceutical and healthcare companies in risk management for new drug design. In the long run, Brittany would eventually like to earn a Ph.D. in applied mathematics and statistics.