5-2011

Understanding the thermodynamics of enzyme-antibiotic interactions with Aminoglycoside phosphotransferase-3’-IIIa

Michele K. Miller
mmille73@utk.edu

Follow this and additional works at: https://trace.tennessee.edu/utk_chanhonoproj

Part of the Biochemistry Commons

Recommended Citation
Miller, Michele K., "Understanding the thermodynamics of enzyme-antibiotic interactions with Aminoglycoside phosphotransferase-3’-IIIa" (2011). University of Tennessee Honors Thesis Projects. https://trace.tennessee.edu/utk_chanhonoproj/1408

This Dissertation/Thesis is brought to you for free and open access by the University of Tennessee Honors Program at Trace: Tennessee Research and Creative Exchange. It has been accepted for inclusion in University of Tennessee Honors Thesis Projects by an authorized administrator of Trace: Tennessee Research and Creative Exchange. For more information, please contact trace@utk.edu.
Understanding the thermodynamics of enzyme-antibiotic interactions with Aminoglycoside phosphotransferase-3’-IIIa

Michele Miller
Chancellor’s Honors Program Senior Thesis

Faculty Research Mentor:
Dr. Engin Serpersu, Department of Biochemistry and Cellular and Molecular Biology
University of Tennessee

May 6, 2011
Abstract

Aminoglycoside phosphotransferase-3’-IIIa is an important enzyme that covalently modifies many aminoglycoside antibiotics by phosphorylation, causing the antibiotic to become ineffective. In an effort to better understand these interactions, isothermal titration calorimetry (ITC) is being used to study the thermodynamic properties of enzyme-aminoglycoside complexes. The enthalpy change ($\Delta H$) upon antibiotic binding to a complex of APH and CaATP, (a non-catalytic form of the required cofactor), has been determined at 37°C and 25°C in order to determine the heat capacity change ($\Delta C_p$) of complex formation. Experiments at additional temperatures are ongoing, and upon completion will complement the data acquired previously in the absence of CaATP and provide a more thorough thermodynamic understanding of this system.

Introduction

The aminoglycoside phosphotransferase is a dynamic enzyme that is approximately 31 kDa. It is found in pathogenic bacteria, such as *E. coli*, and serves as a survival tool for bacteria against aminglycoside antibiotics. The mechanism of action of this enzyme involves the covalent modification of the antibiotic. APH phosphorylates a hydroxyl group of the antibiotic at either the 3’ or 5” position, depending upon whether the antibiotic is a kanamycin or a neomycin, thus rendering it ineffective. The reaction is catalyzed with the help of magnesium ATP. Phosphorylation specifically prevents the antibiotic from binding to the 16S rRNA, where it normally inhibits protein synthesis and results in cell death. This particular phosphorylation can occur with at least ten different aminoglycoside antibiotics, and thus, understanding these interactions is very important because aminoglycoside antibiotics are key in treating many common bacterial infections (1).
Previous studies of APH using nuclear magnetic resonance (NMR) have helped determine the dynamics of APH and have indicated a change in structural dynamics from the apo-enzyme to the enzyme bound with an antibiotic. This work demonstrated the enzyme’s flexibility. Kinetic and crystallographic studies have also been completed with complexes involving APH and MgADP with kanamycin A and neomycin B.

In addition to studies involving the structure of APH, thermodynamic studies involving binary APH-aminoglycoside and ternary APH-CaATP-aminoglycoside complexes were conducted in 2004 using isothermal titration calorimetry (ITC). This previous research yielded negative enthalpy (\(\Delta H\)) values, indicating an exothermic reaction that is enthalpically driven. The exothermic nature of the reaction indicates favorable binding contacts between aminoglycosides and APH. This study concluded that the structure of the antibiotic does affect the thermodynamic properties of the enzyme-antibiotic relationship (1).

Moving forward with the thermodynamic characterization of APH, this paper focuses on the thermodynamic properties of a different complex involving the enzyme, ligand, and CaATP. As with previous experiments, ITC is used here to collect the thermodynamic data of antibiotic association to a pre-formed complex of APH and CaATP to determine the effect that cofactor has on the thermodynamic properties of antibiotic binding. ITC is the only available technique to directly and accurately measure the binding energetics of various biological processes. The parameters that can be determined using ITC include: enthalpy (\(\Delta H\)), the association constant (\(K_a\)), and binding stoichiometry (n). From these values, the Gibbs free energy (\(\Delta G\)), entropy (\(\Delta S\)), and heat capacity changes (\(\Delta C_P\)) can be calculated. All of these parameters are associated with binding. Previous ITC experiments indicate that there are dramatic differences in \(\Delta C_P\) between complexes of APH with two antibiotics, kanamycin A and neomycin B (2). The goal of
this research is to provide information about the $\Delta C_P$ of APH association with these two antibiotics in the presence of the cofactor, CaATP.

**Materials and Methods**

*Overexpression of APH(3')-IIIa*

The cells used for APH expression were *Escherichia coli* BL21(DE3) cells containing the overexpression plasmid pETPCR6, which was provided by Dr. Gerard D. Wright. A glycerol stock containing this cell line was streaked out on plates with 10 mL of Luria broth (LB) agar containing 10 g tryptone/L, 5 g yeast extract/L, 10 g NaCl/L, 10 uL of 100 mg/mL ampicillin, and 16.5 uL of 52 mg/mL kanamycin A and allowed to grow overnight at 37°C. An isolated cell colony was then inoculated into a subculture of 1 mL Luria broth containing 1 uL of 100 mg/mL and incubated at 37°C for 6-8 hours. After sufficient growth the subculture was transferred to a larger volume of LB and incubated overnight at 37°C. 35 mL of this subculture was then added to each of five flasks containing 800 mL of LB. The flasks were incubated at 37°C until the $OD_{600}$ was approximately 0.6. To induce expression, 8 mL of 100 mM IPTG were added to each 800 mL flask and incubated for three to four hours at 37°C. The cultures were then placed in 500 mL centrifuge bottles in 400 mL increments and centrifuged at 6,000 rpm for fifteen minutes. The cells were resuspended in 100 mL of 0.85% NaCl and centrifuged again. Approximately 0.8 liters worth of cells were transferred into a falcon tube and stored at -80°C.

*Protein Purification*

Using the guidelines and procedure provided by Adrianne Norris and Can Ozen, the process of purifying APH to use in ITC experiments involved several steps, and in order to produce a quality protein that yielded good experimental results, many modifications were made to this process of purification. Solutions were prepared as follows from stock solutions of 500
mM TrisHCl (pH 7.5 at 4°C and 50 mM dilution), 2 M NaCl, and 250 mM EDTA: wash buffer (1 L of 50 mM TrisHCl + 1 mM EDTA), lysis buffer (200 mL of 50 mM TrisHCl + 200 mM NaCl + 5 mM EDTA), salt elution 1 (1 L of 50 mM TrisHCl + 500 mM NaCl + 1 mM EDTA), salt elution 2 (1 L of 50 mM TrisHCl + 800 mM NaCl + 1 mM EDTA), and column stripper (500 mL of 50 mM TrisHCl + 1.5 M NaCl). All solutions were set to a pH of 7.5 at 4°C. 0.8 L of frozen cells from the -80°C freezer were suspended in 10 mL of lysis buffer containing 100 uL of 100mM PMSF and 1 mM DTT. The cells were lysed open with three passes through the French press. The lysate was then centrifuged on 17,000 rpm for one hour. After centrifugation, the lysate was separated into three portions of approximately 3.3 mL of lysate each. Each portion of lysate was then combined with an equal amount (3.3 mL) of lysis buffer. This dilution helped facilitate better protein yields. The following steps were performed separately for all three portions of lysate. The lysate was loaded to a strong anion exchange, MacroPrep column (1.5 cm x 20 cm). The column was then washed with 2-3 column volumes of wash buffer. To elute and collect the fractions of protein, a salt gradient was set up and each salt solution contained 1mM of DTT to maintain the monomeric state of the APH. The salt gradient was set up in a large gradient maker with 175 mL of salt 1 in the inside chamber and 175 mL of salt 2 in the outer chamber. A magnetic stirring bar was placed in the center and the gradient maker was placed on a stir plate in the 4°C cabinet. To begin elution, the stir plate was turned on and the valve of the gradient maker was opened to allow the two salt solutions to mix. A fraction collector was used to collect approximately 3-4 mL fractions of protein. A BSA dye test consisting of 400 uL ddH₂O, 100 uL BSA dye, and 10 uL of each fraction was used to determine which fractions contained protein. Protein purity was determined using an SDS-Page gel. The gel set-up included samples of molecular weight standards, the crude lysate, the pellet, and the eluted fractions.
To determine purity from nucleic acids, the 280/260 light absorptive ratio of the pure APH fractions was measured with a Cary-Win UV spectrophotometer. Fractions of equal or similar ratio were combined and concentrated using a Sorvall bench top centrifuge for approximately thirty minutes at 3,000 rpm. This concentration step helped ensure stability of the protein throughout the next steps of purification. Likewise, concentrating the protein improved the 280/260 ratio of the protein and yielded the desired 1.4 ratio.

Next, the protein was dialyzed against three changes of the desired buffer. Each part of the dialysis process lasted approximately 1.5 hours. The pure fractions of concentrated APH were placed in a 23 nm dialysis bag that was thoroughly washed. The buffer used in dialysis varied according to the desired experiment. TrisHCl buffer was initially used during the process of perfecting the purification process, but MOPS buffer was used for all final ITC experiments because the pKₐ of this buffer does not change with temperature. The pH of all buffers was 7.5 at 4°C. The buffers consisted of the following formulas: Tris (1500 mL of 50 mM TrisHCl and 100 mM NaCl) and MOPS (1500 mL of 50 mM Mops and 100 mM NaCl).

Following dialysis, the 280/260 ratio and concentration were checked again prior to protein storage. Under ideal conditions, the protein was used immediately for ITC experiments to ensure the best results. If storage was necessary, the protein was stored at -80°C in an eppendorf tube with Argon on top to prevent exposure to oxygen and help maintain the monomeric form of APH.

Finally, the Macroprep column was cleaned by running 2-3 column volumes of column stripper through and storing the column with wash buffer containing 0.02% NaN₃.

Isothermal Titration Calorimetry
In order to prepare samples for ITC experiments, several steps had to be taken. The enzyme (APH), ligand (antibiotic, tobramycin or kanamycin A), and all activity assay components were thawed on ice. The final dialysate buffer from the protein purification steps was set out at room temperature and the pH was set to 7.5. All components of the ITC machine (syringe and cell) were thoroughly cleaned with ddH₂0 and detergent prior to starting the experiment. Dialysis buffer was placed in the cell and allowed to equilibrate just prior to injecting the enzyme solution.

Enzyme preparation began by centrifuging the protein on a countertop centrifuge for 5 minutes at 13.2 rpm in order to remove any precipitated aggregates. The concentration of the protein was then determined using a UV-Vis spectrophotometer to measure the 280 nm absorbance and an extinction coefficient of 1.54. This concentration was then used to calculate the correct amount of protein needed to complete the activity assay, which ensures the protein is in its native, active state. After these, the enzyme solution for the ITC experiment was prepared using the final dialysate buffer, CaCl₂, ATP, and the purified enzyme to a final volume of 2200 uL. Final concentrations were 30 uM APH, 400 uM ATP, and 600 uM CaCl₂. The ligand solution consisted of 750 or 400 uM Tobramycin or Kanamycin A, respectively, depending on the experiment and desired concentration. After preparing both the enzyme and ligand solutions and placing into brown bottles with a small magnetic stirring bar, both bottles were placed in the thermovac and equilibrated at the experimental temperature for approximately fifteen minutes. The pH of both solutions was checked and matched within +/- 0.03 units of 7.5. Another brown bottle was filled with ddH₂0 and used for the reference water. All three bottles were then degassed in the thermovac for ten minutes at five degrees below the desired experimental temperature.
Following established ITC set-up procedures, the reference water, enzyme solution, and ligand solution were all carefully loaded in the ITC machine. The enzyme-CaATP solution was placed in the cell, while the ligand solution was injected into the syringe. All experimental parameters were carefully set and kept consistent throughout the experiment. These parameters were 29 injections, reference power of 10, injection volume of 10 uL, initial delay of 120 seconds, stirring speed of 307, duration of 20 seconds, spacing of 240 seconds, and filter period of 2. However, later experiments involving the MOPS buffer had 50 injections with an initial injection volume of 10 uL and all additional injections of 5 uL.

Following the ITC experiment, activity, concentration, and pH of the enzyme solution were checked to ensure that there were no major changes.

**Results**

Based upon all experiments conducted, APH yields good thermodynamic information when it is in a high concentration and freshly prepared. Experiments with protein of concentration below 50 uM and activity below 0.02 uM/min. did not provide interpretable data. Graphs of APH at 37°C and 25°C have been obtained and plotted for comparison. See Figure 1, Table 1, and Figure 2.

**Discussion**

A large portion of the time spent on this project focused on perfecting the purification process of APH in order to have a stable protein at a low concentration that would yield interpretable results from ITC experiments.

One of the initial problems with these experiments was a large baseline drift that yielded an inconsistent graph. In order to combat this, DTT, which once served as a way to stabilize APH and prevent the formation of disulfide bonds, was reduced in concentration in several
puriﬁcation steps and completely removed from the MOPS dialysis buffer. Speciﬁcally, the concentration of DTT in Salt Solution 1, Salt Solution 2, and wash buffer was reduced from 10 mM to 1 mM during the puriﬁcation process. DTT was also no longer used for the long-term storage of APH in the -80°C freezer or for maintaining stability during puriﬁcation.

Another consistent problem at the beginning of this experiment was a low 280/260 ratio as well as a low concentration. The ﬁrst step in an attempt to improve these values was experimenting with the effect of using two new ion exchange chromatography columns. The ﬁrst column prepared was a DE52 ion exchange column that is cellulose-based. The second column was a freshly prepared Macroprep column, which is the same resin used in the initial experiments. Both columns had equal dimensions of 1.5 cm x 20 cm. 10 mL of lysate was divided in half and one half was run on each column under the same conditions. An SDS-PAGE gel was run with four fractions eluted from each column. The bands on the gel revealed that two fractions from each column had pure APH. The concentration and 280/260 ratio of each of these fractions was checked and calculated. The #8 and #9 fractions from the DE52 column yielded 280/260 ratios of 0.5375 and 0.5101. The #11 and #12 fractions from the Macroprep column resulted in ratios of 0.8493 and 1.0684. The results of this experiment indicated that the original Macroprep column was the better resin to use in the APH protein puriﬁcation process.

Several additional methods were tried to deal with the issue of improving the 280/260 ratio, but the most effective method developed was to use a centrifugal ﬁltration system to concentrate the protein. This gave a lower overall yield, but the quality of the protein as well as the 280/260 ratio were greatly improved. Prior to the concentration step, ratios were around 0.7 to 0.8, but improved dramatically to the range of 1.2-1.5. As stated in the materials and methods section, the pure portions of lysate, usually about 6-8 mL were placed in a concentrator tube and
centrifuged at 3,000 rpm for 15-30 min. The amount of time varied depending on the desired concentration and initial volume. A centrifugation period of 30 minutes typically resulted in a reduction of volume from the initial 6-8 mL to around 200-500 uL. The drastic loss of protein associated with this method of concentration made it difficult to perform multiple ITC experiments with protein from the same purification preparation. DNase treatments were also utilized at one point in an attempt to improve the 280/260 ratio, but these efforts were not effective.

Initially, the dialysis step in the protein purification process was completed prior to concentration. However, multiple experiments revealed that concentrating the protein sooner yielded better stability and activity. Along this same line, the speed of the purification process also became very important. In order to insure the stability of the protein and prevent the use of DTT and multiple freeze-thaw cycles, it became necessary to complete the entire purification process in approximately four days. Throughout this process, the protein derivatives and fractions were constantly kept on ice in the refrigerator (4°C).

Other attempts to improve the overall yield of APH during the elution process involved testing various dilutions of cell lysate to load to the ion exchange column. After lysing the cells open with the French press and centrifuging them for an hour, the resulting lysate was separated into three equal portions of approximately 3.3 mL. Portion 1 was loaded directly to the column and the protein elution step was carried out according to the normal procedure. The same process was completed for Portion 2 (2x dilution), which consisted of 3.3 mL + 3.3 mL of lysis buffer and Portion 3 (3x dilution), which consisted of 3.3 mL lysate + 6.6 mL lysis buffer. The results of this experiment revealed that diluting the lysate helped reduce the presence of the 260nm contaminant prior to loading the lysate to the column. Additionally, the amount of time
for the lysate to run through the column was significantly higher for Portion 1 and reduced as the
dilution increased. Analysis of the concentration and 280/260 ratios of the resulting protein
fractions revealed that Portion 2 (2x dilution) provided the best results and facilitated a method
to recover a greater amount of protein per portion of original lysate.

Throughout the process of developing a reliable and effective method for purifying APH,
several ITC experiments were conducted. Initially, TrisHCl dialysate buffer was used in the
experiments. However, after evaluating the effect of this buffer’s heat of ionization, an
experiment comparing the difference between using TrisHCl buffer and PIPES buffer was used
to evaluate the effect of the buffer on the results of ITC experiments. TrisHCl has a higher heat
of ionization, which means that the heat it gives off will have a greater additive effect on the
overall change in enthalpy and can interfere with interpreting the overall ∆H occurring as a result
of the ligand binding to the enzyme complex. PIPES has a lower heat of ionization, and therefore
would reduce the additional change in enthalpy that occurs as a result of the buffer. Results did
not reveal significant differences between these two buffers when all other parameters were kept
constant. The signal for each injection of ligand into the enzyme complex did not decrease
significantly enough to make PIPES a more affordable or favorable option. The fact that both
experiments revealed the initial injection and then saturation indicated that it was a protein
stability issue and not an issue involving heat of ionization.

Once a reliable ITC graph was produced using tobramycin, which binds more tightly to
the enzyme, experiments involving kanamycin A and CaATP could begin. Early ITC
experiments also showed a short period of increasing saturation, and in order to get more defined
points, the total number of injections as well as the amount of ligand injected were adjusted to
provide better data. The last two experiments completed reveal that this is potentially helpful, but
more studies will provide a better indication of the effect of these adjustments. Ligand concentrations were also adjusted to reflect the tightness of binding depending on the temperature of the experiment. Calculations using the equation APH-KanA ↔ APH + KanA were used to determine a ligand concentration that was 25x the concentration of the enzyme. This calculation was ideal at higher temperatures because the molecules have greater kinetic energy and movement and are not likely to bind as easily. Lower temperatures result in a lower kinetic energy, which facilitates binding more quickly and more easily. The ligand concentration was reduced to account for this change in temperature and at 25°C, a concentration of about or 13x was used. 750 uM kanamycin A was used for experiments at 37°C, whereas a lower concentration of 400 uM was utilized for the tighter binding that occurs at lower temperatures.

The data displayed in Figure 1 was fit to a single site binding equation in order to get the \( \Delta H \) and \( K_a \) values of -23.95 kcal/mol and 1.79 \( \times \) 10⁶. These numbers were similar to those previously obtained in Can Ozen’s 2008 paper. Plotting the \( \Delta H \) values calculated at 25°C and 37°C versus the temperature generated a line with a slope of -1.6 (Figure 2). This slope represents the change in heat capacity \( \Delta C_p \) (\( \Delta H/T = \Delta C_p \)). This number can be compared to Can Ozen’s 2008 paper where a break at 30 °C was observed in the APH-kanamycin complex yielding \( \Delta C_p \) values of -0.7 kcal-mol-1-deg-1 and -3.8 kcal-mol-1-deg-1 below and above 30 °C, respectively. The \( \Delta C_p \) measured in this experiment is not complete without enthalpy values from other temperatures between 25°C and 37°C. In the binary experiments completed in 2008, there was a break at 30°C, and therefore more points need to be plotted to know whether or not a similar break occurs when CaATP is present. The \( \Delta C_p \) in general is the amount of heat energy that can be introduced into a system and absorbed before the additional heat raises the temperature of the system. Knowing the \( \Delta C_p \) for protein-ligand interactions is useful because
rearrangements of water molecules during the reaction or changes in the conformation of the protein upon ligand binding can contribute to the sign and magnitude of $\Delta C_p$.

Future studies with kanamycin binding to APH and CaATP will continue at other temperatures and will then be repeated using neomycin so that a comparison to previous experiments can be made.

**Reflection**

The completion of this paper marks the end of two and a half years of research in the biochemistry department and three semesters of research in Dr. Serpersu’s lab. Prior to this research experience, my education largely consisted of learning from textbooks, attending lectures, and studying for tests. Research, on the other hand, has been an entirely different experience and one that has taught me a lot about patience, perseverance, hard work, and consistency.

I have had the opportunity to work in two different labs, and thus have been exposed to a variety of biochemical machinery, techniques, and processes. I learned about many of these in my classes and through my coursework, but the practical application of this information has taught me more than any book ever could.

This experience has challenged me to move beyond the idea that all information is contained in a book and has pushed me to think in a very different way. I feel that this shift in thinking has improved my ability to think critically and reason through more complex problems. In addition, this experience has challenged me due to the fact that there was a tremendous amount of trial and error. This project required me to repeat experiments and processes in a logical way in order to determine what changes could help facilitate improved results. I believe
that the “failures” I experienced taught me even more and required me to obtain an even better understanding of my project.

This experience has increased my understanding of a variety of topics from thermodynamics and protein structure to the broader application of understanding antibiotic resistance and mechanisms by which this occurs. I feel confident that the practical experience as well as the technical foundation I have gained will help me tremendously in my future endeavors as a medical professional.

Acknowledgements

I would like to especially thank Dr. Serpersu for all of his time and commitment in helping me learn and grow through this experience. Without his patience, expertise, and understanding, I would not have been able to complete this project. I would also like to thank Adrianne Norris for her help with this project. Her time, dedication, and collaboration throughout this process are greatly appreciated. This has been a wonderful opportunity and I am truly grateful for all the support I received. I would like to thank UT, the biochemistry department, and the Chancellor’s Honors Program for providing me the means and motivation to complete my senior thesis project through two and a half years of research. The practical knowledge and experience I have gained are immeasurable.

References


**Figure 1:** The top panel is the thermogram and the bottom is the isotherm. The red line represents the fit.

<table>
<thead>
<tr>
<th></th>
<th>$K_a$</th>
<th>$\Delta H$ (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>kanamycin A</td>
<td>$2.1 \times 10^5$</td>
<td>-4.2</td>
</tr>
<tr>
<td>(25°C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>kanamycin A</td>
<td>$1.8 \times 10^6$</td>
<td>-24.0</td>
</tr>
<tr>
<td>(37°C)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 1:** Thermodynamic Parameters for binding of kanamycin A to APH(3′)-IIIa with CaATP at 25°C and 37°C at pH 7.5.
Figure 2: Plot of enthalpy versus temperature constructed by linear regression of data from Table 1.