

Effects of Antibiotic binding on the overall structure of the
Aminoglycoside *N3*-acetyltransferase-IIIb; an antibiotic resistance enzyme

Selamawit Tesema

Advisor: Dr. Engin Serpersu

INTRODUCTION:

Antibiotic resistance is a growing problem in the medical field. One group of antibiotics which are widely used for the treatment of bacterial infections are aminoglycosides (AG). They are commonly used to treat infections such as tuberculosis and meningitis, thus understanding the biological basis of antibiotic promiscuity by AG-modifying and uncovering molecular details by which they select their ligands holds importance in both medicinal and scientific communities (Serpersu and Norris, 2013). Aminoglycosides exert their antibacterial effect by binding to the 16S RNA of the 30S ribosome and interfering protein transcription in the bacteria and eventually causing cell death. Bacteria have developed different mechanisms for resistance, one such mechanism is the rise of aminoglycoside modifying enzymes. AG-modifying enzymes are a large family of proteins where each member modifies a unique hydroxyl or amino functional groups in different aminoglycosides (Serpersu and Norris, 2013). These enzymes modify aminoglycosides by transferring phosphate, acetyl or nucleotidyl functional groups from co-factors such as MgATP or Acetyl-CoA. This modification reduces the affinity of the aminoglycoside to the bacterial ribosome rendering it ineffective. The aminoglycoside *N*3-acetyltransferase-IIIb (AAC-IIIb) is an antibiotic resistance enzyme which catalyzes an acetylation reaction which involves transferring an acetyl group from an acetyl-CoA to an amine in the C3 position in all aminoglycosides. AAC-IIIb is one of the most promiscuous AG-modifying enzymes. It has a wide range of substrates. In a study by Norris et al., (2010), steady state kinetic analysis revealed that tobramycin, kanamycin A, kanamycin B, sisomycin, neomycin B, paromomycin, ribostamycin and lividomycin are all substrates with measurable rates. In this study, we analyzed AAC-IIIb peptides generated by trypsin digestion. Trypsin is a protease which cleaves proteins at Arginine and Lysine residues. Following trypsin digestion, the

sample is run through a high-performance liquid chromatography (HPLC) column to determine cleavage pattern and to separate tryptic peptides. Trypsin digestion was performed on the enzyme in its apo- and ligand-bound state to determine the structural changes that occur in the enzyme when the antibody is bound. The ligand in this case was neomycin which is one of the substrates for AAC-IIIb. The chromatograms from the two states indicate that there are ligand binding causes some structural changes in AAC-IIIb.

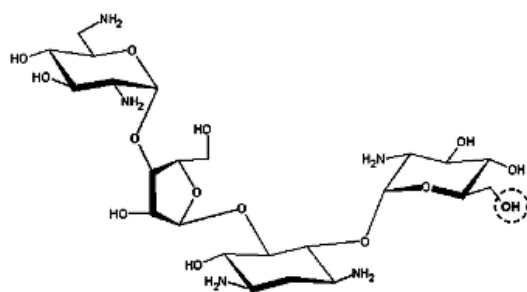


Figure 1: Structure of Neomycin. Neomycin was the ligand used in this study.

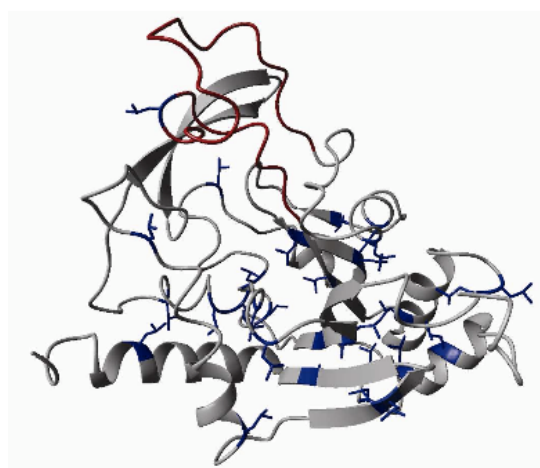


Figure 2: Homology-derived structure of AAC-IIIb

(Norris et al, 2010). Loop involved in aminoglycoside binding is highlighted in red

METHODS:

Stock preparation:

The material for the study included AAC-IIIb, trypsin solution, neomycin, acetonitrile and trifluoroacetic acid (TFA). The AAC-IIIb used for this experiment was over-expressed and purified from *Escherichia coli* BL21(DE3) cells by Adrianne Norris and Sharin Raval. For the first few experiments we used a 100 μ M of AAC-IIIb which was in 50 mM MOPS pH 7.5. Fresh trypsin solution was prepared daily for each experiment by dissolving lyophilized trypsin powder in 0.01M HCL to a final concentration of 200 μ M. 1 μ L of the trypsin solution was added to a 100 μ L of the enzyme solution. The final trypsin-to-enzyme ratio was 1:50. After the digestion had run for 10 minutes, we added 1 μ L of 10% TFA to a final concentration of 0.01% to stop the reaction. The sample was then injected into the HPLC column and fragments were collected.

Ligand bound solution:

Neomycin bound enzyme was prepared by adding 0.4 μ L of 502 μ M de-sulfated neomycin to 100 μ L of 100 μ M AAC-IIIb. This solution was allowed to equilibrate for 5 minutes. Then 1 μ L of trypsin solution was added to the ligand bound enzyme and allowed to react for 10 minutes. Trypsin-to-enzyme ratio was kept at 1:50 to match it with the treatment of the apo-enzyme. TFA was added to a final concentration of 0.01% to stop the reaction. The sample was then injected into the HPLC column and fragments were collected.

HPLC preparation and collection:

The stationary phase of the column was C-18 bound Silica. We used two solutions for the mobile phase of the HPLC. Solution A is the aqueous solution which contained 0.1% TFA dissolved in

water. Solution B was the nonpolar solution which contained 0.1% TFA dissolved in acetonitrile. Prior to the application of the sample solution, the column was calibrated using a series of steps. First, we ran 50% of each solution for about 10 minutes. After that we ran 100% of solution B through the column for 10 minutes. Then the ratio was changed to 2% solution B and 98% solution A for 20 minutes. This was the ratio used at the start of the run. Throughout the run, the ratio of solution A and B was reversed so that at the end of the run the final solution was 2% of solution A and 98% of solution B. Thus, the mobile phase became more nonpolar as we continued through the run. Signal detection was at 280nm and 214nm. Each time there was a peak, fragments were collected. The total run time was 65 minutes.

RESULTS:

High-performance liquid chromatography (HPLC) separates the peptides by size and hydrophobicity. Since the mobile phase is the non-polar acetonitrile, small hydrophilic peptides are eluted first; while long hydrophobic peptides have a longer retention time and are eluted later. The chromatogram of each run represents each peak that was recorded during the run. Below are the chromatograms for the peaks at 280nm. The chromatograms at 214nm are not shown here. Figure 3 represents the chromatogram of the apo-enzyme and figure 4 is the chromatogram of the neomycin-bound enzyme. From the apo-enzyme, 17 peptides were collected and 12 were collected from the ligand bound enzyme. Most of the peaks from the apo-enzyme have corresponding peaks in the ligand bound form. One of the peptides of interest is the last peptide that was recorded in the apo-enzyme. In the ligand bound form, it appears that this peptide is divided into two peptides due to the presence of two peaks where there was only one in the apo-enzyme. From the results, we suspect that a lysine or an arginine in that peptide was

exposed during ligand binding, allowing access to cleavage by trypsin. We attempted to analyze these fragments further using NMR, however the sample concentrations were too low for a meaningful data acquisition. In the future, we hope that increasing the concentration of the enzyme used can help us get a better result from NMR.

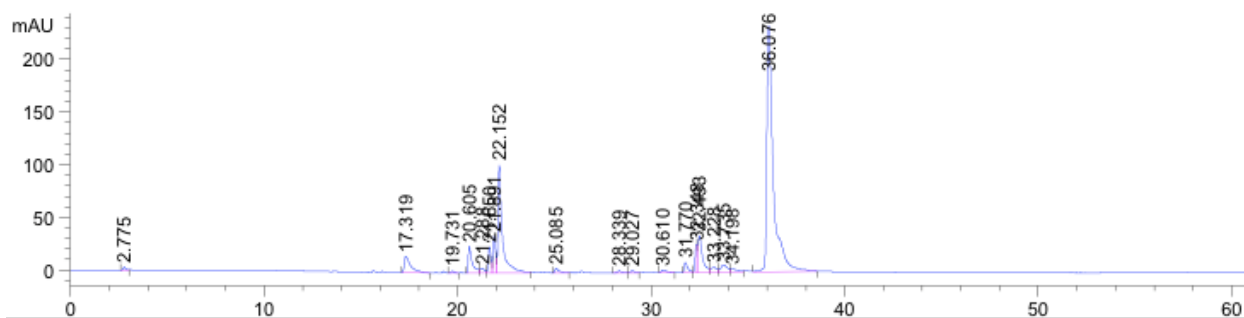


Figure 3: Chromatogram of apo-enzyme at 280nm

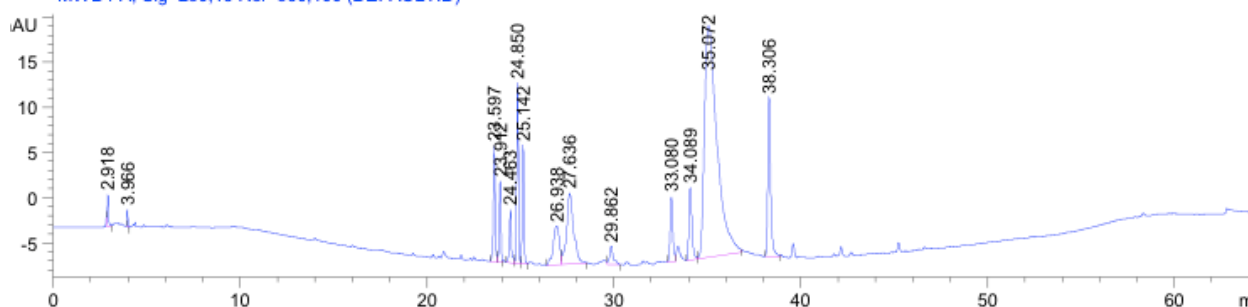


Figure 4: Chromatogram of the enzyme-ligand complex at 280nm

DISCUSSION:

Studies on the structure of AAC-IIIb have found that the enzyme has a rigid structure in its apo- and ligand-bound forms except for a flexible loop that is found near the AG binding site. This loop is comprised of ~35 amino acids. Examining the differences in the chromatograms of the apo and ligand bound AAC-IIIb can help us potentially determine parts of the protein that

changes exposure to solvent upon ligand binding, which awaits determination of peptide sequences by NMR.

The chromatograms show that the peaks are concentrated around the same area in both samples. However, the apo-enzyme and ligand bound enzyme differ in the number of peptides they produce during tryptic digestion. From the apo-enzyme 17 peptides were collected, while only 12 peptides were collected from the tryptic digestion of the enzyme-ligand complex. This suggests that ligand binding hides some of the Lysine and Arginine residues reducing the cleavage sites. Additionally, in the peptides that appeared comparable in the apo- and ligand-states that was a slight shift in the retention times. For example, the peptide that appeared at 22.15 in the apo-state, was collected at 24.85 in the ligand-bound state. This suggests that ligand binding exposes different amino acids shifting the hydrophobicity of the peptide. These differences in the chromatography profiles suggest that aminoglycoside binding causes conformational changes in AAC-IIIb.

We examined the amino acid sequence of AAC-IIIb to give us an idea of the possible peptides that are generated by trypsin digestion. Below is the amino acid sequence for AAC-IIIb grown in *E. coli* BL21(DE3) cells. The possible cleavage sites are highlighted in yellow (lysine) and green (arginine). Hydrophobic side chains, tryptophan (W) and tyrosine (Y), with an absorbance of 280nm are also highlighted. Peptides without tryptophan or tyrosine are not represented in the above chromatograms which show the peaks at 280nm.

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MTSATASFAT  RTSLAADLAA  LGLAWGDAIM  VHAAVSRVGR  LLDGPDTHA
ALRDTVGP GG  TVLAYADWEA  RYEDLVDDAG  RVPPEWREHV  PPDPQRSRA
IRDNGVLPEF  LRTTPGTLRS  GNP GASLVAL  GAKAEWFTAD  HPLDYG YGEG
SPLAKLVEAG  GKVLMLGAPL  DTLTLLHAE  HLA DIPGKRI  KRIEVPFATP
TGTQWRMIEE  FDTGDPIVAG  LAEDYFAGIV  TEFLASGQGR  QGLIGAAPSV
LVDAAAITAF  GVTWLEKRFG  TPSP

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Figure 5: Possible trypsin cleavage sites for AAC-IIIb. Lysine(K) and Arginine(R) which are amino acids recognized and cleaved by trypsin are highlighted. Tryptophan(W) and Tyrosine(Y) are highlighted in grey since those are the amino acids with an absorbance of 280nm.

Potential amino acid sequence of the peptide at which there is some structural change:

IEVPFATP TGTQWRMIEE FDTGDPIVAG LAEDYFAGIV TEFLASGQG

We propose that the peptide shown above may be the one that elutes at 36.07 in the apo-enzyme. Since this peptide is hydrophobic due to the presence of a number of phenylalanine residues,, we expect that it would have a longer retention time than other peptides. In the apo-enzyme we propose that the arginine in the middle of this peptide is hidden and it elutes as one peptide. In the ligand-bound enzyme, on the other hand, this arginine is exposed and available for cleavage by trypsin. Thus, this peptide is cleaved and elutes as two smaller peptides. This hypothesis can be tested further using NMR analysis of these samples.



Figure 6: Structure of AAC-IIIb. The peptide

shown above is highlighted yellow and the arginine is highlighted red. The loop involved in ligand binding is highlighted in green.

This structure of AAC-IIIb supports our data. In the apo-enzyme, the flexible loop shields the highlighted arginine from cleavage by trypsin. Therefore, the peptide highlighted in yellow

elutes as one long peptide. However, when neomycin is bound the loop folds around the ligand exposing this arginine to cleavage. This suggests that ligand binding causes structural changes in AAC-IIIb.

CONCLUSION:

Structural analysis of aminoglycoside-modifying enzymes has significance in scientific and medical communities. Understanding protein-ligand interactions will help us design antibiotics that are resistant to the action of AG-modifying enzymes such as AAC-IIIb. With the rise of strains of multidrug-resistant Tuberculosis, it is becoming more important to design novel antibiotics which can combat bacterial growth and are not susceptible to modification by resistance conferring bacterial enzymes. The present study investigates the protein-enzyme interaction between AAC-IIIb and neomycin by comparing trypsin digestion patterns in the apo- and ligand-bound state of the enzyme. To further investigate protein-ligand interactions, more experiments analyzing conformational changes in AAC-IIIb can be done using ligands which are structurally different from neomycin such as kanamycin A.

REFERENCES

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