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COLLEGE SCHOLARS PROJECT APPROVAL

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Molecular Determinants of Binding of a Spin-Labeled Analog of a Coenzyme A to Aminoglycoside Acetyltransferase(3)-IIIb Using Electron Paramagnetic Resonance Spectroscopy
Project Title

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DATE COMPLETED 4/30/09

Andrew Hunter Wheeler

College Scholars Senior Project

Mentor: Prof Engin Serpersu

Defended: 30 April 2009

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Abstract

One of the common mechanisms for aminoglycoside resistance arises from a reaction involving the transfer of an acetyl group from acetyl coenzyme A (AcCoA) to the 2-deoxystreptamine ring of aminoglycosides catalyzed by aminoglycoside acetyltransferase(3) (AAC). The present study was intended to study AAC-ligand interactions by utilizing a spin-labeled analog of AcCoA for studies using Electron Paramagnetic Resonance (EPR) spectroscopy. It was found that spin-label Coenzyme A (CoA) binds very weakly to the enzyme in absence and presence of aminoglycoside antibiotics.

Introduction

Aminoglycosides are a class of antibiotics derived from the Streptomyces genus that target the 30S ribosomal subunit in gram negative bacteria (Llano-Sotelo et al, 2002). Binding of aminoglycosides to 16S RNA causes mistranslations and premature stops in protein synthesis resulting in cell death. There are a few subclasses of aminoglycosides, such as the kanamycins and neomycins, all with distinct structural differences. All aminoglycosides, however, are amine-modified pseudosaccharides, each antibiotic compound containing

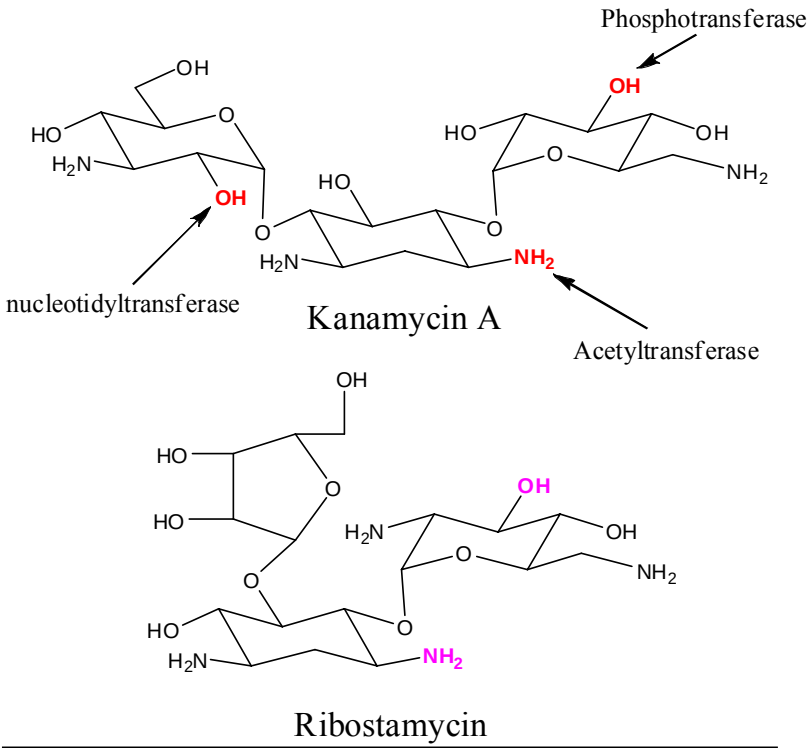

Figure 1. Examples of Aminoglycoside antibiotics revealing different sites of modification by AGMEs, namely AAC(3) (acetyltransferase) , APH(3') (phosphotransferase) , and ANT(2") (nucleotidyltransferase)

one non-sugar ring designated as the deoxystreptamine ring (DOS ring). An interesting feature of all aminoglycosides is that each of them contains two superimposable rings, the DOS ring and the ring designated as "primed." Still more interesting, the functionality of these antibiotics is very sensitive to modifications which can render them biologically inactive, particularly on the two superimposable rings. This is significant because it implies that every group (except the 5-position on the 2-DOS ring) on aminoglycosides is important to the aminoglycosides' roles in binding to bacterial ribosomes (Owston and Serpersu 2002).

Unfortunately, many bacteria are becoming resistant to aminoglycosides by producing Aminoglycoside Modifying enzymes (AGMEs) that modify them by group transfer. The enzymatic modifications of aminoglycosides occur as *N*-acetylation, *O*-nucleotidlylation, or *O*-phosphorylation. Our laboratory studies one representative aminoglycoside-modifying enzyme from each catalytic group. One of these enzymes is the aminoglycoside nucleotidyltransferase(2")-Ia (ANT2"), which catalyzes the ATP-dependent adenylylation of some aminoglycosides. This particular enzyme, however, cannot modify pentose rings (Wright and Serpersu 2005). Another AGME uses a 2^+ metal divalent cation to transfer the gamma phosphate of metal-bound ATP to the 3' position of aminoglycosides, aminoglycoside phosphotransferase(3')-IIIa (APH3'). The third enzyme is the aminoglycoside acetyltransferase(3)-IIIb (AAC), which is the subject of this work. AAC serves to covalently modify a number of aminoglycoside antibiotics by transferring the acetyl group from acetyl coenzyme A to the 3-NH_2 on the 2-DOS ring of aminoglycosides (Owston and Serpersu, 2002).

Kinetic and thermodynamic characterizations have been conducted on enzymes that can modify this class of antibiotics by nucleotidylation and phosphorylation, but no such data has been available on an enzyme that modifies the 2-deoxystrptamine ring. This study is directed to

thermodynamically characterize AAC by determining stoichiometry and dissociation constants of several enzyme-ligand complexes. The method chosen to attempt to describe these interactions entails a synthesis of a paramagnetic substrate analog of Acetyl Coenzyme A for analysis by Electron Paramagnetic Resonance (EPR).

Currently, there is no crystal structure of AAC(3)-IIIb (although there do currently exist crystal structures on acetyltransferases that modify the 2-DOS ring at other positions) , and the residues involved in the covalent modification of aminoglycosides by AAC are not known. The goal of this work is to initiate the characterization of thermodynamic properties of AAC-ligand complexes in order to dissect ligand-enzyme interactions. Results of this work will not only aid in better understanding of thermodynamic properties of AAC-ligand interactions but also may be helpful in development of new drugs to overcome bacterial resistance to the action of aminoglycoside antibiotics.

The complete thermodynamics of AAC-ligand interactions can be described by three binary and three ternary simultaneous equilibria in the following manner:

K_1= [AcCoA][aminoglycoside]/[AcCoA-aminoglycoside]
K_D=[AAC][AcCoA]/[AAC-AcCoA]
K_2=[AAC][AcCoA-aminoglycoside]/[AAC-AcCoA-aminoglycoside]
K_A'=[AAC-aminoglycoside][AcCoA]/[AAC-AcCoA-aminoglycoside]
K_3=[AAC-AcCoA][aminoglycoside]/[AAC-AcCoA-aminoglycoside]
K_S=[AAC][aminoglycoside]/[AAC-aminoglycoside]
These equilibria are interdependent and related to each other as shown below:
$K_1K_2=K_3K_D=K_A'K_S$ (Serpersu et al, 1987)

When four of the relevant constants are known, the remaining two constants can be calculated from the latter equation.

This project is aimed to determine the dissociation constants involving CoA by preparing a spin-labeled CoA analog (SL-CoA) to be used in electron paramagnetic resonance spectroscopy. Binding of the spin-labeled CoA to AAC causes broadening of the EPR signal, and the amount of spin-labeled CoA bound is directly proportional to the change in relative intensity. Thus, the free and bound spin label concentrations can be determined. However, since EPR can detect only paramagnetic species, then only then equations containing free CoA can be experimentally determined, which are K_1, K_D, and K_A'. Ks can be determined by isothermal titration calorimetry, allowing calculation of the remaining two constants. By repeating experiments altering ligand concentrations, Scatchard plot analyses can be performed to determine the stoichiometry of several different enzyme-ligand complexes. This work will be repeated for several different aminoglycosides in order to quantitatively compare AAC-antibiotic complexes with two different classes of aminoglycosides.

Experimental

i. Preparation of Spin-Label Coenzyme A

The compound used in the synthesis of spin-label CoA (SL-CoA), 3-carboxyamide-2,2,5,5-tetramethylpyrrolidine-1-oxyl (TMPO) contains a paramagnetic oxygen as can be seen in Figure 2. The spin label was prepared by converting the amide to a carboxylic acid and finally to an acid chloride. The prepared material was then placed in an aqueous solution with Coenzyme A such that the terminal SH of CoA covalently attached to the carbonyl carbon of TMPO. The scheme for preparation of the spin-labeled material is what is demonstrated in Figure 2. The

TMPO

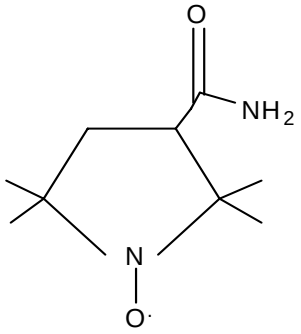

Figure 2. Structure of TMPO used to synthesize SL-CoA with a paramagnetic oxygen designated by the lone electron.

conversion of TMPO to the acid radical was used as described by Rozantzev et al (1965). Both the conversion of the material to the acid chloride and the purification of SL-CoA were taken from Weidman et al (1973) and optimized as described below.

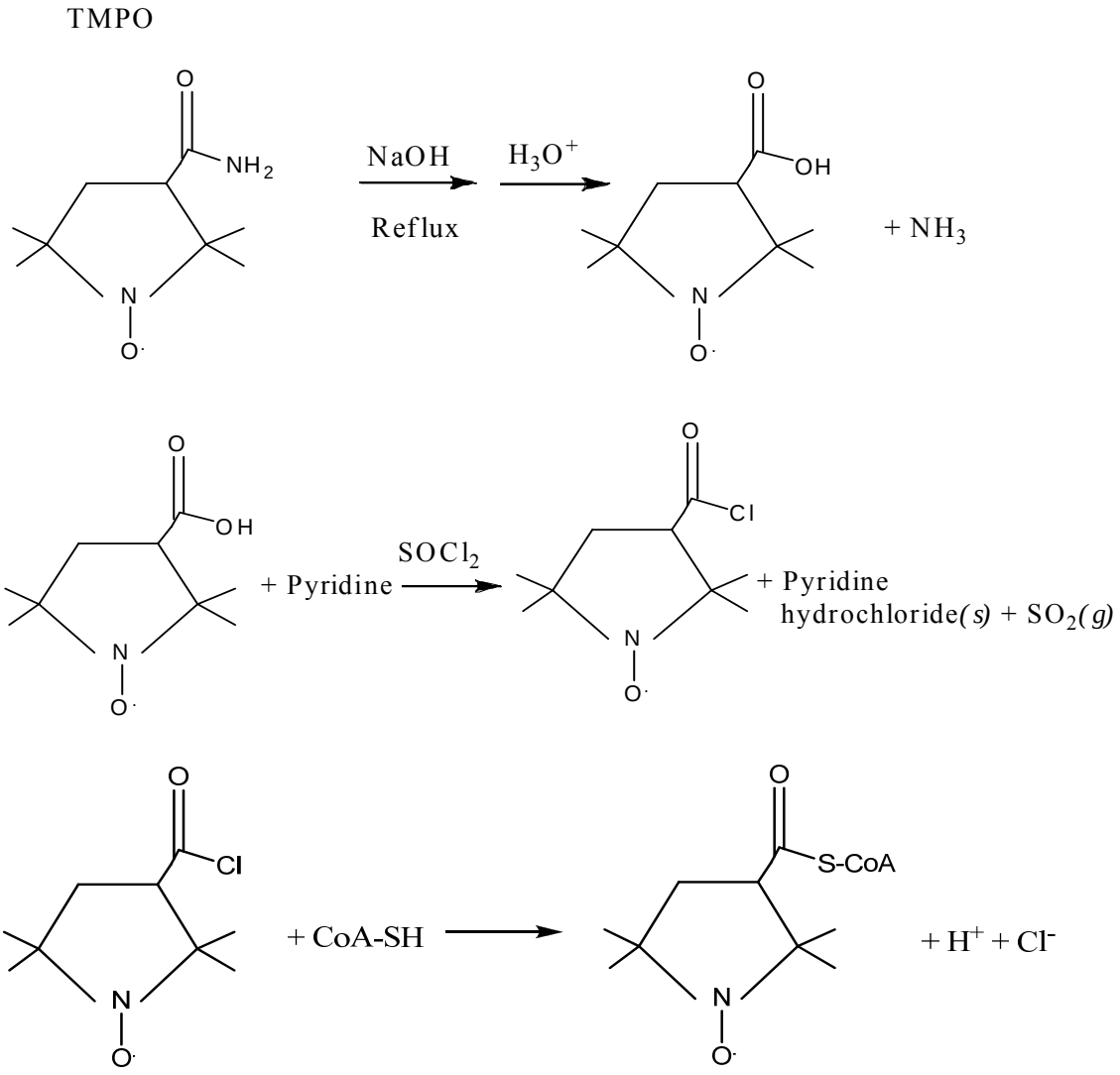

Figure 3. Synthetic scheme for the synthesis of SL-CoA.

Preparation began by placing 1 gram of TMPO in 11.1 ml 10% NaOH in a reflux condenser, and the solution was heated until no more ammonia evolved. When no more ammonia gas was detected, 3.4 ml of 12 N HCl was added to the reaction mixture, and the spin-label material was extracted by suction filtration using a ceramic filter and allowed to dry. The carboxylic acid radical formation was confirmed by IR spectroscopy with the -OH appearing at 3100/cm.

Next, argon gas was vented into a 2 ml graduated cylinder containing 18.6 mg TMPO acid radical at 0℃. 300 µl distilled anhydrous pyridine was then added to the graduated cylinder and mixed until the acid radical was completely dissolved. 12 µl (165 µM) $SOCl_2$ was then added to the reaction mixture and was mixed for 15 minutes to convert the acid radical to the acid chloride. This was a deviation from the procedure described by Weidman et al (1973) where only 9 µl of thionyl chloride was added. The additional thionyl chloride was necessary to convert the acid radical to the acid chloride which proved to maximize the yield of spin-labeled CoA in the final synthetic step. Finally, 120 µl (40 µM) of the acid chloride was added to a 1ml solution of 10 µM CoA-SH, 40 mg $NaHCO_3$, and 1.2 mg EDTA at 0℃. The nitroprusside test for free SH was performed as suggested by Weidman et al (1973), but none of the tests were ever negative. One could only add more TMPO acid chloride to reduce the intensity of the color of the test (Discussed in "Results and Discussion").

The reaction mixture was then passed on a 20 x 1.5 cm Sephadex G-10 column at pH 7.4 at 4℃ to remove salt impurities and free coenzyme A from spin-labeled CoA. Adjusting pH to 7.4 was a deviation from the procedure described by Weidman et al (1973) and was found to be necessary to prevent acid-catalyzed hydrolysis of the thio ester linkage. The fractions with CoA

were found by reading the 260 absorbance. The fractions with CoA and SL-CoA were separated by 4ml, ensuring that free CoA-SH and SL-CoA were separated. The earlier fractions of CoA were pooled and passed through a Macro-Q strong anion exchanger at pH 7.4. A linear NaCl gradient was used with an initial salt concentration of 25mM and a final salt concentration of 400mM. The fractions containing SL-CoA were pooled and lyophilized to near dryness. The lyophilized material was brought to a volume of 800 µl and passed on a 1 x 10 cm sephadex G-10 column at pH 7.4 at 4℃. The fractions were again pooled and lyophilized to near dryness, and the material was brought to a final volume of 1ml. The method for synthesis and purification typically gave rise to yields of SL-CoA between 20-25%.

ii. Purification of AAC(3)-IIIb

AAC(3)-IIIb was overexpressed in *BL-21 Escherichia coli.* This bacteria was used for overexpression because the gene for AAC has been genetically engineered to include a Histidine Tag (Histag), a short repeat of histidine residues, at the N-terminal end for purification purposes. The cells were treated with PMSF and lysed at 1500 psi. After centrifugation to remove cell debris and other insoluble material, the cell lysate was passed through a Ni^{2+} affinity column at 4℃ equilibrated with 100 mM NaCl, 50 mM Tris-HCl, and 20 mM imidazole at pH 8.2. A linear 30 ml gradient of 100 mM initial and 350 mM final imidazole concentration was passed through the column, and the fractions containing AAC-Histag as found by reading the 280/260 Absorbance ratio were pooled and dialyzed 3x in 500 ml 100 mM NaCl and 50 mM Tris-HCl at pH 8.2 and 4℃. The dialyzed material was treated with thrombin for one hour at 24℃ and passed through the Ni^{2+} affinity column in the same salt, buffer, pH, and temperature conditions as before. The column was washed with the 50 mM Tris-HCl and 100 mM NaCl solution, and the immediate fractions of AAC without Histag were collected. To eliminate Thrombin, the fractions

containing AAC were passed through a 1.5 x 20 cm Macro-Q strong anion exchanger with a linear NaCl gradient 300 mM-1000 mM. AAC typically eluted at approximately 600 mM NaCl. The fractions containing pure AAC were pooled and dialyzed three times as described earlier.

The purity of AAC was confirmed by SDS-PAGE gel electrophoresis. The activity of AAC was checked by determining enzymatic activity using kanamycin as the substrate. The activity assay is based on observation of the formation of pyridine-4-thiolate, which absorbs at 324 nm (19,800 $M^{-1}cm^{-1}$) from 4,4'-Dipyridyl disulfide. The reaction was initiated by the addition of 10 nm AAC (final concentration) into a mixture containing 25 mM Tris at pH 7.6, 1 mM EDTA, 100 mM NaCl, 750 μM 4,4'-Dipyridyl disulfide, 100 μM AcCoA, and 100 μM Kanamycin A. Purified AAC was confirmed to have an activity of 60 units/mg with kanamycin A as the substrate.

iii. Electron Paramagnetic Resonance Spectroscopy

The instrument used for the EPR experiments was the BRUKER EMX X-Band (9.78GHz). A quartz tube with an 80 μl volume capacity was filled with water, and an EPR spectrum with attenuation of 30 and a gain of 5E4 was taken to ensure that no paramagnetic noise would come from the EPR tube. The experiments were performed in pairs in the following way: One EPR run was conducted with 50 mM Tris-HCl, 100 mM NaCl, and SL-CoA at pH 7.8; the second, all other conditions held constant, with 150 μM AAC present. The sets of experiments were repeated in this manner while changing the fraction of SL-CoA present with and without enzyme.

Results and Discussion

Although a procedure that was given in literature was followed (Weidman et al 1973), we encountered several difficulties which required that the synthetic procedure be optimized. In the synthesis of SL-CoA, the reaction of the CoA-SH with R-Cl involves a nucleophile that attacks the carbonyl carbon to form a tetrahedral addition intermediate, which then breaks down with the loss of the halide (Loudon 2002). Because of the tetrahedral intermediate mechanistically required for the reaction to occur, it was initially thought that the phosphate group oxygens on CoA would not be favorable for this reaction due to the strong electron withdrawing power of the phosphates themselves. It was also thought that the two secondary alkyl OH groups on CoA would not be reactive with the acid chloride. The reason for this is that the acid chloride is not an alkyl halide, implying that no substitution reaction could take place because the reactions S_N1, S_N2, E1, and E2 explicitly require an alkyl halide, and secondary alcohols and thiols are much less reactive than their primary counterparts (Loudon 2002). The test for unreacted CoA after the reaction had been performed, however, was always positive. The observed phenomenon was due to the fact that the substitution of CoA-SH for Cl competes for the intermediate addition of OH for Cl, and OH is present in great excess of the acid chloride; therefore, CoA-SH conversion to SL-CoA could not ever be driven to completion, so there was always some detectable free CoA-SH.

MALDI-TOF mass spec was used to confirm a single addition of the TMPO moiety to Coenzyme A, as shown in Figure 4. Since nitroxides and carbonyl groups can ionize in MALDI experiments, then the isotopic peaks are consistent with a single spin-label event. The data was used only to confirm a single spin-label event, not to attempt to confirm the site of CoA modification (discussed in “Future Directions”).

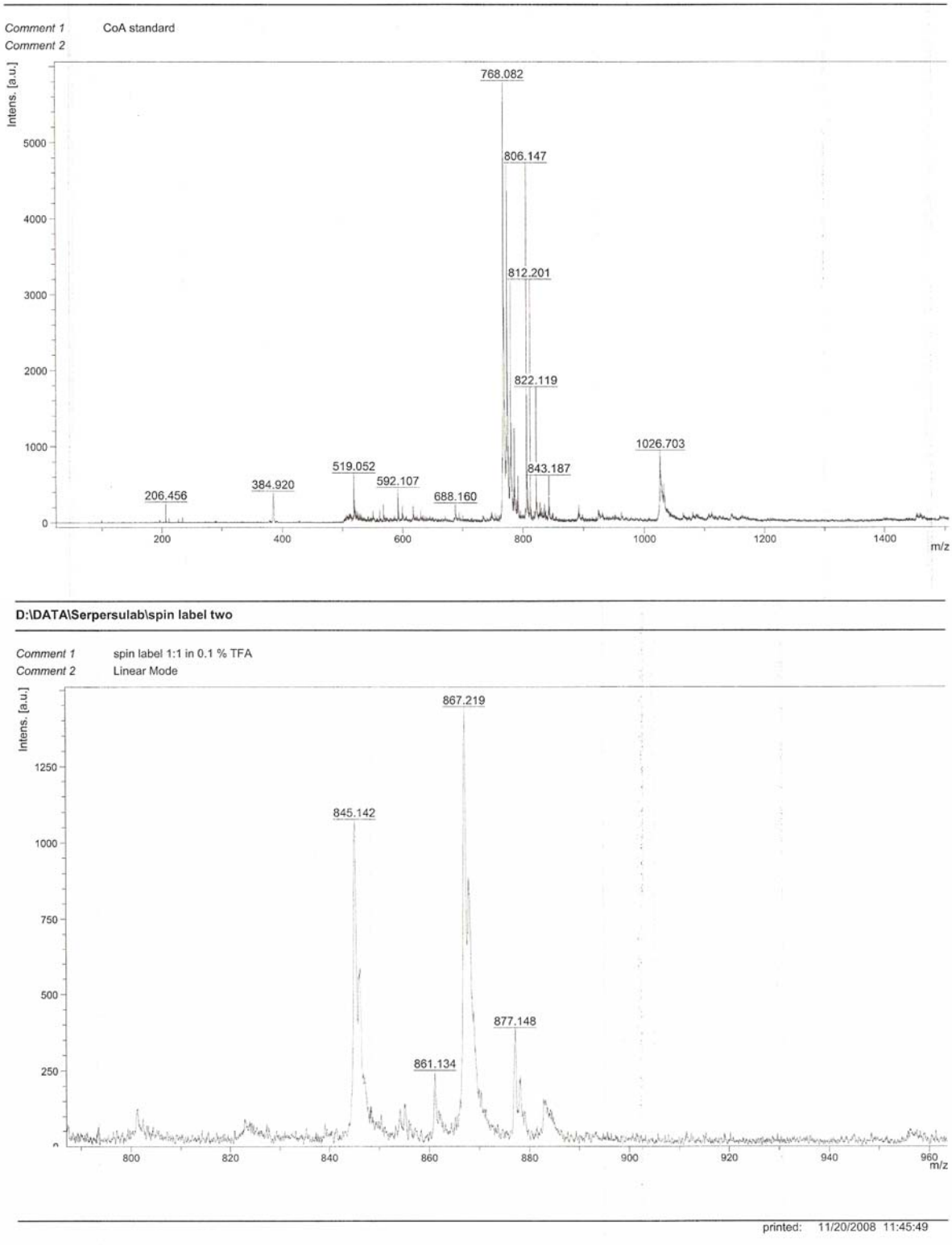

Figure 4. Mass spectrometry data from coenzyme A (top) and spin-labeled coenzyme A (bottom).

Beginning this work, it was first thought that Isothermal Titration Calorimetry (ITC) could be used to discover KD, KA', and Ks. EPR work could have potentially been used to discover K1, KD, and KA'; so four relevant constants would have been obtained, and K2 and K3 could have been calculated. In this way, the EPR work would have served both to complement ITC data and to confirm the reproducibility of the results. It was determined in ITC experiments that the presence of aminoglycosides increases the affinity of CoA to AAC. Also, when malonyl CoA was substituted for Acetyl CoA in the presence of antibiotic, K_A' increased 50 fold to 100μM. Therefore, it was expected that the affinity of AAC for SL-CoA would be very weak. If it were possible to detect binding of SL-CoA to AAC, then one would be able to see the binding by increasing the ratio of AAC present, thereby increasing the amount of CoA bound and, as a consequence, detecting a change of paramagnetic signal height.

The first set of EPR experiments involved observing paramagnetic signal arising from 10 μM SL-CoA and comparing that signal to paramagnetic signal received by the addition of 150 μM final concentration AAC and 400μM neomycin B. 400 μM final neomycin concentration was used because earlier ITC experiments showed that AAC becomes saturated with neomycin with neomycin concentrations two and a half fold AAC concentration. Despite the addition of antibiotic, there was no observable broadening in the EPR signal, and, therefore, no observable binding. A second set of experiments then used 2 μM SL-CoA with all other conditions held constant for the purposes of introducing 75 fold excess concentration of AAC compared to SL-CoA. Figure 5 show the EPR signal as a result of adding 2 μM SL-CoA only and of adding 2 μM SL-CoA, 400 μM final concentration neomycin B, and 150 μM final concentration of AAC, respectively.

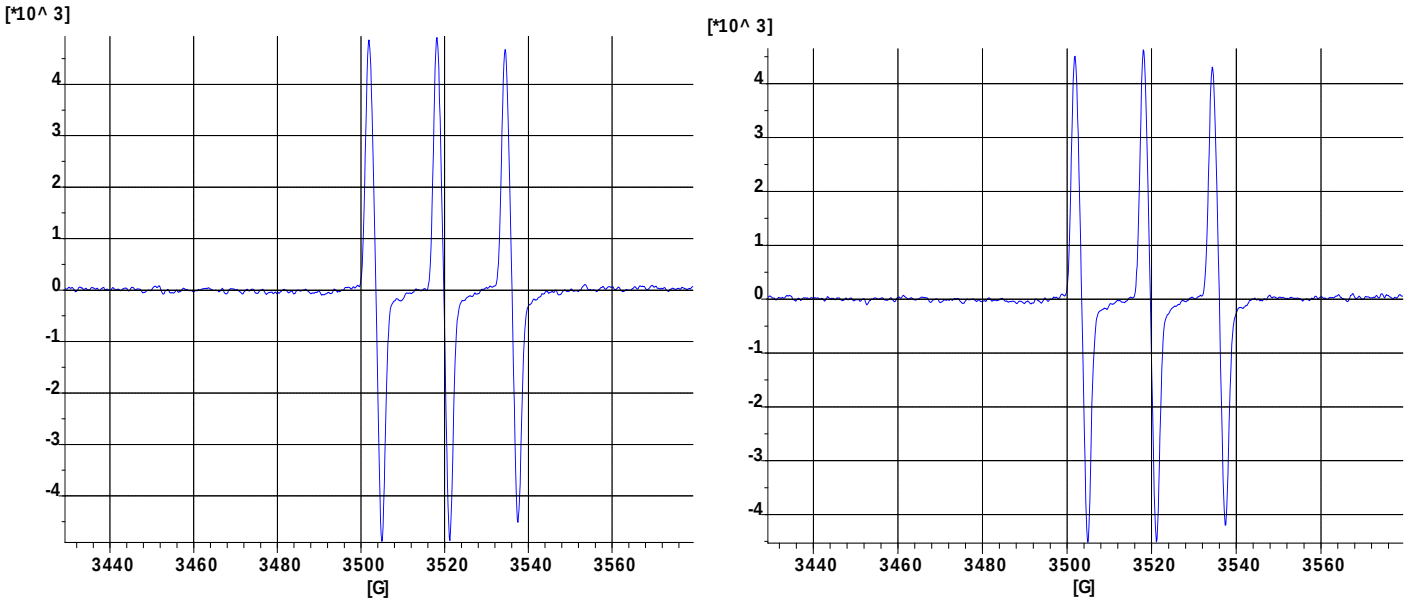

Figure 5. EPR spectra of relative intensity vs [G] of 2 µM SL-CoA only (left) and 2 µM SL-CoA in the presence of 150 µM AAC and 400 µM neomycin B (Right).

As seen here, there was no observable change in paramagnetic signal even with enzyme concentrations of 75 fold excess of SL-CoA. This may suggest that the dissociation constant, $K_{A'}$, is in the millimolar range when SL-CoA is substituted for Ac-CoA. It could be the case that the paramagnetic group on SL-CoA is outside the active site of the enzyme and freely rotating, resulting in no change in paramagnetic signal even if SL-CoA is bound to the enzyme-aminoglycoside complex. It is also possible that the bulky spin label moiety is causing unfavorable steric interactions with the enzyme itself such that SL-CoA binds very weakly or, more likely, that the pyrrolidine ring attached to the spin-label itself is clashing with the 2-deoxystreptamine ring of the bound aminoglycoside, resulting in low affinity of SL-CoA to AAC-aminoglycoside complex.

Future directions

Recent ITC data has shown that there is an observable binding of coenzyme A to aminoglycoside (personal communication of unpublished data). Additional EPR experiments will be conducted using at least 100 fold excess aminoglycoside to SL-CoA to determine whether or not K_1 can be measured using this technique. Two possibilities are foreseen: Either there will be no observable change in paramagnetic signal that arises from the suspected weak interaction of SL-CoA-aminoglycoside complex formation, or there will be an observable change such that K_1 can be measured. If K1 can be measured, these experiments will serve to complement ITC data.

Additional EPR work will also be done using very high concentrations of AAC in the absence of antibiotic. The reason for conducting this work, even knowing that the dissociation of SL-CoA from AAC greatly increases in the absence of antibiotic, is to see if there is an observable binding in the absence of antibiotic—because, if the pyrrolidine ring system is clashing with the 2-deoxystreptamine ring, then the absence of antibiotic may allow for one to observe a binding of SL-CoA to AAC in the presence of high AAC concentrations.

In addition to the latter experiment, kinetic inhibition experiments will be done with the spin-labeled analog to check whether or not the Spin-labeled analog of AcCoA can compete with AcCoA for the active site of AAC. If a competition is observed, then this would suggest that the spin-label itself is attached to the sulfer atom, and, therefore, would serve to support that SL-CoA has been successfully synthesized with the spin-label molecule attached at the suspected position. If no inhibition is detectable, then the experiment will serve neither to support nor to give doubt as to the success of the synthesis. Since it is expected that the bulky spin-label is

clashing with the 2-DOS ring, then one could more usefully defend why an inhibition may not have been visible.

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