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Mutagenic Studies of a Unique Cysteine Ligase Enzyme

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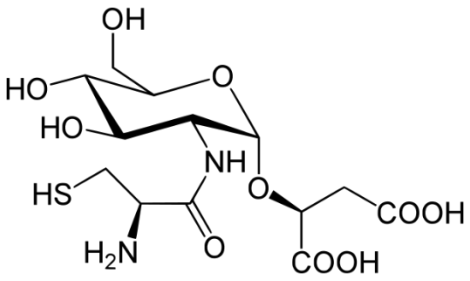
Abstract

BshC is the final enzyme in a three step pathway for the synthesis of bacillithiol, a compound that enables resistance to fosfomycin in Gram-positive bacteria. BshC is unique from other enzymes of its kind because of an additional ADP binding site and inactivity when studied in the laboratory. To explore BshC function, several site-directed mutants have been selected within the ADP binding pocket. Fluorescence assays have been utilized on the wild-type BshC and one mutant, Y510Q. We determined that Y510Q does not bind ATP as effectively as wild-type BshC. These fluorescence assays will be utilized on W506L, E384A, and H386A mutants and structural analysis of all the mutants will be initiated. Gaining more understanding of the structure of these mutants and how they bind ATP will give a better understanding of how BshC binds its substrate, which will allow the development of inhibitors to combat fosfomycin resistance.

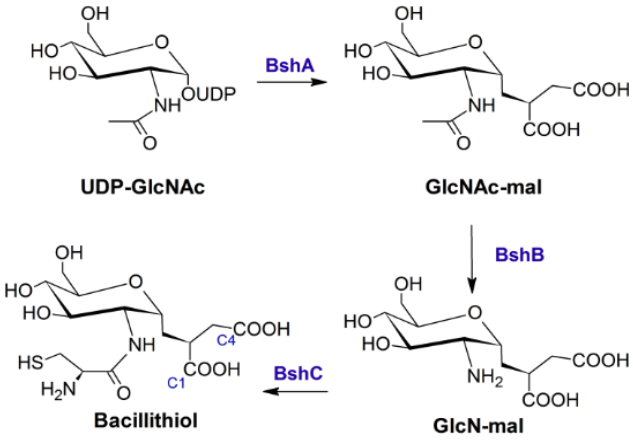
Low molecular weight (LMW) thiols play an important role in maintaining redox balance and detoxifying xenobiotic agents in cells.[1,2] Examples of these LMW thiols are mycothiol (MSH) and bacillithiol (BSH) (Scheme 1). The LMW thiols, MSH and BSH, are similar in shape and function; however, there are some differences. MSH is a molecule produced and used by mycobacteria, while BSH is a molecule that is produced by some pathogenic Gram-positive bacteria like *Staphylococcus*, which causes skin infections, and *Bacillus anthracis*, which causes anthrax.[2] The drug Monurol is commonly used to treat such infections, as well as being commonly prescribed for urinary tract infections; the active ingredient being fosfomycin.[3] Fosfomycin is a compound that inhibits the biogenesis of the pathogen's cell wall. The problem at hand is these bacteria are resistant to the drug, due to their production of the compound



Scheme 1 *The structure of bacillithiol (BSH)*

bacillithiol.[4] Through the study and understanding of the enzymes that biosynthesize bacillithiol, the development of an effective inhibitor can be created to not only reduce the production of BSH, but also increase the effectiveness of fosfomycin drugs once again.

Bacillithiol is produced through a pathway involving three different enzymes (Scheme 2): a glycosyltransferase (BshA), a deacetylase (BshB), and a putative cysteine ligase (BshC).[5] BshC is the enzyme that is responsible for the completing step of attaching the cysteine to the acceptor (GlcN-mal), using ATP to drive the reaction to completion. The final step in this biogenesis pathway has proven to be unique from the others in its pathway because it cannot be replicated in a laboratory setting, by taking the BshC enzyme and providing all of the substances needed to create bacillithiol. This presents an interesting trait of the BshC enzyme and causing question to what is missing.



Scheme 2 *The three step pathway of the production of bacillithiol starting with BshA, followed by BshB, and ending with BshC.*

The Cook lab has determined the structure of BshC.[1] The key domains of BshC include: the Rossman fold, two connection peptide motifs (CP1 and CP2), and an alpha helical coiled-coil. The portion of the enzyme that plays the most important roles in the production of BSH, is the Rossman fold domain, which is the putative active site where citrate, a bacillithiol analog, is bound in the structures that have been determined so far (Figure 1). The

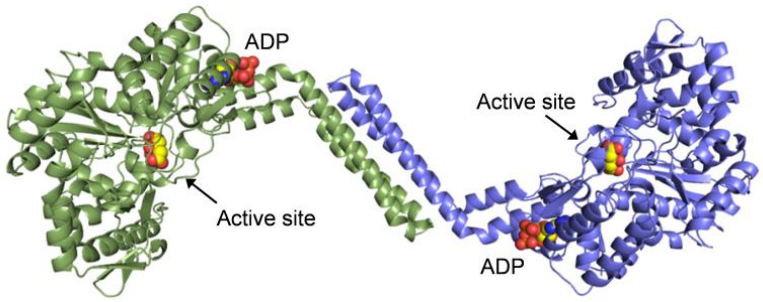


Figure 1 *BshC dimer: there is reason to believe that this is how BshC forms in its natural environment.*

alpha helical coiled-coil is important in creating the dimer that BshC is found naturally in solution. In a pocket between the coiled coil and CP2 domains an adenosine diphosphate (ADP) molecule was observed. The additional ADP binding site is unique from similar enzymes of its kind, including MshC.[2] Fluorescence assays have proven that BshC from *B. subtilis* does bind adenosine triphosphate (ATP); nonetheless, there is now the question as to whether ATP is binding in the Rossman fold active site or the additional ADP binding pocket (Cook Lab, unpublished).

This problem will be approached by, not only looking at purified BshC as found in the species of *Bacillus subtilis* (BshC WT) which will be expressed using the *E. coli* strain BL21 (DE3) and is optimal for expressing enough protein to study. Site-directed mutagenesis will allow us to create four mutant forms of BshC such as BshC Y510Q and BshC W506L in using the *E. coli* strain XLI Blue, this will be optimal for plasmid production. In this process we will be altering the amino acid residues in areas we believe to be binding sites. The effect of these changes will be assessed and compared to the BshC WT and help us identify where the ATP/ADP are binding what amino acid residues are critical for such binding to occur. The pure form of both the wild type and mutant type of the enzymes will be purified using nickel-affinity chromatography. Using the purified form of both types of enzymes, we will examine the function and structure of each of the types through x-ray crystallography and fluorescence binding assays. With the BshC WT as the "control group", we will be able to compare the different proteins' results and form a better understanding of the final step in the biogenesis of bacillithiol.

Materials and Methods:

Growing BL21 (DE3) E. coli Transformed with BshC WT. The LB Broth solution was prepared and autoclaved into two 2 L baffled flasks, each with 1 L of broth, and one, one liter graduated

flask with 250 mL of broth solution as a starter flask. Approximately 50 µL of thawed out chemically competent BL21 (DE3) *E. coli* cells were transformed with the addition of 1 µL of plasmid pPDC005 (160 ng/µL) to the cell solution. The cells were allowed to rest for 7 minutes on ice, placed in a Precision warm water bath set at 42°C for 90 seconds, and allowed to recover for 10 minutes on ice. LB broth was added to the cellular solution in 250 µL amount and shook for an hour at 37°C. After an hour of allowing the cells to grow, cellular solution was plated onto two LB-agar plates treated with kanamycin and placed in 37°C environment overnight for to allow for more growth.

Kanamycin was added to the 250 mL starter flask for a final concentration of approximately 25 ng/µL. About 5 µL of the LB and kanamycin solution was flooded onto the plate and scraped with a sterile wand. The cells were transferred from the plate and into the starter flask by pipette. The starter flask was shaken at 200 RPM at 37°C for approximately one hour. Kanamycin was added to both of the 2 L flasks to make a final concentration of 1000 ng/µL. After the cells in the starter flask were shaken for an hour, they were inoculated with the rest of the media with 20-50 mL of the starter flask per 1 L of final culture. The two 1 L of final cultures were shaken at 200 RMP at 37°C for a few hours while monitoring the OD_{600}. When the OD_{600} value reached the value of 1.0 the cells were cooled to a temperature of 18°C and IPTG was added to the flasks at a final concentration of 1 mM. The flasks were shaken overnight at 200 RPM at 18°C. The cells were harvested by centrifugation using the centrifuge at 5000 xg for 15 minutes and stored at -20°C.

Purification of BshC WT: Nickel Affinity Chromatography and TEV Cleavage. The transformed *E. coli* cell paste expressing the BshC WT was re-suspended in approximately 3 mL of lysis buffer

(20 mM Tris, pH 8.0 + 300mM NaCl + 10mM Imidazole) per gram of cell paste. After the cell paste was completely thawed, EDTA was added to a final concentration of 0.5 mM. Lysozyme was then added to a final concentration of 0.1 mg/mL. The cells were allowed to sit on ice and swirled occasionally. The cells became viscous over a 30 minute waiting period for lysing and then $MgCl_2$ was added to a final concentration of 5 mM, and then DNase I to a final concentration of 5 μg/mL. The cells were allowed to sit at room temperature of 15 minutes, allowing enough time for the suspension to reduce in viscosity. The cells were then sonicated for 2 cycles of 10 seconds. The lysed cells were centrifuged at 13,000 xg for 20 minutes at 4°C and the supernatant was loaded onto a 10 mL Ni-NTA column equilibrated with lysis buffer (20 mM Tris, pH 8.0 + 300 mM NaCl + 10 mM Imidazole). The column was washed with wash buffer (20 mM Tris, pH 8.0 + 300 mM NaCl + 25 mM Imidazole) until the solution coming off the column was nearly negative on a Bradford spot test. Elution buffer (20 mM Tris, pH 8.0 + 300 mM NaCl + 250 mM Imidazole) was added to the column. The solution coming off of the column was collected in approximately 3 mL fractions until the buffer coming off of the column is nearly negative on the Bradford spot test. The fraction that contained the target protein BshC WT was collected.

After completing the Ni-NTA Column, 2 mL of rTEV was added to the falcon tube of purified BshC WT. Then, DTT was added to the standard dialysis buffer (20 mM HEPES (pH 7.5) + 50 mM NaCl) to make a final concentration of approximately 0.5 mM. The rTEV BshC WT solution was dialyzed in the standard dialysis buffer overnight at room temperature to remove excess imidazole and the his-tag. This prep was run back over the Ni-NTA column the next morning, the run off collected into a falcon tube.

Determining Protein Concentration of BshC WT. The solution of the rTEV BshC from the previous method was transferred into a centrifugal filter test tube. The swinging bucket centrifuge was used at a speed of 3.0 x 10 g for 25 minute cycles at 4°C. After each cycle, a Bradford spot test was run on the solution that passed through the filter to ensure that there were no leaks in the membrane of the centrifugal test tube. This process was repeated until BshC was fully concentrated in the membrane of the filter test tube and the solution from dialysis was used up completely. Throughout the process of centrifugation, 10 μL samples were taken of the concentrated protein solution and the absorbance was measured using the Agilent Cary 60. After the solution was concentrated completely to 8 mg/mL, the solution was pipetted into test tubes in 200 μL increments and stored at -80°C. An Amresco SDS-PAGE gel was made using 12.5% Next Gel, Coomassie stain, and de-stain (10% MeOH, 10% AcOH). The Amresco SDS-PAGE gel was run to ensure the purification of our protein and the success of the TEV Cleavage.

Growing BshC WT Crystals: Hanging Drop Vapor Diffusion. Protein prep number 065 (BshC WT *B. subtilis* on 5/26/15) was used to set up six trays for growing crystals via Hanging Drop Vapor Diffusion. The first two trays had Hampton Research Peg Ion I as the well solution, the third and fourth try had Hampton Research Peg Ion II. The fifth and the sixth trays had the Hampton Research Crystal Screen. AMP (2 mM) was added to the protein prep and the final protein solution made up half of the drop, the other half well solution were the total volume was 4 μL. Only one well had developed crystals and was replicated when a Refine tray was created through seeding. These crystals were frozen in liquid nitrogen directly and taken to LS-CAT at Argonne National Labs.

Mutagenesis for BshC Y510Q. The primers 132 and 133 were brought to 125 μg/mL using sterile MQ water, the concentrations and A_{260}/A_{280} ratio were determined on the Thermo Scientific NanoDrop 2000c. The following ingredients were combined into a small test tube and placed into the Thermocycler: 10 ng of the plasmid, pPDC005, 125 ng of the primers 132 and 133, 5 μL of dNTP mix (stock 2 mM), 5 μL of buffer (10x), 3 μL of DMSO, 1 μL of Turbo Pfu Polymerase, and sterile MQ water to reach the total reaction volume of 50 μL. After program ran for 19 cycles, 1 μL of Dpn1 was added and allowed to incubate at 37°C for an hour. Approximately 50 μL of competent XL1 Blue *E. coli* cells were transformed with BshC Y510Q following the same method used to transform BL21 (DE3) *E. coli* transformed with BshC WT.

MiniPrep for BshC Y510Q. The Thermo Scientific Gene JET Plasmid MiniPrep Kit was used to isolate the BshC Y510Q plasmid. Exactly six individual colonies were plucked using a pipette tip from the plate of XL1 Blue *E. coli* transformed with BshC Y510Q and placed into six individual test tubes, each filled with 7 mL of LB broth solution and 7 μL of kanamycin antibiotic. The test tubes, including the pipette tip, shook at 37°C and left over night. The rest of the Mini Prep procedure was in accordance with the manufacturer's instructions. A DNA Gel was then run to confirm that there was indeed BshC (known from the DNA Sequencing Ladder) in the sample. It was confirmed that the sample might contain the desired mutation, 6 templates were made, however, only two of them were sent in for sequencing. All of the templates contained a dilution of the MiniPrep DNA and DI water with a final concentration of 50 ng/mL and 10 μL per template.

Growing BL 21 (DE3) E. coli Transformed with BshC Y510Q. This method followed the method, "*Growing BL21 (DE3) E. coli Transformed with BshC WT*," the only difference was using the plasmid from BshC Y510Q mutagenesis in the transformation.

Purification of BshC Y510Q: Nickel Affinity Chromatography and TEV Cleavage. The method "*Purification of BshC WT: Nickel Affinity Chromatography and TEV Cleavage"* was repeated, the only difference being the cell paste to run over the column for purification was not BshC WT, but BshC Y510Q from BL 21(DE3) *E. coli*.

Transformation of XL1 Blue E. coli with BshC Y510Q and Plasmid Propagation. The 50 µL of XL1 Blue *E. coli* cells were transformed with BshC Y510Q according to the procedure, "*Mutagenesis of BshC Y510Q.*" After the plate was able to go overnight in the 37°C, four individual colonies were "picked" off the agar plate and each placed into a sterile disposable culture tube with 7 mL of LB medium and treated with 7 µL of kanamycin. These tubes shook at 200 RPM overnight in a 37°C environment. The tubes were then centrifuged (along with the pipette tip) at 2000 RPM (a serological centrifuge at maximum speed) for 8 minutes, then discard the supernatant. The "*MiniPrep for BshC Y510Q"* method was followed, the only change being the desired protein for concentration. After the MiniPrep was completed, the protein's concentration was determined through absorption and Beer's Law. Once the final concentration and the A_{260}/A_{280} ratio was determined, the concentrated protein was stored in the -20°C freezer.

Growing BshC Y510Q Crystals: Hanging Drop Vapor Diffusion. The method, "*Growing BshC WT Crystals: Hanging Drop Vapor Diffusion*" was followed to grow crystals of BshC Y510Q.

The same 6 trays and Hampton Research solutions were used. This method required two drops of protein on each well. One drop contained 2mM AMP and the other contained 2mM glcNAC.

Fluorescence Assays of BshC WT and BshC Y510Q. Using the fluorimeter, BshC WT and BshC Y510Q were tested in the following way at 280 nm emission and observed at 332 nm. The 2 mL cuvette was soaked in 6 M HCl for approximately five minutes to ensure the cuvette was clean. There were 1000 µL of filtered Standard Dialysis Buffer (20 mM HEPES (pH 7.5) + 50 mM NaCl) and 1 µL of 20 mg/mL gelatin used as the initial solution, then 2 µL of BshC Y510Q (PROT #067 15mg/mL) was added to the cuvette. 1 µL of 0.25 mM ATP was added to the cuvette in the same manner as the enzyme. After about a minute 3 µL of 0.25 mM ATP was added, after another minute 5 µL of 0.25 mM ATP was added, and then after another minute or two 7 µL of 0.25 mM ATP was added. Following using the 0.25 mM ATP, 5.0 mM ATP was added in 1 µL increments every minute for the next three additions.

Mutagenesis for BshC E384A, BshC H386A, & BshC W506L. The method, "*Mutagenesis for BshC Y510Q.*" was followed for all three mutations. The only difference being the primers 136 and 137 were used for BshC E384A. The transformation of the *E. coli* and MiniPrep was followed exactly as the method describes for all three mutations. The primers 138 and 139 were used for the mutagenesis for BshC H386A. The transformation of the *E. coli* and "*MiniPrep for BshC Y510Q."* method describes. Repeat method, "*Growing BL21 (DE3) E. coli Transformed with BshC WT"* using the correctly sequenced plasmid from the method, "*Mutagenesis for BshC H386A."* Primers 130 and 131 were used for the mutagenesis of BshC W506L. After the mutagenesis process was completed, 50 µL of XL1 Blue *E. coli* cells were transformed with BshC

W506L. After the colonies grew overnight, the method, "*MiniPrep for BshC Y510Q*" was followed.

Growing BL21 (DE3) E. coli Transformed with BshC W506L. Repeat method, "*Growing BL21 (DE3) E. coli Transformed with BshC WT"* using the correctly sequenced plasmid from the method, "*Mutagenesis for BshC W506L."*

Results

Determining the Purified Protein Concentration of BshC WT. The final absorbance reading and Beer's Law was used to calculate the concentration of protein to be 8.0 mg/mL. Through using a derived form of the Beer's Law equation, the final concentration of BshC WT was calculated to be 8 mg/mL. This will allow the making of crystals and taking both fluorescence and functional assays of this protein. The Amresco SDS-PAGE also revealed that the protein sample from BshC WT concentration 8 mg/mL is almost completely pure.

The Amresco SDS PAGE (Figure 2) was used to assess the purity of the protein BshC WT. Lanes one and nine were about 5 µL of protein ladder. The second and third lanes have the concentrated BshC WT (8.0mg/mL). The fifth and sixth lanes were form a sample taken off of the Ni-NTA column after the TEV Cleavage. Lanes 2 and 5 have approximately 5 µL of sample, while lanes 3 and 6 have approximately 10 µL of sample. The Amresco SDS PAGE helped us to know that the BshC WT protein was the size expected compared to the protein ladder scale for protein size. This test also helped us know the protein was pure because there were no thick bands in places unexpected.

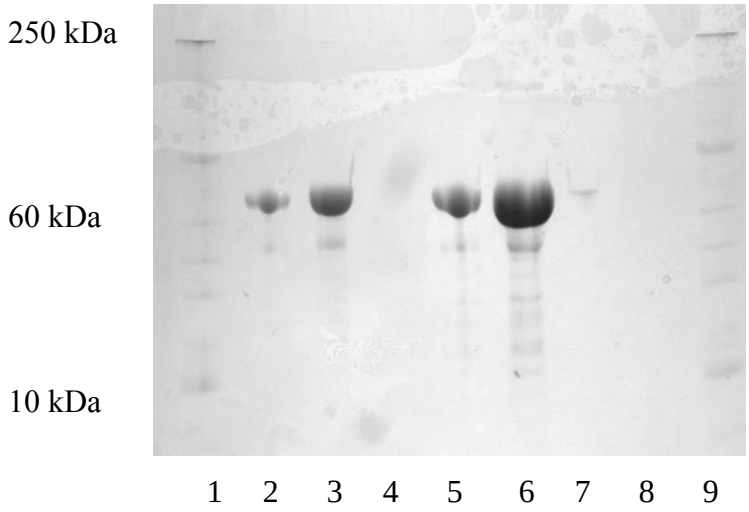


Figure 2 Amresco SDS PAGE BshC WT. *The gel was stained with Coomassie and the protein was purified and isolated using nickel affinity chromatography. A molecular weight standard was placed in lanes 1 and 9. Concentrated BshC WT protein (8mg/mL) was loaded into lanes 2 and 3. BshC WT protein from the flow through of Ni NTA column after the TEV Cleavage procedure was loaded into lanes 5 and 6. Lanes 2 and 5 contain approximately 5 µL of sample while Lanes 3 and 6 contain approximately 10 µL of sample. Lanes 4 and 8 were left blank intentionally. The SDS PAGE revealed that the desired protein was present in the sample and highly pure. This same test was run on the mutant BshC Y510W with similar results*

Growing BshC WT Crystals: Hanging Drop Vapor Diffusion. After a couple days cell #44 in Tray #2 produced small "flake-like" crystals. After a trip to Argonne National Laboratory, determined that the crystals growing in Hamptons Research Peg Ion I, 44th solution, were indeed salt crystals despite the ability to break and replicate them.

Determining Correct Mutation in BshC Y510Q. The DNA Agarose Gel, stained with ethidium bromide, shows that the gene is indeed BshC in size (Figure 3). This gel also helps to identify the purity of the protein and that they are valid for sequencing. Though the DNA gel does prove purity and size, it does not prove that the mutation was successful but more so proves that something did not go completely wrong. The sample from lane 2 in the DNA Gel was prepared and sent to the University of Michigan for sequencing analysis. According to the DNA sequencing results from the University of Michigan's DNA Sequencing services, the site-directed mutagenesis was successful in replacing the tyrosine to a glutamine in the putative active site of BshC Y510Q (Figure 4). Though

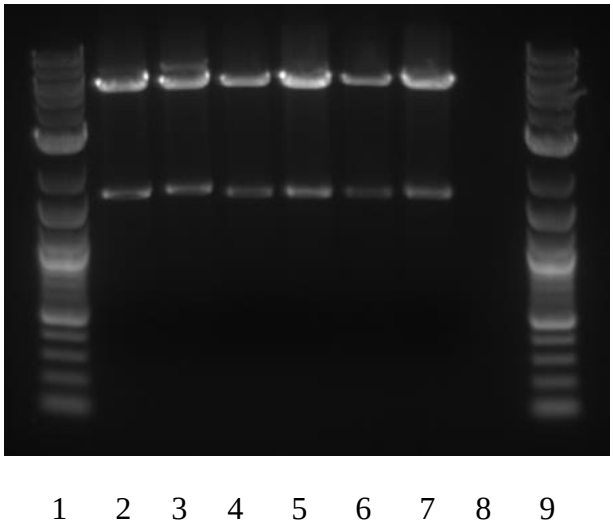


Figure 3 DNA Agarose Gel: BshC Y510Q. *The agarose gel was stained with ethidium bromide. A DNA ladder was placed in lanes 1 and 9. Six different plasmids of the mutant BshC Y510Q were isolated and cut with the restriction enzymes NdeI and XhoI and loaded into lanes 2 through 7. Lane 8 was intentionally left blank. This analysis confirmed the presence of a gene the size of BshC.*

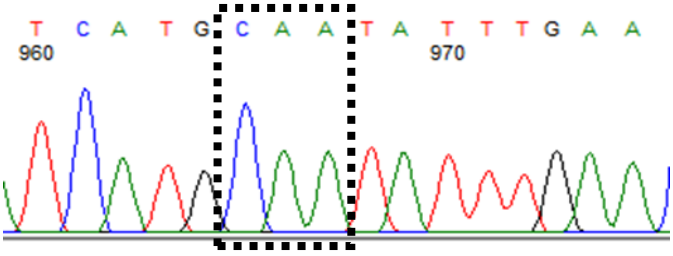


Figure 4 Sequencing Chromatograph: *The chromatogram from that analysis confirmed that the desired mutation from tyrosine (UAC) to a glutamine (CAA) at location 510 on the BshC gene.*

there was a single silent mutation, the clone was accepted as valid to use for plasmid propagation and further testing to allow greater insight into BshC.

Fluorescence Assays of BshC and BshC Y510Q. The fluorescence assays revealed that this mutated enzyme seemed to be unaffected as ATP was added to the solution of buffer, gelatin, and enzyme (Figure 5). Though there did seem to be a much long equilibration period for the mutated enzyme then the wild type, there is reason to believe that this mutant may not be binding ATP in its putative active site where the mutation was made. When comparing the BshC WT to BshC Y510Q from Table 1 it is clear to see that there is something very different happing when ATP is added to BshC Y510Q. An SDS PAGE was run for BshC Y510Q, however, the gel was smeared and did not turn out.

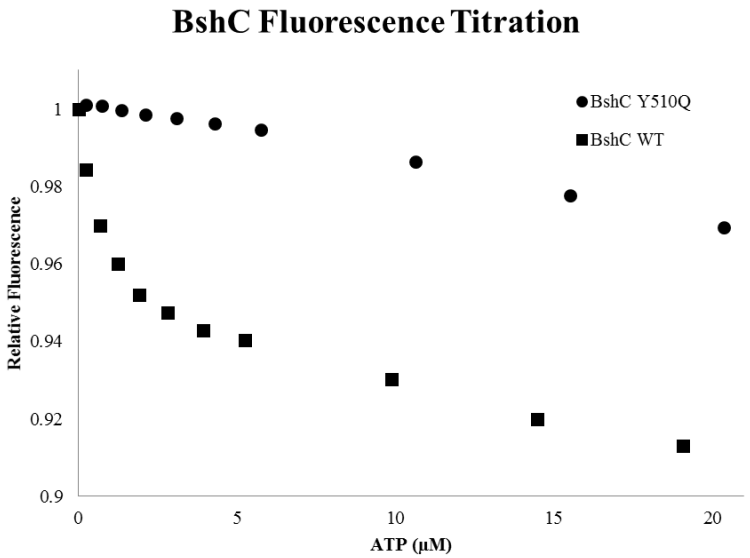


Figure 5 Fluorescense Assays of BshC WT and BshC Y510Q. *The graph above shows the change of intrinsic tryptophan fluorescence upon adding ATP. The sample was excited at 280 nm and fluorescence was observed at 332 nm. The BhsC WT was used as a control to compare with the mutant results. Fluorescence decreases dramatically when ATP is added to BshC WT, whereas BshC Y510Q is barely affected.*

Structural Analysis. With the mutation confirmed, crystal trays were set up to determine how the mutation affected the binding and structure of the enzyme. The crystals in Figure 6 were taken to Argonne National Laboratories and analyzed via X-ray diffraction. Figure 7 (above) shows the structure of the ADP binding pocket for BshC WT where the ADP is clearly bound in the pocket, while the bottom figure shows the structure of the ADP binding pocket for BshC Y510Q from the crystals in Figure 6 produced low resolution structures (3 Å and 4 Å, respectively), but it was clear that no ligand bound within the binding pocket.

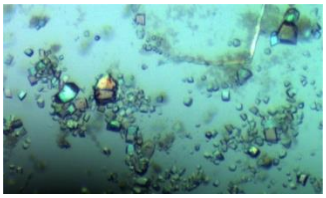


Figure 6 BshC Y510Q *The BshC Y510Q crystals were hanging in a Hampton Research Peg Ion II in 2mM glcNAc with 0.2 M ammonium citrate tribasic pH 7.0, 20% w/v Polyethylene glycol 3,350.*

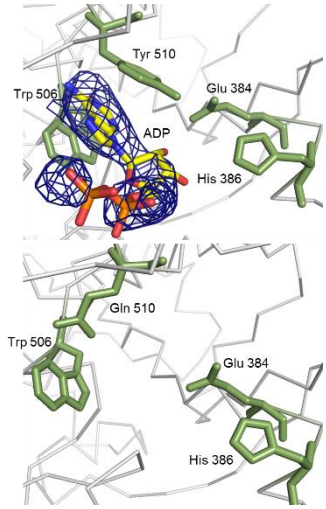


Figure 7 ADP Binding Pocket*: BshC WT (above) & BshC Y510Q (below)*

Conclusions and Future Work

The results from this project have given insight into how the amino acids around the active site and ADP binding site of BshC affects how the enzyme binds ATP. The fluorescence assay allows the inference that BshC WT binds to ATP more effectively than BshC Y510Q. The crystallographic analysis of BshC Y510Q further indicates the dramatic effect the site directed mutation has on the enzyme. We can understand from these two assays that the tyrosine in the ADP binding pocket plays an important role in ATP binding in the ADP binding pocket. There is now the question of how BshC WT binds with ATP within the ADP binding pocket, assuming

ADP is already bound there. There is also the question of how does this mutation affect the enzyme's ability to produce BSH.

Our future work will involve making three more mutations to the three other amino acids believed to be key in ADP binding: BshC W506L, BshC E384A, and BshC H386A. The mutants BshC W506L and BshC H386A have been successfully mutated and are in the process of being purified via nickel affinity chromatography. There is need to grow crystals of all four BshC mutants to obtain, hopefully, higher resolution structures. Performing more fluorescence assays on all four BshC mutants is required to gain more understanding as to how the mutations affect the ADP binding pocket through titrating with different compounds (eg. GTP). Through understanding the properties of these four amino acids and how they affect ATP binding we can gain information regarding how BSH is produced by this enzyme and the role of the ADP binding pocket. With a greater understanding of how this process works, our data can be used to develop and characterize inhibitors.

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