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Determining Function of Rv2173 in Biosynthesis of Menaquinone in Mycobacterium Tuberculosis

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Appendix C

RESEARCH / CREATIVE PROJECT ABSTRACT / EXECUTIVE SUMMARY
FINAL REPORT FORM

Title of Project
Determining Function of Rv2173c in Biosynthesis of Menaquinone in Mycobacterium tuberculosis.

Student Name Jessica Sempf

Faculty Sponsor Dr. Mann

Department Chemistry

Abstract

Mycobacterium tuberculosis, which is transmitted from human to human via respiratory droplets, is one of the leading killers among bacterial diseases in the human body (Center for Disease Control and Prevention, 2012). Although the cure for this disease is unknown, much advancement has been made in discovering one. A current focal point in research is how this bacterium produces ATP via menaquinone pathways, and how it is able to flourish even in stressful environments. We worked with a specific gene, Rv2173, which encodes for a product with an unknown chain length. It is believed that this product plays a role in the biosynthesis of menaquinone. Gas chromatography-mass spectrometry data indicated that the chain length of the product is 30 carbons long with a molecular weight of around 420. Discovering how menaquinone is synthesized can be useful because it can lead to the ability to target and inhibit synthesis, which Mycobacterium tuberculosis cannot survive without.

The end product of this project in electronic format has been submitted to the Provost/Vice President for Academic Affairs via the Office of Grants & Sponsored Projects Officer (Maxwell 161, npeterson@winona.edu).

Student Signature ______________________ Date ____________

Faculty Sponsor Signature ______________________ Date ____________

Determining Function of Rv2173 in Biosynthesis of Menaquinone in *Mycobacterium tuberculosis*

Jessica Sempf, Larissa Luhring, Kaila Kovac, and Francis M. Mann
Department of Chemistry
Winona State University

Abstract

Mycobacterium tuberculosis, which is transmitted from human to human via respiratory droplets, is one of the leading killers among bacterial diseases in the human body (Center for Disease Control and Prevention, 2012). Although the cure for this disease is unknown, many advancements have been made in the right direction . A current focal point in research is how this bacterium produces ATP via menaquinone pathways, and how it is able to flourish even in stressful environments. We worked with a specific gene, Rv2173, which encodes for a product with an unknown chain length. It is believed that this product plays a role in the biosynthesis of menaquinone. Gas chromatography-mass spectrometry data indicated that the chain length of the product is 30 carbons long with a molecular weight of around 420. Discovering how menaquinone is synthesized is useful because it can lead to the ability to target and inhibit synthesis, which would render *Mycobacterium tuberculosis* dead.

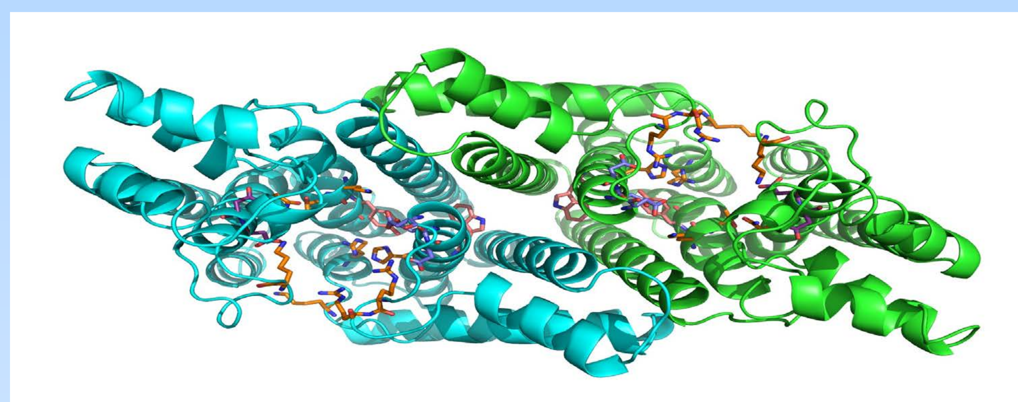


Figure 1: Isolated crystal structure of Rv2173 dimer (Courtesy of J.Johnston)

Introduction

Tuberculosis is a bacterial infection that generally affects the lungs, but can affect other organ systems as well (Center for Disease Control and Prevention, 2012). *Mycobacterium tuberculosis* is the group of bacterial cells that cause both the latent and active form of Tuberculosis disease in the human body. *Mycobacterium tuberculosis* is so dangerous because it can be aerosolized, meaning that the physical bacterial particles can be dispersed into air or gas. This bacterium can only be contracted if the bacterial droplets are inhaled, where they either lie dormant, the latent form, or begin to reproduce rapidly, the active form. The cases in which the bacterium becomes active only occurs in people who have compromised immune systems prior to coming in contact with the bacterial droplets.

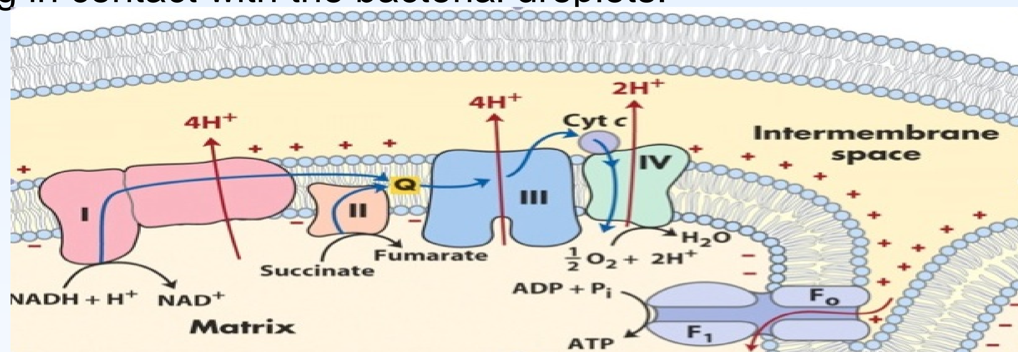


Figure 2: Pathway of oxidative phosphorylation including the utilization of quinones (Nelson & Cox 2005) .

Quinones, which are compounds that are chemically stable, are an important aspect in oxidative metabolism where they easily accommodate electrons. *Mycobacterium tuberculosis* uses oxidative phosphorylation to create ATP through electron transport chain. A key component in the production of ATP via this chain is the use of Menaquinone, the specific quinone for bacteria (As seen in Figure 2). An unclear aspect to this pathway is how *Mycobacterium tuberculosis* is able to create ATP successfully under any type of environment, including environments with low oxygen. Through the characterization of the specific gene, Rv2173, researchers can determine how this gene plays a role in the biosynthesis of Menaquinone.

Acknowledgements

- Winona State University for Student Research Grant to J.Sempf
- Winona State University Department of Chemistry for use of facilities
- Jodie Johnston, University of Aukland, Aukland, NZ for crystal structure

Methods

Protein Purification of Rv2173 in *E. coli* Cells

Transformation of the Rv2173 gene from *Mycobacterium tuberculosis* into *Escherichia coli* C41 cells. 25 µL of *E. coli* and 2 µL of the DNA were added to a tube and vortexed, then incubated on ice for 30 minutes. The mixture was heat shocked for 45 seconds using a 42˚ C heat block. The bacteria were fed 250 µL of NZY medium, consisting of 10 g NaCl, 10 g casein, 5 g yeast extract, and 1 g anhydrous $MgSO_4$ per liter. The tubes were shaken and incubated at 37˚ C for an hour and then plated on 50 mg/mL Carbenicillin. To induce protein expression from the cells, another bacterial transformation was performed into Lucigen C41 cells of *E. coli.* After growth on plates was achieved, one colony was inoculated into 5 mL NZY medium containing 50 mg/mL and grown overnight. Then 1mL of the suspension was inoculated in 50 mL NZY and grown for 2 hours. Additionally, 50 mL of NZY was inoculated to 500 mL NZY (50 mg/mL) and were grown to OD_{600} = 0.6 - 0.8. The suspension was then put on a shaker at 16˚ C . 0.5 mM IPTG was added to allow protein expression, and it was grown for 16-18 hours. The last step was to harvest by centrifugation at 5000 rpm.

To actually purify the protein, batch purification with Ni-NTA was performed under denaturing conditions according to manufacturer protocol. The first step was to add lysis buffer to our tube of cells to re-suspend, and then transfer the contents to a 50 mL centrifuge tube to vortex. A buffer containing 8M Urea was used. Then 3 mL of 50% Ni-NTA His-Bind slurry was added to the tube of lysate and put on a shaker for one hour. The lysate-resin mixture was then loaded into the column and the flow-through was collected in a centrifuge tube. The protein was then washed with 8 mL Buffer C and collected, followed by elution with 2 mL Buffer D and 2 mL Buffer E. The contents of the Buffer E wash was placed in a piece of 15 kD dialysis tubing (Spectrum Laboratories) and placed in a beaker containing 8M urea. Every 8 hours the tubing was moved to a decreasing concentration of urea: 6M, 4M, 2M, and 0M.

SDS-PAGE

Denaturing SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) was performed to separate the proteins according to individual size and charge. Lane 1 contained the marker, followed by lysate, flow-through, Buffer C wash, Buffer D wash, and Buffer E wash in lane 6. The system was run at 100 V, and the gel was removed and stained with Commassie Blue G-250.

Prenyltransferase Assay

There were four total assays, each containing a combination of IPP and another diphosphate, which was known as the substrate. Each assay also contained a phosphate buffer, $MgCl_2$, enzyme solution and distilled water. The first assay contained IPP and DMAPP, the second contained IPP and GPP, the third contained IPP and FPP and the fourth contained IPP and GGPP. The amount of diphosphate required was determined based on their individual molecular weights: 3.14 µL of GPP, 2.46 µL of IPP, 9.01 µL of GGPP and 2.46 µL of DMAPP. Each assay contained 0.01 mL of $MgCl_2$ and 100 µL of enzyme solution. Based on the different amounts of substrate that were water was added to make the total assay volume equal to 1 mL. Assay 1 contained 785.07 µL , assay 2 contained 784.4 µL , assay 3 contained 783.72 µL and assay 4 contained 778.52 µL of distilled water. At the designated time, the enzyme solution was added to the cocktail, vortexed and placed back in 37 C heat bath. After 30 minutes in the heat bath ~1 mL of 10% Methanol/1 M HCl was added to each assay and they were placed in a heat block at 95 C for 5 minutes. The samples were then ready to be analyzed by the GC-MS.

Prenyltransferase Assay Conditions and Results

Assay	Substrate 1	Substrate 2	Product m/z
1	IPP	DMAPP	412
2	IPP	GPP	412
3	IPP	FPP	412
4	IPP	GGPP	412

Methods

Protein Assay for Menaquinone Synthesis Analysis

A protein assay including 22 samples was made. Each tube contained a total volume of 1 mL with 100 µL of cells or protein. Varying amounts of buffer and different substrates, as demonstrated on chart, were added to each of the tubes. The samples were done in duplicate, so there were 44 tubes total. Odd numbered tubes contain cells, while even numbered tubes contain protein. The exact contents of each tube can be seen in Figure 1. Each substrate had a set amount whenever it was used; IPP = 2.46 µL, GPP = 3.14 µL, FPP = 3.82 µL, GGPP = 9.01 µL, and DMAPP = 2.46 µL. The tubes sat in a 30 C water bath for 30 minutes and then 100 µL of Alkaline Phosphatase was added to each tube.

Table 1: Substrate amounts for HPLC Assay

Sample	Buffer (µL)	Cells/Protein	S1	µL S1	S2	µL S2	S3	µL S3
12	880	100	DHNA	20	x	x	x	x
14	880	100	2HQ	20	x	x	x	x
16	870.99	100	DHNA	20	GGPP	9.01	x	x
18	870.99	100	2HQ	20	GGPP	9.01	x	x
20	888.53	100	IPP	2.46	GGPP	9.01	DHNA	20
22	888.53	100	IPP	2.46	GGPP	9.01	2HQ	20

HPLC Assay

Samples are analyzed by injecting 250 µL into the Shimadzu SPD-10AV-VP HPLC equipped with an octadecyl column (25 cm x 4.6 mm). Once the sample has been injected the run is started with ten minutes of 0% methanol at 0.200 mL/min. A ramp of 3%/min is used until 90% methanol is reached, then a ramp of 2%/min is used to 100% methanol. The run is reset by decreasing to 0% methanol for 10 minutes.

Results

Tagged purification of protein

Figure 4: SDS PAGE of tagged protein during purification.

Figure 5: SDS PAGE of tagged Protein during purification.

Figure 4 indicates a darker band stretch across the four lanes to the right. These were all samples from the buffer E wash, which was determined to be the most purified wash. Lane 1 to the left was the marker lane, followed by 2 washes with buffer C. As you can see, other proteins show on the buffer C lanes because they were not as purified as the Buffer E washes, which had our specifically tagged protein purified. Each lane on this gel contained 15 µLof sample.

Figure 5 shows the tagged purification of protein. Starting from the left is the marker lane. 5 lanes from the left shows a darker dyed spot around the middle of the gel; this lane contained 10 µLfrom Buffer E, which is the most purified. This gel also allows for identification of molecular weight by comparing to marker lane. The gel indicates that the protein of interest was successfully tagged.

Enzyme assay resulting in product molecular weight of 412 m/z.

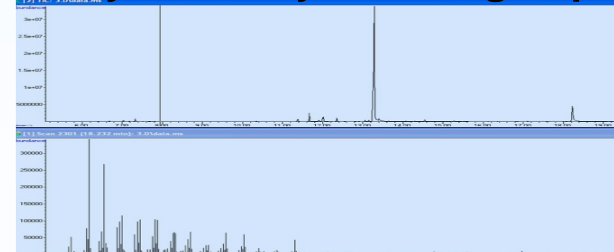


Figure 6: GC-MS analysis of prenyltransferase activity

The GC-MS spectra indicates a peak around a molecular weight of 415 m/z, which corresponds to a prenyltransferase product of 30 carbon atoms and one alcohol. However, as this product was observed regardless of the substrate available to the enzyme, it is questionable whether this is the true enzymatic product of Rv2173.

Results

HPLC Spectra

Figure 7: Menaquinone

Figure 8: DHNA & GGPP

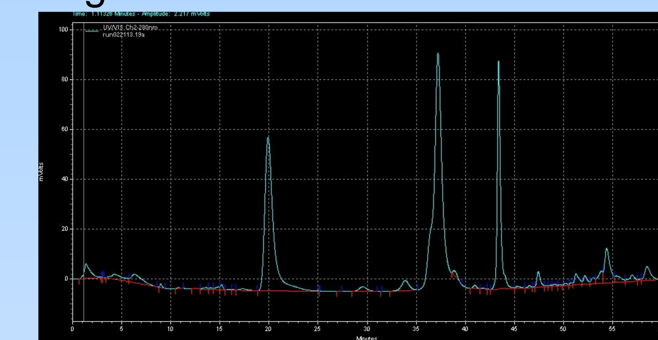
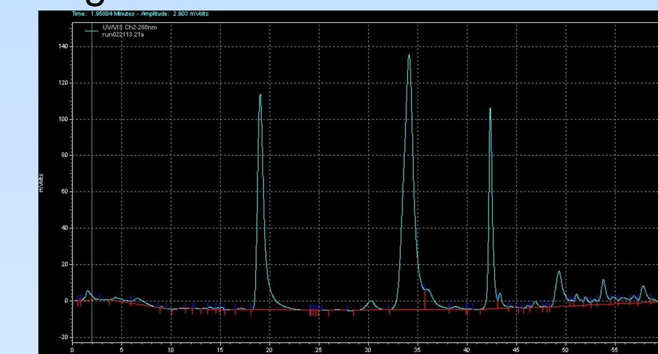


Figure 9: 2HQ & GGPP



HPLC Spectra allow for identification of products in a specific sample. Figure 6 indicates the spectra of menaquinone, the specific Quinone that *M. Tuberculosis* uses in its electron transport chain, shows a specific peak at about 53.8 minutes. Different substrates with similar structures to menaquinone (seen in Table 2) were combined to produce a product that includes menaquinone. Figure 7 indicates the spectrum of DHNA combined with GGPP, which produced a peak at 53.8 minutes, the same as menaquinone. Figure 8 indicates the spectrum of 2HQ and GGPP which also indicates a peak at approximately 53.8 minutes. Figures 7 & 8 show that there are different substrates that the bacteria may use in order to make the menaquinone necessary for its survival. This knowledge may help in the discovery of a drug which can inhibit the formation of the menaquinone therefore killing the bacteria.

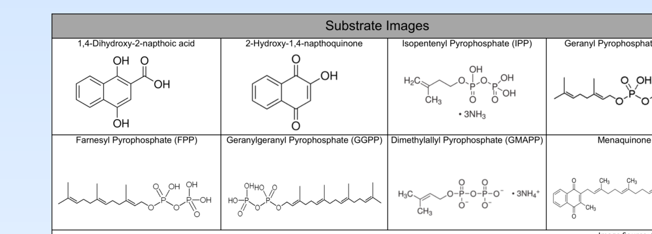


Table 2: Substrate names and structures

Conclusion & Future Directions

Conclusion:

- *M. tuberculosis* Rv2173 uses isoprenyl substrates to make a 30-carbon isoprenyl alcohol
- Rv2173 attaches GGPP to a napthaquinone ring structure to synthesize menaquinone-like product

Future Directions:

- Further purify product of 1,4-Dihydroxy-2-napthoic acid with GGPP and 2-Hydroxy-napthaquinone with GGPP and run HPLC method to determine if product is indeed like menaquinone
- Send purified product to a lab for a MALDI-TOF MS to be completed
- Research mechanism by which product is formed
- Research how a methyl bisphosphonate drug could inhibit synthesis of menaquinone

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