Mutagenesis-Based Active-Site Characterization of the Diabetes Drug Target MitoNEET

Lisa Mickley
lmm165@zips.uakron.edu, lmm165@zips.uakron.edu

Please take a moment to share how this work helps you through this survey. Your feedback will be important as we plan further development of our repository.

Follow this and additional works at: http://ideaexchange.uakron.edu/honors_research_projects

Part of the Analytical Chemistry Commons, Medicinal-Pharmaceutical Chemistry Commons, and the Other Chemistry Commons

Recommended Citation
http://ideaexchange.uakron.edu/honors_research_projects/278

This Honors Research Project is brought to you for free and open access by The Dr. Gary B. and Pamela S. Williams Honors College at IdeaExchange@UAkron, the institutional repository of The University of Akron in Akron, Ohio, USA. It has been accepted for inclusion in Honors Research Projects by an authorized administrator of IdeaExchange@UAkron. For more information, please contact mjon@uakron.edu, uapress@uakron.edu.
Mutagenesis-Based Active-Site Characterization of the Diabetes Drug Target MitoNEET

Lisa Mickley
Biochemistry Major (Department of Chemistry)
Project Sponsor: Dr. Thomas C. Leeper
Number of Credits: 4
April 21, 2016
Abstract: This project investigates the cluster stability of an outer mitochondrial membrane protein mitoNEET under mutational stress and drug binding analysis. MitoNEET is a part of a class of proteins that coordinate an Fe-S molecule into its tertiary structure. This protein is believed to facilitate the oxidative capacity of the electron transport chain by reversible loss of its coordinated Fe-S molecule. The research of this paper uses site directed protein mutagenesis to selectively alter the three cysteine, one histidine cluster coordination into more common coordination patterns such as two cysteine, two histidine or four cysteine. The ultimate goal is to optimize conditions for future research and analysis by UV-Vis spectrometry. Research led to the conclusion that optimal conditions for kinetic analysis under acidic conditions were in a pH 5.5 20 mM citric acid reaction buffer with 100 mM NaCl and 5% DMSO. To the reaction buffer 100 μM protein and 100 μM drug were added. This reaction was carried out using the Shimadzu Spectra apparatus (λ = 459 nm) and the reaction cuvettes were temperature controlled by a water bath at 27°C to yield complete cluster loss within 6 hours.

Introduction

MitoNEET is a 17-kDa protein containing 106 amino acid residues found on the outer mitochondrial membrane (OMM). It was discovered in 2004 by Colca, et. al while investigating the side effects of the diabetes drug pioglitazone (Colca, et al. 2004). It is anchored to the OMM by its amino terminus leaving its carboxy terminus oriented toward the cytoplasm (Wiley, Murphy, et al. 2007). As identified by Paddock, et. al in 2007 there are two distinct regions of the mitoNEET homodimer – the β-rich domain, which was
coined the β-cap, and the helical 2Fe-2S binding, or cluster binding, domain (Paddock, et al. 2007). Its two protomers are interrelated by an unusual β strand swap that forces contacts between residues on opposing protomers and increases cooperativity (Baxter, Jennings and Onuchi 2011). According to research done by Baxter, et. al in 2012, the protein’s strand swap causes cooperative folding and assembly and results in dimerization preceding final folding (Baxter, Jennings and Onuchic 2012).

This protein has sparked researchers’ curiosity through the unique coordination of its iron-sulfur cluster. Iron sulfur cluster containing proteins using two iron atoms and two sulfur atoms (Fe₂S₂) are most commonly found with two coordination patterns. The Rieske centers are characterized by a two cysteine, two histidine coordination. Comparatively the ferredoxin family of iron-sulfur proteins is characterized by a coordination complex of four cysteines. The cluster coordination in mitoNEET (mNT) could be considered an intermediate between the two most commonly known coordinations, utilizing a three cysteines and one histidine complex coordination, and has led to the discovery of a new class of CDGSH Fe-S domain proteins (Lin, et al. 2007) (Wiley, Paddock, et al. 2007).

Figure 1: Structural representation of mitoNEET; data obtained from the Protein Data Bank (PDB ID 2qh7). This figure shows the distinctive features of the protein of interest – its strand swapping, β-cap, cluster binding domain, and cluster coordination.
The coordinating histidine residue has been shown to be critically important during redox chemistry of the protein's iron-sulfur cluster. A simple mutation of the residue from His to Cys decreases the redox potential ($E_m$) by more than 300 mV, increasing the stability of the cluster (Conlan, et al. 2011) (Bak and Elliot 2013). Though the mutational stresses of this mutation on cluster stability have been well studied, the stresses introduced by altering the remaining coordinating residues has yet to be determined. Therefore, testing the stability of the cluster under mutational stress shows investigative promise. Studying these effects requires a library of mNT active site mutants such as those listed in Table 1. With a library such as this, comparisons can be made across each unique coordination pattern within mNT. Independent of mutational stress the redox potential has also been found to be sensitive to variation in pH with stability decreasing at pH values below 8 (Bak, Zuris, et al. 2009) (Zuris, et al. 2011).

The focus of my project was to investigate the stability of mitoNEET's iron-sulfur cluster in mildly acidic conditions and its rate loss over time. MitoNEET has been implicated as a mitochondrial Fe-S trafficking protein and understanding rate kinetics of its redox potential is essential to the advancement in our understanding of this diabetes drug target (Wiley, Murphy, et al. 2007) (Lin, et al. 2007). Unique to many other proteins the iron cluster of this homodimeric protein causes mitoNEET to take on a red color in its holo-

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Cluster Coordination</th>
<th>Familiar Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>C72, C74, C83, H87</td>
<td>mitoNEET (CDGSH)</td>
</tr>
<tr>
<td>H87C</td>
<td>C72, C74, C83, C87</td>
<td>ferredoxin</td>
</tr>
<tr>
<td>C72H</td>
<td>H72, C74, C83, H87</td>
<td>Rieske</td>
</tr>
<tr>
<td>C72H, H87C</td>
<td>H72, C74, C83, C87</td>
<td>mitoNEET (CDGSH)</td>
</tr>
<tr>
<td>C74H</td>
<td>C72, H74, C83, H87</td>
<td>Rieske</td>
</tr>
<tr>
<td>C74H, H87C</td>
<td>C72, H74, C83, C87</td>
<td>mitoNEET (CDGSH)</td>
</tr>
<tr>
<td>C83H</td>
<td>C72, C74, H83, H87</td>
<td>Rieske</td>
</tr>
<tr>
<td>C83H, H87C</td>
<td>C72, C74, H83, C87</td>
<td>mitoNEET (CDGSH)</td>
</tr>
</tbody>
</table>

Table 1: A list of all the active site mutants and which coordination pattern they follow post directed mutagenesis.
form. Because of this property, the loss of its iron-cluster can be monitored over the course of time using UV-Vis spectroscopy. As the protein begins to lose its iron to the solution, its color fades and the decrease in absorbance can be correlated to an increase in the concentration of the apo-form in solution (Lin, et al. 2007). Comparing absorbance curves we are able to understand relative stabilities of the mutant library.

Another way that the stability of the cluster can be altered is by adding a drug that will target mNT to the reaction solution. This can be used in addition to protein mutagenesis to provide a broader scope to investigative results. MitoNEET was discovered by researchers while investigating the effects of pioglitazone that could not be explained via PPAR-γ (Geldenhuys, Leeper and Carrol 2014) (Rizos, et al. 2009). As previously mentioned, pioglitazone is a diabetes drug commonly used today to treat type 2 diabetes, along with diet and exercise. This drug is a part of the thiazolidinedione class of medications that work to increase the body’s sensitivity to insulin by enhancing oxidative capacity and normalizing lipid metabolism (Paddock, et al. 2007). Previous to the discovery of mNT, the mechanism by which pioglitazone enhanced oxidative capacity had yet to be determined. As a protein of the OMM involved in electron transfer and associated with the activity of complex I of the cellular respiratory chain, binding of pioglitazone to this protein effects oxidative capacity by stabilizing its cluster coordination and therefore decreases oxidative stress (Wiley, Murphy, et al. 2007) (Brunmair, et al. 2004). Since the discovery of mNT in 2004 via this drug, pioglitazone has been used repeatedly to study the properties of mNT and therefore can be a means by which to validate novel technique and results.
obtained from such techniques. Though it has become a standard drug used to study mNT, its limited solubility in water of only 0.00442 mg/mL presents challenges to studying proteins, such as mNT, that are water soluble (Bieganski and Yarmush 2011) (Wishart, et al. 2006). Because of this limitation and the stabilizing effects DMSO had on cluster stability, we began to search for an alternative drug. Dr. Geldenhuys of NEOMED has recently begun to use nitrofurantoin (nF) to study mNT – a novel analysis. Nitrofurantoin with a water solubility of 0.415 mg/mL became an appealing alternative to pioglitazone (Wishart, et al. 2006). Nitrofurantoin is an antibiotic commonly used to treat urinary tract infections providing a reasonable explanation for its increased water solubility (American Society of Health-System Pharmacists 2010). Though this drug minimized the need for DMSO in solution, it did not come without its own set of complications. Nitrofurantoin has a characteristic yellow coloration which seemed to interfere with absorbance at 459 nm though previous publications did not indicate this may be the case (Conklin and Hollifield 1965).

Figure 2: This figure shows the chemical structure of both drug compounds. Highlighted in red is their structural similarity and possible interaction site with mNT.

As investigations for this project evolved, the focus of the assay turned more toward drug analysis and away from mutational stress due to available investigative resources, not all mutants were successfully synthesized. This paper details the investigative process toward the final results and the motivations that led toward those results.
**Materials and Methods**

**Primer Design / PCR:** For each of the desired mitoNEET mutants Agilent QuickChange primer design was used to design appropriate primers for the mutants of interest (H87C, C83H, C72H, C74H, H87C C83H, H87C C72H, and H87C C74H). The ExPASy ProtParam web tool was used on each mutant strain to evaluate the predicted effects these mutations would have on the molecular weight, pl, extinction coefficient, and E. coli half-life. Finally, primers were ordered from Integrated DNA Technologies (Coralville, IA). After primers arrived, PCR reactions were run in microcentrifuge tubes for each mutant of interest. To the tube appropriate amounts of ddH₂O, reaction buffer, plasmid DNA template, sense and anti-sense primers, dNTP mix, and PfuTurbo DNA polymerase were added to the microcentrifuge tube (see table 2).

The reaction mixture was then thermocycled using a PCR program for appropriate amplification cycling through sequential temperatures of 95°C, 60°C, and 68°C respectively. After PCR amplification,

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sterile H₂O</td>
<td>36.5</td>
<td>NA</td>
</tr>
<tr>
<td>10x rxn buffer</td>
<td>5</td>
<td>1x</td>
</tr>
<tr>
<td>Template DNA</td>
<td>1</td>
<td>10 ng/µL</td>
</tr>
<tr>
<td>Sense Primer</td>
<td>1.25</td>
<td>100 ng/µL</td>
</tr>
<tr>
<td>Anti-sense Primer</td>
<td>1.25</td>
<td>100 ng/µL</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Quick Solution</td>
<td>3</td>
<td>NA</td>
</tr>
<tr>
<td>PfuTurbo DNA Polymerase</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2:** PCR reaction conditions.

<table>
<thead>
<tr>
<th>Cycle Step</th>
<th>Number of Cycles</th>
<th>Temperature (°C)</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial Denaturation</td>
<td>1</td>
<td>95</td>
<td>1 min</td>
</tr>
<tr>
<td>Replication</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a) Denaturing</td>
<td></td>
<td>95</td>
<td>50 sec</td>
</tr>
<tr>
<td>b) Annealing</td>
<td></td>
<td>60</td>
<td>50 sec</td>
</tr>
<tr>
<td>c) Extension</td>
<td></td>
<td>68</td>
<td>4.5 min</td>
</tr>
<tr>
<td>Final Extension</td>
<td>1</td>
<td>68</td>
<td>7 min</td>
</tr>
<tr>
<td>Hold</td>
<td>1</td>
<td>4</td>
<td>∞</td>
</tr>
</tbody>
</table>

**Table 3:** Thermal Cycler temperature and run settings.
1 µL of Dpn1 restriction enzyme was added to the mixture and incubated in a 37°C water bath for an hour.

**Plasmid Propagation, or Transformation of XL10-Gold Cells:** Protocol was taken from QuickChange II XL Site-Directed Mutagenesis Kit instruction manual (Agilent Technologies 2013). XL10-Gold ultracompetent cells were thawed on ice and 45 µL aliquots were added to prechilled 15 mL falcon tubes. β-ME mix (2 µL) was added to the aliquots and contents were incubated on ice for 10 minutes with gentle swirling every 2 minutes. The Dpn1-treated DNA (2 µL) were then transferred into the aliquots of ultracompetent cells and heat pulsed in a 42°C water bath for 30 seconds before a 2 minute incubation on ice. Preheated NZY+ broth (0.5 mL) was then added to each tube for an hour incubation at 37°C with shaking at 225-250 rpm. Post incubation the transformation was plated onto LB-ampicillin agar plates and incubated at 37°C overnight.

**DNA plasmid miniprep:** A single colony was isolated from the XL10-Gold transformation plates to inoculate a culture of 5 mL LB-ampicillin. The culture was then incubated at 37°C for 12-16 hours with shaking at 260 rpm. In 1.5 mL microcentrifuge tubes, bacterial cells were pelleted by centrifugation at room temperature and 10,000xg for 1 min. The bacterial pellet was then resuspended in 250 µL of solution I/RNase A solution, mixed by pipetting, and transferred to a new 1.5 mL microcentrifuge tube. Solution II (250 µL) was then added to the new microcentrifuge tube and gently mixed by inversion to obtain a clear lysate. Solution III (350 µL) was added and mixed, again by inversion, to form a white flocculent precipitate. During a 10 minute room temperature centrifugation at 13,000xg the HiBind
DNA Miniprep Column was prepared by adding 100 μL of equilibration buffer and the centrifuged in a 2 mL collection tube for 1 minute at 13,000xg. Post the 10 minute centrifugation, the cleared supernatant was poured into the HiBind column without disturbing the pellet. The lysate was passed through the column by centrifugation at 13,000xg for 1 minute and the flow through was discarded. The next step was repeated twice; 700 μL of DNA wash buffer was added to the column and centrifuged at 13,000xg for 1 minute. To dry the column matrix the empty column was then centrifuged at 13,000xg for 2 minutes. The column was then placed in a clean 1.5 mL microfuge tube and 50 μL of elution buffer was added directly onto the column matrix and centrifuged for 1 minute at 13,000xg. Yield and quality of DNA were obtained by reading UV absorbance at 260 nm and 280 nm.

**Transformation of C43 DE3 Cells:** Transformation of the C43 DE3 cells was similar to that of the XL10-Gold but with some modifications to optimize transformation for these cells. Miniprepped plasmid DNA (50 ng) was added to 50 μL of the thawed C43 DE3 cells on ice and gently stirred with a pipette tip before a 30 minute incubation. After incubation, the cells were heat shocked in a 42°C water bath for 45 seconds. Following heat shock the cells were returned to ice for 2 minutes. Room temperature expression recovery medium (950 μL) was added to the solution mixture before being placed in the shaker for one hour at 250 rpm and 37°C. The transformed cells were then plated in two volumes onto ampicillin-LB agar plates to ensure variation in colony density and incubated overnight at 37°C.
**Protein Expression:** Transformed C43 DE3 cells were used to inoculate an overnight culture in 25 mL of 2xYT medium. After incubation, a Fernbach flask containing 500 mL of the same medium was inoculated with the 25 mL overnight. Optical density at 600nm (OD$_{600}$) was monitored and cultures were supplemented with 1 μM Fe(III)Cl$_3$ at an OD$_{600}$ of 0.2. Growth was once again monitored and temperature was decreased to 29°C once an OD$_{600}$ between 0.6 and 0.7 was obtained so as to allow the cells to adjust to the final induced growth temperature change before introducing them to IPTG. At an OD$_{600}$ of 0.8, IPTG was added to a final concentration of 0.5mM to induce mitoNEET expression. After the addition of IPTG the culture was left to inoculate for 10 hours before being centrifuged at 10,000 xg for 8 minutes and pelleted. Pellets were stored overnight at 4°C.

**Protein Purification:** Pelleted cells were resuspended in 25 mL of buffer A (50 mM Tris, 300 mM NaCl, 20 mM imidazole; pH 8.5). Cell lysis was accomplished via French pressure cell, taking advantage of the cell’s inability to appropriately adjust to rapid decreases in pressure. Lysed cells were centrifuged for 15 min at 14,000xg and 4°C. The supernatant was retained for further purification using immobilized metal affinity chromatography (IMAC). These proteins were designed to contain a 6xHis-tag that will bind nickel and can be utilized for purification. Columns used for the purification of these proteins were HisTrap (General Electric) columns and were stored in 20% v/v ethanol. Since ethanol solutions can denature any protein the columns had to be prepped with consecutive washes of water, buffer A, buffer B, and finally buffer A again. After this preparatory sequence the protein sample was injected into the column and washed with buffer A. With the His-tag, elution of the protein from the column required a gradient addition of buffer B.
(50 mM Tris, 300 mM NaCl, 600 mM imidazole; pH 8.5) taking advantage of imidazole’s higher affinity for the Nickel column than a 6xHis-tag. The eluted protein was collected into fractional tubes and pooled for dialysis. The column was washed with water before storage in ethanol. The protein was then dialyzed overnight in 50 mM Tris, 300 mM NaCl pH 8.5 with thrombin added for cleavage of the His-tag. The cleaved protein was once again run through the AKTA with the His-trap column however without the tag, fractions were collected immediately upon injection. Depending on the volume of pooled fractions, a concentration step using the spin column was sometimes required between AKTA runs. After AKTA purification the protein samples were concentrated using the spin column and stored at 4°C for future experimentation.

**Acrylamide Protein Gels:** Throughout protein purification 20 μL aliquots were removed as a means to test efficacy of purification. Post complete purification these aliquots were analyzed on analytical gels. Equal volume of 2xSDS-10% β-mercaptoethanol loading buffer containing Omni-Pure Coomassie® R250 brilliant blue dye (EMD Chemicals, Gibbstown, NJ) was added to the 20 μL aliquots and the samples were placed on the heat block at 95°C for 5 minutes. After removal from the heat block the samples were agitated via washboarding technique along the test tube rack before a brief centrifugation at maximum speed. The Bio-Rad Mini-PROTEAN® Tetra Electrophoresis system was then assembled and prepared using 1x Tris, Glycine, SDS running buffer and 10 μL of sample were loaded into each of 15 wells. Prestained protein ladder (5 μL) was added to the remaining well and the gel was run at 200 V for approximately 30 min, or until there was sufficient separation. Post run the gel was carefully removed and placed in a gel box, rinsed with water, and covered
sufficiently with blue-silver dye stain. After sufficient staining the gels were destained with water and viewed in the lightbox.

**Fe-S Stability Assays:** Purified and concentrated protein mutants were tested for Fe-S cluster stability via two experimental techniques, both similar in methodology however differing in the number of conditions that can be tested in a given trial.

*Protocol 1:* 96 well plate assays with a total volume of 200 µL per well were used to compare Fe-S stability across the mitoNEET mutants. Optimal conditions for the 96 well plates were determined to be 13.75% DMSO, 80 µM mNT in pH 5.5 Fe-S buffer (20 mM CA, 100 mM NaCl) for pioglitazone drug analysis. Drug analysis with nitrofurantoin only required 2% DMSO in solution. Due to the inherent red color of the holo form of mNT’s homodimer, cluster loss could be monitored by UV-Vis Spectroscopy (λ = 458 nm) for a period of 18-26 hours, depending on the mutant of interest, at ambient temperature. Data collected was processed and analyzed using Microsoft Excel.

*Protocol 2:* The Shimadzu spectrometer was also used to measure absorbance loss of the mitoNEET mutants over time in a mildly acidic solution. Purified and concentrated protein solutions stored in buffer A were added to a Fe-S buffer (20 mM citric acid, 100 mM NaCl; pH 5.75) and 5% DMSO solution for cuvette analysis. Absorbance was measured at 459 nm, the temperature was set to a standard of 27°C, and the experiment was set to run on kinetics from between 10-18 hours depending on the mutant of interest. Post completion the data was collected and analyzed in Microsoft Excel.
Results and Discussion

Research is a collaborative effort with many individuals understanding the logistics of a variety of techniques however over time becoming more specialized and capable in specific areas. Due to the nature and focus of my project, I had limited experience with Primer Design, PCR, DNA miniprep, and transformation. I did have broader exposure to protein expression and purification; however, the majority of my experience spent in the laboratory was directed toward optimization of monitoring Fe-S cluster loss of mNT mutants in acidic solution via UV-Vis spectroscopy. These results will elaborate on my methodology of optimization.

Though the original objective for these kinetic assays was to compare relative stability of the cluster mutants, Table 4 shows why the focus and investigative purpose quickly changed. In order to successfully compare mutant stability, the mutants must be available. After months of research and systematically working through the synthesis of each mutant unfortunately, the only mutants successfully synthesized were WT, H87C, C83H, and C83H H87C. With this limited library from which to work it was decided that drug studies would be a great compliment to the available mutants. It is for this reason that

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Expression</th>
<th>Purification</th>
<th>Stock Concentration (≥ 2mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>complete</td>
<td>complete</td>
<td>1.75 mM</td>
</tr>
<tr>
<td>H87C</td>
<td>complete</td>
<td>complete</td>
<td>0.656 mM</td>
</tr>
<tr>
<td>C72H</td>
<td>complete</td>
<td>incomplete</td>
<td>NA; colorless after AKTA 2</td>
</tr>
<tr>
<td>C72H, H87C</td>
<td>complete</td>
<td>incomplete</td>
<td>NA; colorless after AKTA 2</td>
</tr>
<tr>
<td>C74H</td>
<td>complete</td>
<td>incomplete</td>
<td>NA; colorless after AKTA 2</td>
</tr>
<tr>
<td>C74H, H87C</td>
<td>complete</td>
<td>complete</td>
<td></td>
</tr>
<tr>
<td>C83H</td>
<td>complete</td>
<td>complete</td>
<td>0.467 mM</td>
</tr>
<tr>
<td>C83H, H87C</td>
<td>complete</td>
<td>complete</td>
<td>0.554 mM</td>
</tr>
</tbody>
</table>

Table 4: A summary of mutant protein synthesis results.
two drugs – pioglitazone and nitrofurantoin – became a prominent part of my research. Table 4 provides a general overview of the results of synthesis; however, Figure 3 can be utilized to compare relative purities of these four samples.

Previous to my time spent in the lab, publications concerning mNT had indicated that a pH of less than 8 would destabilize the coordination of mitoNEET's cluster; however, the appropriate buffer was yet to be determined (Bak, Zuris, et al. 2009) (Zuris, et al. 2011). Determination of optimal conditions began by testing a range of acidic pH buffers for both

![Figure 3](image)

**Figure 3:** Analytical protein gels run for each synthesized mutant. A) C83H cleaved protein, concentrated flow through, uncleaved; LADDER; H87C C83H crude lysate, concentrated flow through, fraction 6, uncleaved B) C72H crude lysate; H87C C72H flow through, fraction 2; LADDER; C72H cleaved, fractions 2-4, flow through; H87C C72H crude lysate C) LADDER; C74H cleaved, uncleaved, flow through, fraction 7 uncleaved, crude lysate D) LADDER; H87C resuspended, crude lysate, flow through, fractions 2-6 uncleaved, cleaved
Tris and citric acid to determine which would be the most favorable for further studies.

Results can be seen in Figure 4. These data show that at a pH of 5.5 in a 20 mM citric acid (CA) buffer with 100 mM NaCl the 2Fe-2S cluster is destabilized over a reasonable time scale with complete loss occurring before 400 min.

Figure 4: In the kinetic assay above absorbance values ($\lambda = 458$ nm) were collected every 10 min for 18 hours. Conditions for each curve can be determined using the key to the right of the graph. Cluster loss of 40 $\mu$M mNT occurred most effectively in the pH 5.5 20 mM CA buffer with 100 mM NaCl. Data Collected 10-16-2014.

These results gave an appropriate starting point for which to design future drug studies. Since pioglitazone had already been proven to significantly stabilize the 2Fe-2S cluster of WT mNT and was available for our use, experiments were run with pioglitazone to verify the accuracy and robustness of our conditions. In Figure 7 we see that in a 2:1
Figure 7: In the kinetic assay above absorbance values ($\lambda = 458$ nm) were collected every 2 minutes over the course of 18 hours. All sample conditions were run in a 20 mM CA buffer with 100 mM NaCl of varying pH with a standard 5% DMSO and [pioglitazone]:[mNT] ratios varying from 1:1 to 1:2. Data Collected 10-21-2014.

Figure 6: Conditions for the above graph are the same as those listed in Figure 7 however data graphed is restricted to pH 5.5. Data Collected 10-21-2014.

Figure 5: Conditions for the above graph are the same as those enumerated in Figure 7 however data graphed is restricted to pH 5.75. Data Collected 10-21-2014.
ratio (pioglitazone:mitoNEET) the protein’s 2Fe-2S cluster is visibly stabilized. Highlighting specific sets of results, in Figures 5 and 6 only curves of comparable conditions are shown. These results seem to confirm previous studies, showing great promise as we began to proceed.

Though results were promising, reevaluating calculations and procedure it became apparent that the drug wells possessed a subtle DMSO gradient. To verify results were not from the DMSO gradient, a range of DMSO percentages were prepared and run. The results from the 5-25% gradient can be viewed in Figure 8, which compares the relative stabilization of 15%, 20%, and 25% DMSO in solution. From the graph it becomes apparent

Figure 8: In the kinetic assay above absorbance values (λ = 458 nm) were collected every 2 minutes over the course of 18 hours. All sample conditions were run in a pH 5.5 20 mM CA buffer with 100 mM NaCl across a DMSO percentage gradient of 5-25%. Data Collected 10-28-2014.
that at 20% DMSO in solution, the stabilizing effects of DMSO begin to overshadow those of pioglitazone. At 10% however, pioglitazone continues to be the dominant player. These conclusions are drawn from the fact that pioglitazone will bind each monomer of mNT to stabilize its active cluster. In solution at the same concentration of protein – if all pioglitazone is bound to mNT – only half the protein’s potential binding sites are occupied and its active cluster will only be minimally stabilized. At double the concentration of mNT, all sites will be occupied and therefore we should see the full stabilizing effects of pioglitazone. At percentages of 20% or more DMSO in solution, pioglitazone in a 1:1 concentration ratio to mNT already displays noticeable stabilization – more than we would expect – and doubling the drug concentration doesn’t seem to provide additional stabilization. To study the effects of pioglitazone stabilization DMSO percent in solution therefore must be limited to less than twenty percent. Figure 9 shows the stabilizing effect of DMSO in solution. This experimental trial unfortunately confirmed suspicions that in the absence of

Figure 9: The kinetic assay above aims to demonstrate the stabilizing effects of DMSO in solution for 40 μM mNT w/o pioglitazone. Data Collected 10-28-2014.
drug, DMSO has a stabilizing effect on the 2Fe-2S cluster of mitoNEET. In order to perform drug studies with pioglitazone, DMSO must be in solution to solubilize the drug; however, too much will give us a false positive and hinder accurate interpretation of results.

Narrowing the percentage range to between 5% and 15% DMSO yielded similar results confirming the stabilizing effects of DMSO under acidic conditions (Figure 10). A minimum limit of 10% DMSO in solution was discovered to be necessary for complete dissolution of pioglitazone into the reaction solution. So as to reduce the background stabilization by DMSO and allow the stabilizing effects of pioglitazone to predominate, the working concentration for mitoNEET was doubled to yield results in Figure 11.
The results above led to the conclusion that optimal conditions for the assay of interest were at 13.75% DMSO, 80 μM mNT, and room temperature run for 24 hours in the pH 5.5 Fe-S buffer (20 mM CA with 100 mM NaCl). Though running these experiments at room temperature presents a comparative roadblock when trying to explain clinical significance, heating the plate through the length of the assay caused evaporation and/or condensation effects that prevented any useful data from being obtained. Experiments were therefore run at ambient temperature.

**Figure 11:** For the kinetic assay above the reaction was run in a pH 5.5 20 mM CA buffer with 100 mM NaCl for 24 hours with absorbance values (λ = 458 nm) taken every 2 min. DMSO percentages in solution ranged from 10% to 15% and mitoNEET concentrations were increased to 80 μM. Data Collected 11-18-2014.
Concerned that the drug studies with pioglitazone and its DMSO requirements presented too many confounding variables, drug analysis with pioglitazone halted and focus was redirected toward nF, a drug with a much higher water solubility. Similar to pioglitazone, we notice a stabilizing effect for nF on the 2Fe-2S cluster of mNT. Results using the 96 well plates allowed for effective comparison of conditions during optimization; however, temperature control was limited and heating from the bottom of the plate led to significant condensation and/or evaporative effects. Though cuvette assays

**Figure 12**: This kinetic assay was run with nF (nitrofurantoin) instead of pioglitazone. Notice the required percentage of DMSO in solution is much lower for nF solubility. The assay above reveals that nF similar to pioglitazone has a stabilizing effect on the 2Fe-2S cluster of mNT. Also notice the spike in absorbance with the addition of nF even though its absorbance spectra is believed to fall outside the range of 459 nm. Nitrofurantoin concentrations were 125 µM for this trial. Data collected 4-29-2015.
limit the number of conditions that can be tested during an individual trial, or experiment, to one, the remaining data was collected in this manner due to more even temperature control. Results of similar conditions, therefore were compared across consecutive days.

In Figure 13 we see the promising results of cuvette analysis. Run under the same conditions WT mNT in solitude, and in the presence of both pioglitazone and nF respectively, will lose its cluster completely prior to 6 hours into the assay. This time scale is a reasonable period of measure for cluster loss. Due to the limitations on time and complete pioglitazone solubility in 5% DMSO at concentrations greater than 100 μM Figure 13 is not completely representative of potential results from this assay. Pioglitazone is

![Fe-S Cluster Loss: Cuvette Analysis](image)

**Figure 13:** These assays were run on consecutive days under identical conditions using the Shimadzu UV-Vis Spectrometer. Each assay was run for 18 hours using 100 μM mNT in a pH 5.5 Fe-S 20 mM CA buffer with 100 mM NaCl.
known to bind to each monomer of mNT and therefore for an accurate representation its Fe-S cluster stabilization should be in solution as such. It is unknown as to whether or not nF behaves in this way as well; however, I believe treating it in the same manner has merit.

Conclusion

Though the original focus of this project may have been to compare relative cluster stability of mNT active site mutants in an acidic buffer, the results of protein synthesis and purification led the project in a different direction. Focused on optimizing conditions to quantify, compare, and analyze 2Fe-2S cluster loss of mNT over a reasonable time-scale, results are as follows. Using a water bath temperature control (27°C) for cuvette analysis via UV-Vis spectroscopy, complete absorbance decay of WT mNT in pure solution and in the presence of pioglitazone and nitrofurantoin will occur within the first 6 hours or 360 minutes of the assay. Within the cuvette both protein and drug are dissolved in a pH 5.5 Fe-S buffer (20 mM CA with 100 mM NaCl) and 5% DMSO. Protein and drug were each present at a concentration of 100 μM in solution.

As a means of continuing the work I have started but unfortunately did not have the time to finish, cuvette assays could be run with double the drug concentration to the concentration of protein so as to gain further insight on the true effects of both pioglitazone and nitrofurantoin.
References


Appendix

General Safety Considerations

Laboratory protective equipment and clothing were worn at all times, including safety glasses and latex or nitrile gloves. When necessary, a standard laboratory coat was also considered. Long pants and close-toed shoes were worn every day. When performing hazardous activities, such as operating the French pressure cell or handling carcinogens, an authority figure was informed. Insulated gloves were worn when handling superheated or super cooled glassware and reagents. All broken glassware or sharps were disposed of in the appropriate bins.

Handling of Biohazardous Materials

When coming into contact with bacteria and cells, special care was taken to avoid unwanted spreading of living organisms. Pipette tips used to transfer liquid containing cell media were immediately disposed of into designated biohazard pipette tip boxes. Other containers holding such media were cleaned with dilute bleach and disposed of into the lab’s red biohazard waste containers. When appropriate, a Bunsen burner was kept on to destroy aerosolized cells in the air.

Handling of Chemicals and Reagents

All material and safety data sheets (MSDS) are kept in the lab. These resources were consulted before use for any potentially dangerous chemicals. Most of the chemicals in the lab are minor skin and orifice irritants and gloves were worn at all times when handling them. Particularity dangerous chemicals, such as ethidium bromide – a carcinogen and
DNA mutagen – were handled with extreme care and under supervision. Long term exposure to such chemicals were kept to an absolute minimum.

**Instrument Operation**

No pieces of instrumentation were operated without clearance and proper training on its use by an authority figure. Instruments, such as the high-speed centrifuge and French pressure cell, were operated only while under authoritative supervision.