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Akshitha Adhiyaman April 2, 2020

The Structural and Biochemical Studies of the Thioesterase Domains of Them1

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Obesity is an overwhelmingly large health issue across the world and we desperately need new ways to battle this epidemic. Brown adipose tissue can potentially be utilized to treat obesity due to its high capacity to burn fat to drive non-shivering thermogenesis. Thioesterase superfamily member 1 (Them1) is highly expressed in brown adipose tissue and suppresses thermogenesis through hydrolyzing acyl-CoA, antagonizing lipid metabolism. Mice lacking Them1 are protected against diet-induced obesity and metabolic disorders; therefore, inhibitors of Them1 are highly desired. There is currently no structure of the thioesterase domains of Them1 and the enzymatic mechanism of the enzyme remains unknown. In this study, we elucidated the mechanism by which Them1 hydrolyzes acyl-CoA using mutagenesis, and made progress in determining the structure of Them1's thioesterase domains. Collectively, this work advances us one step closer to pharmacologically targeting Them1 to counter obesity and its related disorders.

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I would like to thank Dr. Eric Ortlund for not only providing me with the opportunity to work in his lab, but also for all his support and guidance over the past two years. I would also like to thank Matthew Tillman for his unconditional encouragement and persistent teaching throughout my time in the lab. Finally, I would like to thank Dr. Megan Cole and Dr. Matthew Weinschenk for being great professors and mentors to me throughout my college career.

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Chapter 1: Introduction

The obesity epidemic is an increasingly concerning issue that affects roughly 107 million adults and children across the United States of America [1]. Even though more attention and information has been brought to the public eye about obesity, the problem is not diminishing. Obesity affects women, minority races, and lower socioeconomic classes at much higher rates, making this a compounded issue [2]. Obesity also can further progress health conditions like type 2 diabetes, heart disease, high blood pressure, sleep apnea, and much more [3]. Altogether, obesity can lead to poorer quality of life and be damaging to one's health. The most typical form of treatment for obesity is encouraging healthy lifestyle changes like incorporating exercise into one's daily routine or eating balanced meals; however, these are difficult to implement, especially in lower socioeconomic classes with limited financial resources and leisure time. Therefore, we need to find novel ways to treat obesity.

Excitement has developed around utilizing brown adipose tissue (BAT) as a potential therapeutic target towards treating obesity and its related metabolic disorders. BAT was originally only thought to be found in newborns and infants. Adults can produce heat through the process of shivering, but babies cannot do that due to the lack of development of their skeletal structures. They need prolific amounts of BAT to participate in non-shivering thermogenesis and maintain their body temperatures. It was thought that the excess of BAT in infants regresses and is lost overtime [4]. This was later proven to be incorrect as deposits of BAT were found in adults via fluorodeoxyglucose positron emission tomography (FDG PET). This technique is traditionally used to identify and study metastatic tumors as these tumors exhibit increased

glucose intake. FDG is a glucose analog that can be detected by positron emission tomography. When administered in healthy humans, the FDG uptake was detected in many symmetrical locations, making it unlikely that these measurements were showing a tumor. It was later shown that these metabolically active pools of tissue were in fact BAT located in the supraclavicular region, around solid organs, and many other locations in the adult human body [4][5].

Brown adipocytes, the functional unit of BAT, have many mitochondria which play a key role in producing heat to maintain core body temperature by using up chemical energy from fat [6]. Non-shivering thermogenesis begins when norepinephrine is released from sympathetic nerves. Norepinephrine binds to β-adrenergic receptors, which initiates a signaling cascade, activating Protein Kinase A (PKA). PKA stimulates hormone sensitive lipase, which breaks down triglycerides in a lipid droplet into free fatty acids (FFA). Free fatty acids are then activated by Acyl-CoA Synthetase Long-Chain Family Member 1 (Acsl1), which utilizes coenzyme A and ATP to generate acyl-CoA (Figure 1). In order to be shuttled across the mitochondrial membrane, carnitine exchanges with coenzyme A generating acyl-carnitine that travels through both membranes via carnitine transferases. In the mitochondrial matrix, acyl-carnitine is converted back into acyl-CoA and undergoes β-oxidation to become acetyl-CoA [7]. Acetyl-CoA enters the Krebs cycle in which electron transporters like NADH and $FADH_2$ are produced. These travel across the electron transport chain and cause hydrogen protons to be continuously pumped outside of the mitochondrial matrix, creating an electrochemical gradient. Typically, oxidative phosphorylation occurs and protons will travel through ATP Synthase to

create ATP. However, in non-shivering thermogenesis that occurs in brown adipocytes, the protons travel through uncoupling protein 1 (UCP1) which produces heat instead (Figure 2) [8].

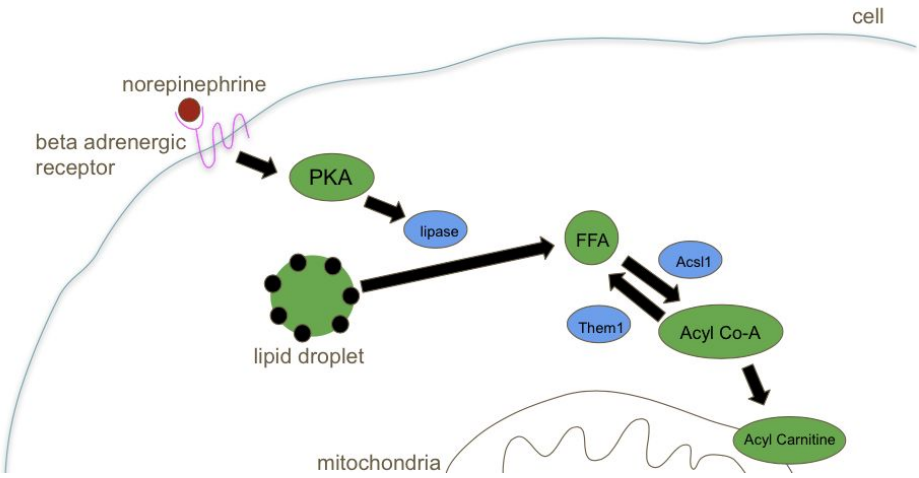

Figure 1. Schematic of Non-Shivering Thermogenesis from Extracellular Signaling to the Mitochondrial Membrane. The sympathetic nervous system releases norepinephrine which activates PKA via a G-protein coupled receptor pathway. This then stimulates lipase to break down the lipid droplet into free fatty acids (FFA). Acsl1 converts these FFA into acyl-CoA which then reacts with carnitine to form acyl carnitine. Now, acyl carnitine can pass through the mitochondrial membranes. The protein of interest, Them1, is also shown.

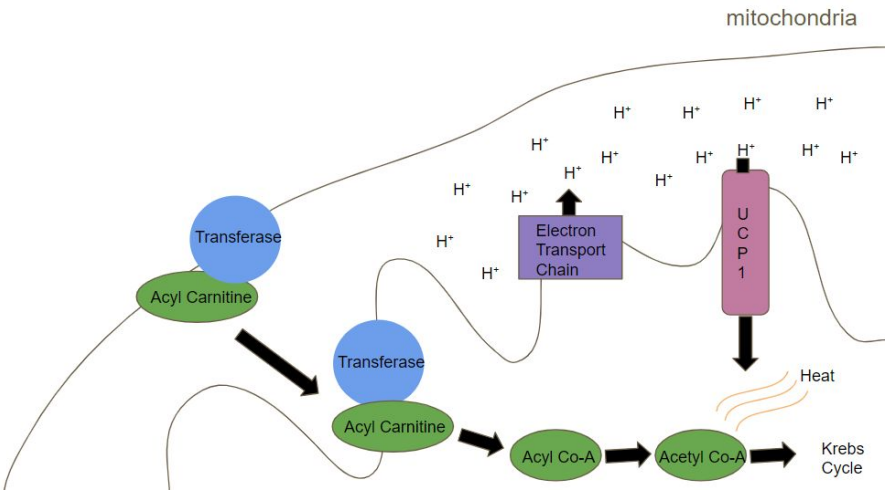

Figure 2. Schematic of Non-Shivering Thermogenesis from Mitochondrial Membrane to the Production of Heat. The acyl carnitine is shuttled across the mitochondrial membranes via transferases. The acyl carnitine is then eventually converted into acetyl Co-A, which will go into the Krebs Cycle. Uncoupling protein 1 (UCP1) produces heat when hydrogen protons are pushed back down the electrochemical gradient.

Many of these proteins, such as Acsl1 and UCP1, are transcriptionally upregulated in BAT once it is activated to expand the thermogenic capacity of the tissue [9]. Thioesterase superfamily member 1 (Them1), is one such protein that is upregulated with the thermogenic machinery following BAT activation, and was originally hypothesized to contribute to non-shivering thermogenesis [10]. However, studies suggest that Them1 antagonizes rather than promotes thermogenesis. It in fact hydrolyzes the acyl-CoA back into free fatty acids, countering the effect of Acsl1 [11]. When Them1 was deleted in mice, these mice exhibited increased levels of thermogenesis and were protected against diet-induced obesity [12]. The knockout mice that were fed the high fat diet weighed 22% less than wild type mice that were fed the high fat diet at

21 weeks. Furthermore, the knockout mice consumed about twice as much as the wild type mice, regardless of the diet [12]. This emphasizes the point that even though the knockout mice consumed about twice as much as the wild type mice, their body weight was significantly less. These data suggests manipulation of Them1 in humans could be protective against diet-induced obesity.

Them1 is just one of the thirteen mammalian proteins that hydrolyzes acyl-CoA compounds. These proteins are split into two types; the type I proteins have an α/β-hydrolase domain while the type II proteins have hot-dog domains [13]. Them1 is a type II protein, containing two hot-dog fold thioesterase domains. Additionally, Them1 contains a C-terminal steroidogenic acute regulatory transfer-related (START) domain. This START domain is hypothesized to stabilize the entire structure as well as facilitate the reaction from acyl-CoA to free fatty acids [12]. Though there is a structure of the Them1 START domain, there is not a structure of the thioesterase domains [13]. Additionally, the enzymatic mechanism of Them1 remains unknown. Therefore, we set out to solve the structure of the Them1 thioesterase domains and determine its enzymatic mechanism, which will enhance our understanding of this metabolically important enzyme. Furthermore, these studies will assist in the development of inhibitors that will potentially increase energy expenditure and treat obesity.

Goals

The goals of this research project are to determine the enzymatic mechanism and structurally characterize the thioesterase domains of Them1.

This will be achieved by:

1) Purifying the thioesterase domains of Them1 (Thio domain) as well as constructs containing mutations in its putative active site residues (D74A, N232A, and the double mutant).

2) Testing the thioesterase activity for the wild type and mutant proteins.

3) Determining the structure of the thioesterase domains of Them1 through crystallizing the thioesterase domains for X-ray crystallography.

Chapter 2: Creating Them1 Construct

Since there is currently no structure of the thioesterase domains of Them1 (also known as ACOT11), we developed a homology model based upon a previously determined structure of a homologous protein, ACOT12, which shares 51% sequence identity with Them1. These two type II proteins also consist of two "hot dog" domains as well as a START domain [14].

To identify residues putatively involved in the enzymatic mechanism of Them1, we structurally aligned our Them1 homology model with known crystal structures of ACOT7, ACOT12, and ACOT13. ACOT7 only consists of two hot-dog domains and ACOT13 only has one hot-dog domain. The evolutionary relationships between these four type II proteins were also studied. Them1 (ACOT11) is most closely related to ACOT12, followed by ACOT7, and then finally by ACOT13. This is also clearly shown through the similarities between the domain organizations of the four proteins [14].

Although ACOT13 was the most structurally different and most evolutionarily distant from Them1, we still used ACOT13 to identify vital residues within the active site of Them1. This was due to the fact that ACOT13's enzymatic mechanism was already discerned. Additionally, there were similarities between the substrate that was used to crystallize ACOT13, undecan-2-one-CoA, and Them1's prefered substrate, palmitoyl-CoA [15].

ACOT13 contains only one hot-dog domain, but dimerizes in order to create two active sites at the interface. Through this alignment, we identified two recognized Them1 active sites,

of which only one was highly conserved. Residues D65 and N50 in ACOT13 structurally aligned with residues D74 and N232 of the highly conserved active site in our Them1 homology model (Figure 3). The two residues in Them1 (D74 and N232) are predicted to be important within the active site as the two residues in ACOT13 (D65 and N50) were shown to disrupt enzyme activity when mutated. Therefore, we mutated both aspartic acid 74 and asparagine 232 to alanine, a nonpolar amino acid. We predicted that the D74 mutation would disrupt the aspartic acid oxygen attack on the hydrogen of the water molecule. Additionally, we predicted that the N232 mutation would disrupt the hydrogen bonding between this residue and the acyl-CoA. Though threonine 91 is also conserved in Them1, we did not mutate this residue as mutating its structurally analogous residue, serine 83, in ACOT13 did not significantly alter enzyme activity [16].

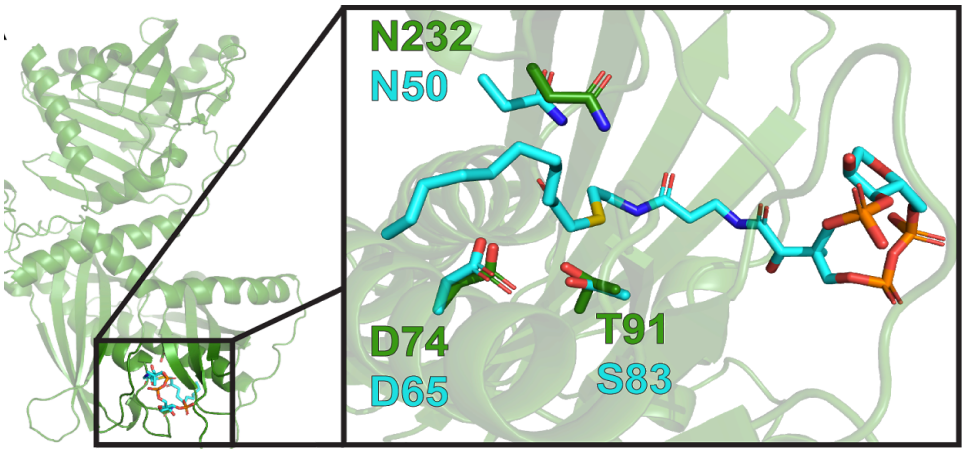

Figure 3. Schematic of the Overlapping Residues of Them1 and ACOT13. There are three conserved residues in one of the active sites of Them1 and ACOT13. The green structure is Them 1. The cyan structure is the undecan-2-one-CoA substrate from the ACOT13 structure. The Protein Data Bank (PDB) code is 35FO.

The full length protein was attempted to be purified, but was extremely challenging. To facilitate the purification, a construct was made with only the two thioesterase domains of Them1 (Thio domain). To further facilitate purification, we removed the first 40 residues of Them1 that were predicted to be intrinsically disordered. The thioesterase domains (residues 41-364) of wild-type and mutant (D74A; N232A; D74A & N232A) *Homo sapiens* Them1 in the pMCSG7 vector were recombinantly expressed in *Escherichia coli* strain BL21 pLysS cells. The thioesterase domains were expressed as a His_6 fusion containing a tobacco etch virus (TEV) protease cleavage site to facilitate tag removal (Figure 4).

Figure 4. Construct of the Them1 Thioesterase Domains. This construct starts with six histidine residues followed by a TEV site and the two thioesterase domains of Them1.

Chapter 3: Purifying the Them1 Thioesterase Domains

The Them1 construct was recombinantly expressed in *Escherichia coli* strain BL21 pLysS cells. These cells were then grown in 6 liters of lysogeny broth media at 37°C. Protein expression was induced with 0.5 mM isopropyl β-d-1-thiogalactopyranoside at about an OD_{600} of 0.7, about two hours after the start of the growth. The bacterial cells then continued to grow overnight at a lowered temperature of 18°C. Next, the cells were harvested by spinning at 3500 rpm for 25 minutes. The cells were lysed by adding five components lysis buffer (20 mM Tris, 1 M NaCl, 5% glycerol, and 25 mM imidazole, lysozyme, DNase, phenylmethylsulfonyl fluoride (PMSF), and 2-mercaptoethanol) to the harvested cells. The mixture was stirred until it was homogenous. The cells were then sonicated for 30 minutes, followed by spinning at 16,000 rpm for 45 minutes to separate the insoluble and soluble lysates.

The thioesterase domains were first purified using nickel affinity chromatography with a 3-step elution profile (5%, 50% 100% 500mM imidazole). The eluted protein was collected, concentrated, and further purified through size exclusion chromatography using a HiLoad 16/60 Superdex 200 column. The Thio domain purified as a trimer. It eluted from the size exclusion chromatography at 90 mL. Based upon standards, this is where the trimer of the Thio domains would be. Gel electrophoresis was used to confirm the protein purity at about 37 kDa (Figures 5-9). A bicinchoninic acid assay (BCA) was used to determine the concentration of the protein.

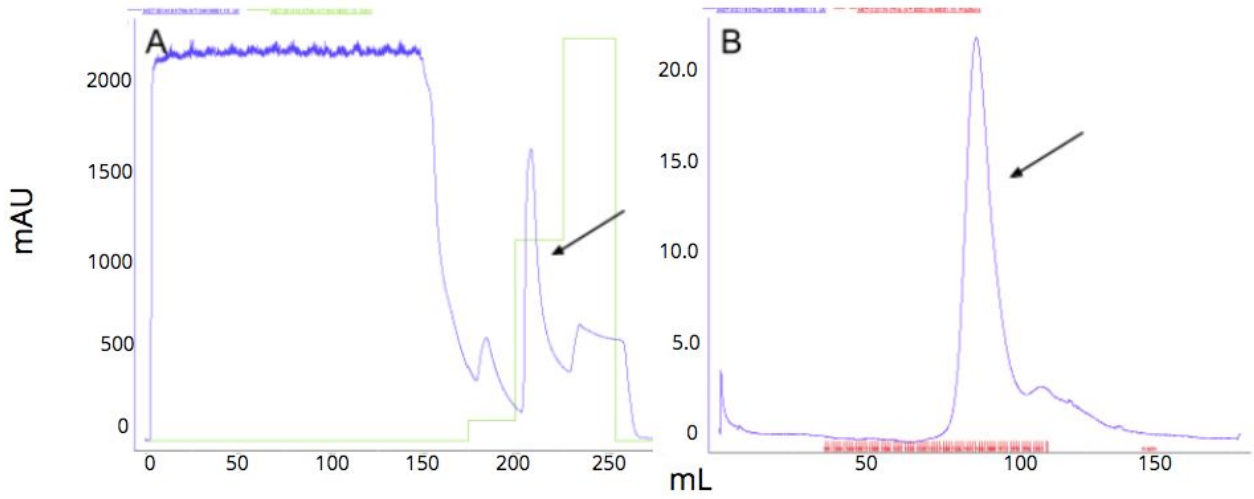

Figure 5. Nickel Affinity Chromatography and Size Exclusion Chromatography of Wild Type Them1 Thioesterase Domains. ***A.*** Nickel affinity chromatography of wild-type Them1 thioesterase domains. The blue line represents the UV absorption at 280 nm. The green line represents the percent of the Buffer B (500 mM imidazole). The three steps were set to 5%, 50%, and 100% of 500 mM of imidazole. The arrows represent where the protein was eluted and collected. *B.* Size exclusion chromatography of wild-type Them1 thioesterase domains using SHiLoad 16/60 Superdex 200 column. Protein elutes at 90 mL, which corresponds with a molecular weight of 111 kD, meaning the thioesterase domains purify as a trimer.

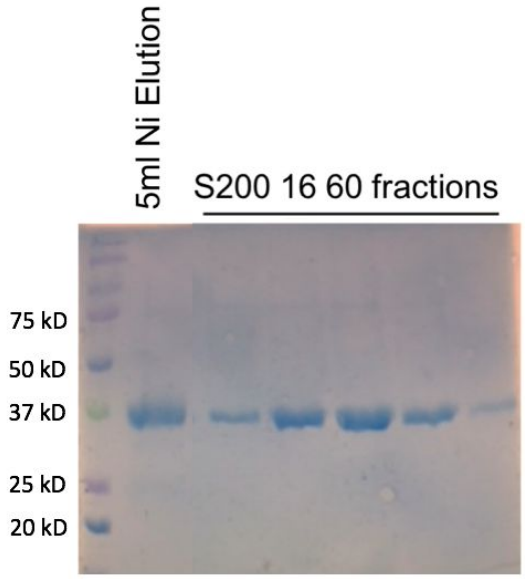

Figure 6. Gel Electrophoresis of Nickel Affinity and Size Exclusion Chromatography of Wild Type Them1 Thioesterase Domains. SDS-Page protocols were used. The thioesterase domains are about 37 kDa. There is a lot of protein in all of the fractions collected from both peaks of the nickel affinity column and the sizing column.

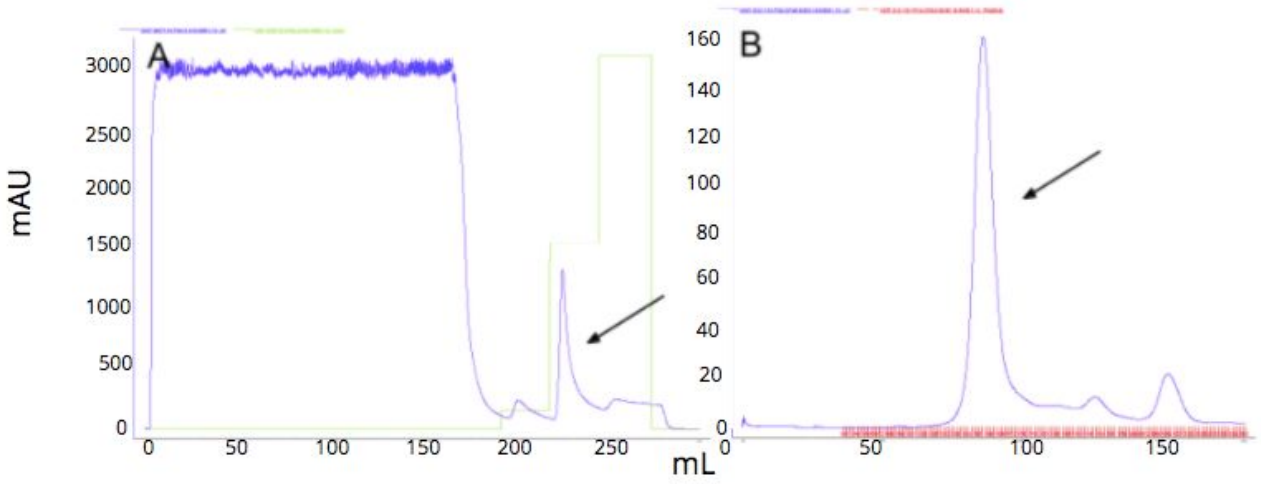

Figure 7. Nickel Affinity Chromatography and Size Exclusion Chromatography of Them1 Thioesterase Domains D Mutant. *A.* Nickel affinity chromatography of Them1 thioesterase domains D mutant. The blue line represents the UV absorption at 280 nm. The green line

represents the percent of the Buffer B (500 mM imidazole). The three steps were set to 5%, 50%, and 100% of 500 mM of imidazole. The arrows represented where the protein was eluted and collected. *B.* Size exclusion chromatography of Them1 thioesterase domains D mutant using Superdex 200 16/60 column. Protein elutes at 90 mL, which corresponds with a molecular weight of 111 kD, meaning the thioesterase domains purify as a trimer.

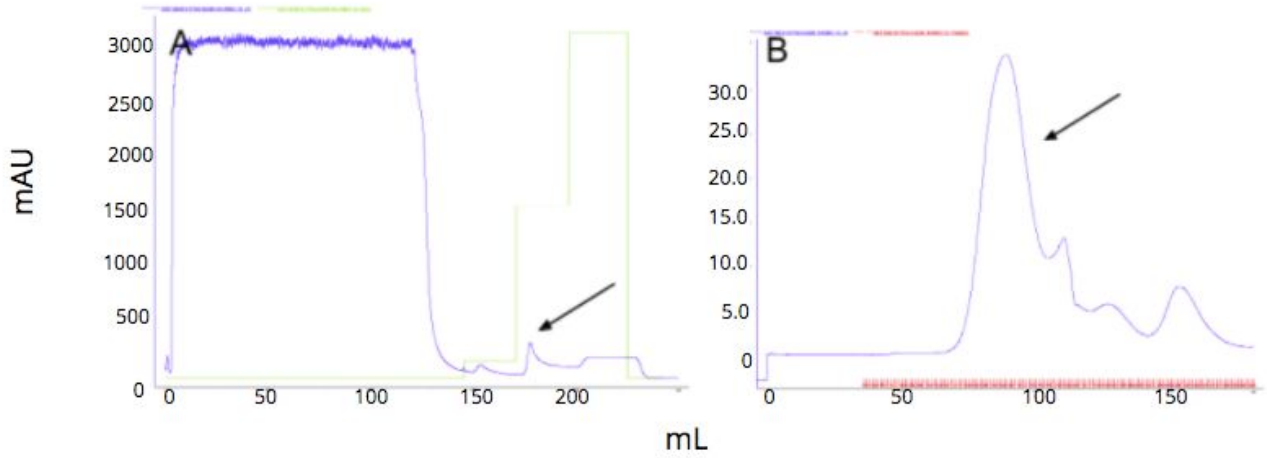

Figure 8. Nickel Affinity Chromatography and Size Exclusion Chromatography of Them1 Thioesterase Domains N Mutant. ***A.*** Nickel affinity chromatography of Them1 thioesterase domains N mutant. The blue line represents the UV absorption at 280 nm. The green line represents the percent of the Buffer B (500 mM imidazole). The three steps were set to 5%, 50%, and 100% of 500 mM of imidazole. The arrows represent where the protein was eluted and collected. *B.* Size exclusion chromatography of Them1 thioesterase domains N mutant using Superdex 200 16/60 column. Protein elutes at 90 mL, which corresponds with a molecular weight of 111 kD, meaning the thioesterase domains purify as a trimer. Compared to the wild type Them1 thioesterase domains and D mutant, the peaks are not as clean. The protein was only collected from the 90 mL peak and ran on an SDS-page gel to ensure purity. It is possible that

the mutations in the thioesterase domains are slightly disrupting the folding of the protein which make it more difficult to purify.

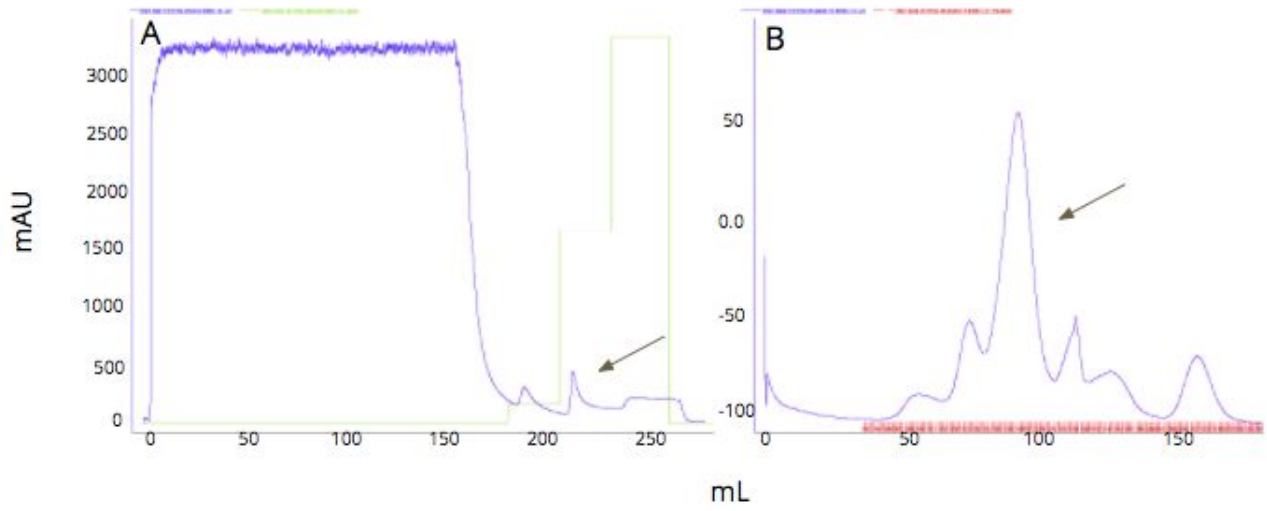

Figure 9. Nickel Affinity Chromatography and Size Exclusion Chromatography of Them1 Thioesterase Domains DN Mutant. ***A.*** Nickel affinity chromatography of Them1 thioesterase domains DN mutant. The blue line represents the UV absorption at 280 nm. The green line represents the percent of the Buffer B (500 mM imidazole). The three steps were set to 5%, 50%, and 100% of 500 mM of imidazole. The arrows represent where the protein was eluted and collected. *B.* Size exclusion chromatography of Them1 thioesterase domains DN mutant using Superdex 200 16/60 column. Protein elutes at 90 mL, which corresponds with a molecular weight of 111 kD, meaning the thioesterase domains purify as a trimer. Compared to the wild type Them1 thioesterase domains and D mutant, the peaks are not as clean. The protein was only collected from the 90 mL peak and ran on an SDS-page gel to ensure purity. It is possible that the mutations in the thioesterase domains are slightly disrupting the folding of the protein which make it more difficult to purify.

Chapter 4: Thioesterase Activity Assays

Purified wild-type and mutant Them1 thioesterase domains were used in a thioesterase activity assays in order to determine the enzymatic mechanism of Them1. 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) was used to probe acyl-CoA hydrolysis, as it reacts with the free thiols on CoA, which is a product of acyl-CoA hydrolysis, generating 2-nitro-thiobenzoate (TNB) that absorbs at around 412 nm [17]. This allows for a quantitative measure of product formation. The protein was incubated with 0.3 mM DTNB in a buffer containing 30 mM Hepes, 150 mM NaCl, and 5% glycerol at 37°C for 30 minutes to allow the colorless DTNB to react with the free thiols in the Thio domain. This removes any bias in measurement caused by the free thiols in the protein and creates a baseline.

The total reaction volume was 200 µl, with the protein held constant at 1 uM and the substrate, myristoyl-CoA, titrated in (range: 0 µM to 20 µM). This was run in a plate reader at an absorbance of 412 nm at 37°C at 10 second intervals for about 30 minutes. The absorbance of the yellow TNB, which is a readout of product formation, was measured and the initial velocity of the reaction was calculated for each substrate concentration. Three technical replicates were conducted for each construct of Them1 (WT, DN Mutant, D Mutant, and N Mutant) and a control as well, which did not contain any protein. Background absorbance of buffer and substrate were removed by subtracting out the absorbance of the control wells. A total of three biological replicates were completed. Data was fit with the Michaelis-Menten equation, which calculates enzyme kinetics of each Them1 construct (Figure 10).

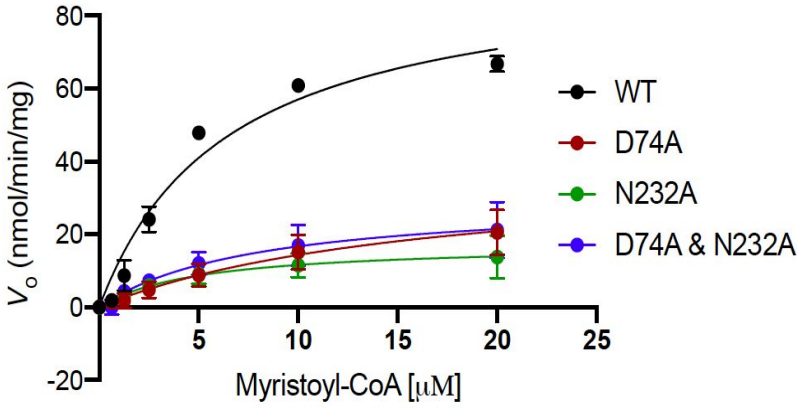

Figure 10. Michaelis-Menten Curve of Myristoyl-CoA with Various Constructs of the Thio domain. The wild type Thio domain has the greatest rate of reaction and activity compared to the mutants. Baseline subtraction was done by using a control of just DTNB and the myristoyl-CoA.

Just as hypothesized, the wild type Thio domain protein had the greatest amount of activity, followed by all of the mutants. This shows that these two residues are vital in the active sites of the Thio domain. Figure 10 also shows that there is still some low level activity in the Thio domain mutants. This suggests that there is either minimal activity occurring in that active site or that the other active site is contributing to the activity shown in the Michaelis-Menten graph. We see that the highly conserved active site is much more efficient than the other active site as there is such a significant change in activity between the wild type Thio domain and the mutant Thio domains.

Since ACOT13 dimerizes, mutation of one residue in its active site would render both sites inactive. In contrast, mutating a residue in one active site of Them1 would not disrupt the

other active site as both hot dog domains are unique. The evolutionary pressure to maintain two functioning active sites in Them1 is likely low, potentially explaining this unbalanced activity in active sites of Them1. Overtime, the selective pressure could cause for only one of the active sites to be functioning as both active sites have the same role.

The results from the thioesterase assays further emphasize the similarities between the enzymatic mechanism between Them1 and ACOT13. The enzymatic mechanism is drawn out to show how water and the three residues in the active site interact with acyl-CoA (Figure 11). The electrons from the single bonded oxygen molecule of the aspartic acid 74 attack the empty hydrogen orbital of the water to form a new bond. Then, the electrons from the oxygen molecule of the water attack the empty carbon orbital of the acyl-CoA, pushing the electrons from its double bond onto the oxygen. This creates an intermediate structure that is stabilized by hydrogen bonding with all three residues. The electrons from the oxygen of the acyl-CoA push back down, forming a double bond with the carbon. The carbon then breaks its bond with the sulfur and coenzyme-A and carboxylic acid are formed. This mechanism shows exactly how Them1 hydrolyzes acyl-CoA into a free fatty acid using coenzyme-A, which is very similar to ACOT13.

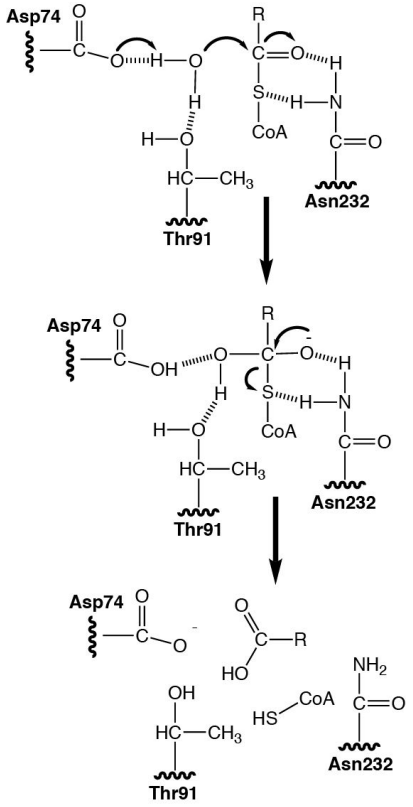

Figure 11. Enzymatic Mechanism of Them1. This schematic shows how Them1 hydrolyzes acyl-CoA. The oxygen from aspartic acid 74 interacts with the water, and the water interacts with the acyl-CoA in order to form an intermediate structure. This intermediate structure is stabilized by hydrogen bonding. The products formed are a carboxylic acid and coenzyme-A.

Chapter 5: Purifying the MBP Tagged Them1 Thioesterase Domains

Purification of the Thio domain was difficult as it was quite unstable at higher protein concentrations. After purifying the protein using nickel affinity chromatography, the protein would crash out if left in the fridge for more than one hour. This made it necessary to run the size exclusion column immediately after the nickel affinity column to prevent the crash out from occurring. Additionally, concentrating the protein led to protein precipitation. Many attempts were made with different buffers in order to stabilize the protein. We tried different pHs as well as different salt concentrations. We also tried to change the 3-step elution profile to 5%, 17%, and 100% 500 mM imidazole. All of these complications encouraged us to develop a construct that would be more stable and consistent throughout the purification process.

We generated a new construct of the thioesterase domains with an N-terminal His tag and C-terminal maltose binding protein (MBP) tag (Thio-MBP) (Figure 12). We predicted the MBP tag would act similarly to the START domain, which stabilizes the protein. We inserted a short linker consisting of four amino acids (NAAA) between the thioesterase domains and the MBP tag. We expressed this construct in the same manner as described in Chapter 3.

Thio-MBP was first purified using nickel affinity chromatography with a 3-step elution profile: 5%, 50%, and 100%. The second peak, at 50% of 500 mM imidazole, eluted the most amount of protein (Figure 13). The gel showed that the protein was very impure as there were many other proteins in the collected fractions (Figure 14). To isolate the Thio-MBP, we introduced an additional purification step of ion exchange chromatography. The pKa of Them1 is

8.3 and the protein is stable at higher pHs, therefore an anion column Q column was used (Figure 15). The gel showed that the protein was quite pure across the first peak (Figure 16). The eluted protein was collected, concentrated, and further purified through size exclusion chromatography using a HiLoad 16/60 Superdex 200 column. The Thio domain purified as a trimer. It eluted from the size exclusion chromatography at around 13 mL. Based upon standards, this is where the trimer of the Thio domains would be (Figure 17). Gel electrophoresis was used to confirm the protein purity at about 79 kDa (Figure 18). A bicinchoninic acid assay (BCA) was used to determine the concentration of the protein.

Figure 12. Construct of the Them1 Thioesterase Domains with an MBP Tag. This construct starts with six histidine residues followed by a TEV site, the two thioesterase domains of Them1, a four residue linker, and then the maltose binding protein tag. This construct was developed in order to facilitate the purification process of the Thio domain.

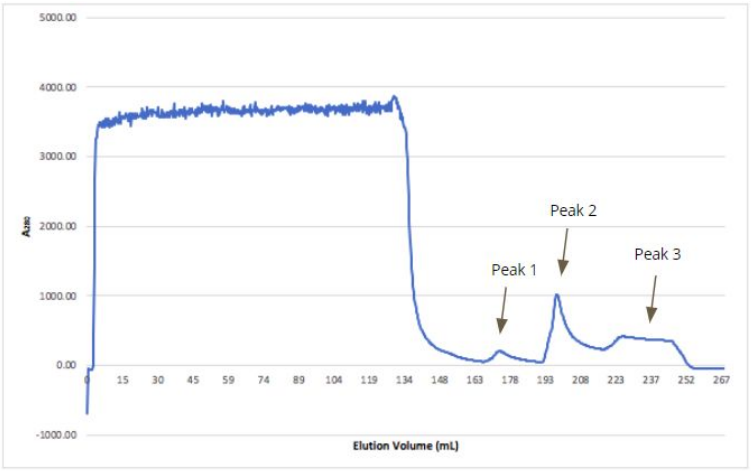

Figure 13. Nickel Affinity Chromatography of Thioesterase Domains with MBP Tag. The arrows represent where the protein was eluted and collected. The blue line represents the UV absorption at 280 nm. The three steps were set to 5%, 50%, and 100% of 500 mM of imidazole.

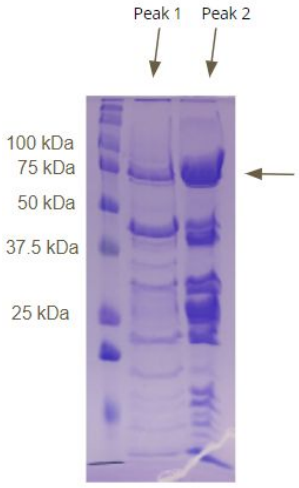

Figure 14. Gel Electrophoresis of Nickel Affinity Chromatography of Thioesterase Domains with MBP Tag. SDS-Page protocols were used. The thioesterase domains are about 79 kDa and typically run a little lower than the 75 kDa marker. There is quite a bit of protein there as shown by the gel, but it is quite impure.

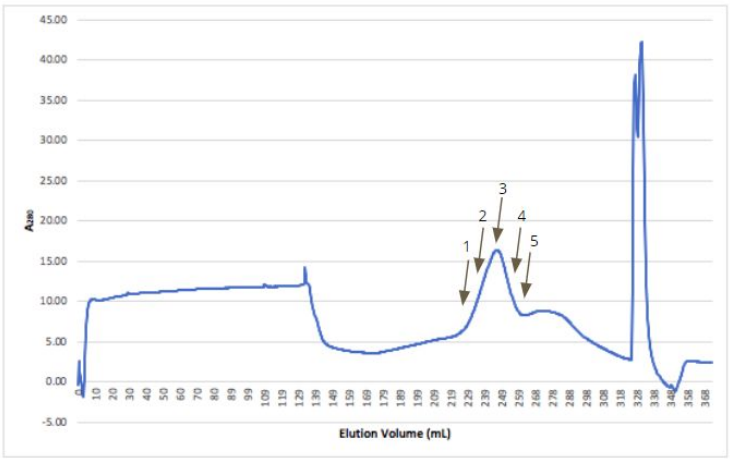

Figure 15. Ion Exchange Chromatography of Thioesterase Domains with MBP Tag. The arrows represent where the target protein was eluted and collected. The large peak on the right represents the elution of the tightly bound target protein. The blue line represents the UV absorption at 280 nm. 1 M NaCl was used to elute the target protein. The pKa of the Them1 thioesterase domain is 8.3.

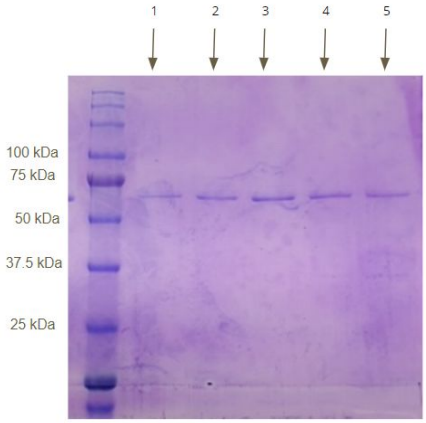

Figure 16. Gel Electrophoresis of Ion Exchange Chromatography of Thioesterase Domains with MBP Tag. SDS-Page protocols were used. The thioesterase domains are about 79 kDa and typically run a little lower than the 75 kDa marker. The protein is pure, but there does not seem to be a large amount of it.

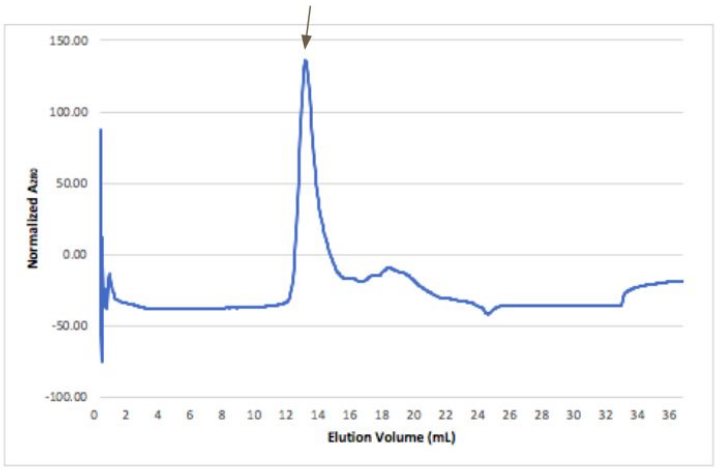

Figure 17. Size Exclusion Chromatography of Thioesterase Domains with MBP Tag. The arrows represent where the target protein was eluted and collected. Protein elutes at around 13 mL, which corresponds with a molecular weight of 237 kDa, meaning the thioesterase domains purify as a trimer. The blue line represents the UV absorption at 280 nm.

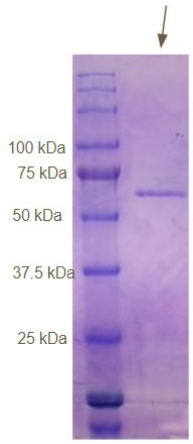

Figure 18. Gel Electrophoresis of Size Exclusion Chromatography of Thioesterase Domains with MBP Tag. SDS-Page protocols were used. The thioesterase domains are about 79 kDa and typically run a little lower than the 75 kDa marker. The protein is pure, but there is not a large amount of it.

Chapter 6: Crystallography Screens with Them1 Thioesterase Domains

In order to better understand Them1's mechanism and how it is regulated, we set out to determine its crystal structure. We first utilized large scale crystal screens via sitting drop vapor diffusion using the Crystal Phoenix robot to identify conditions that crystallized Thio-MBP. We then optimized these conditions, testing protein concentration, buffer pH, salt and polyethylene glycol (PEG) concentrations using hanging drop vaporization in order to improve crystal growth.

Crystals reproducibly formed under these conditions: PEG 400 ranging from 24%-27%, 0.1 M Hepes ranging from pH 7.5, and 0.066 $MgCl_2$. Despite multiple attempts to improve crystal quality, the crystals that formed were small and difficult to loop (Figure 19). The crystals that were looped did not diffract, suggesting this crystal form does not diffract or cryo-freezing the crystal disrupted crystal packing (Figure 20).

Figure 19. Image of the Preliminary Crystals Formed of the Them1 Thioesterase Domains. The conditions of these crystals are PEG 400 25%, 0.1 M Hepes pH 7.4, and 0.066 M $MgCl_2$. The protein concentration is 4 mg/mL. There was a 1:1 ratio set between the buffer and protein.

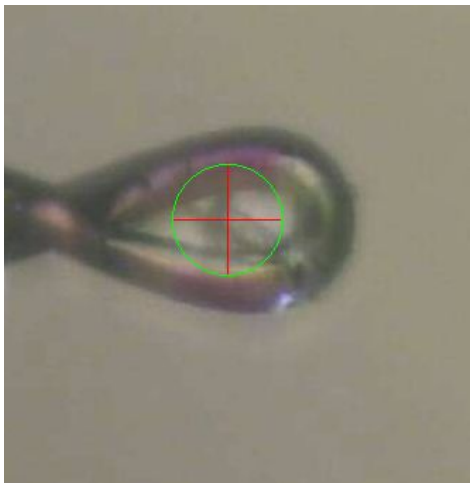

Figure 20. Thio-MBP Crystal within 100 µm Cryo Loop. This image was obtained at the APS (SER-CAT) beam line. The cryo loop is 100 µm in size. There was no diffraction of the crystal.

Chapter 7: Future Directions and Conclusion

The thioesterase assays provided insight about the structure of Them1 and further confirmed its similarity to ACOT13. We see that these different type II proteins have similar enzymatic mechanisms to hydrolyze acyl-CoA. This could lead to challenges in the future as it may become difficult to develop a specific competitive inhibitor. If all the enzymatic mechanisms are so similar to one another, finding an inhibitor that binds specifically to Them1 could be extremely difficult. It may be necessary to turn toward allosteric inhibitors or to manipulate the START domain. This emphasizes our need for more structural information about the Them1 thioesterase domains.

Moving forward, it would be vital to go back to the large scale crystal screens and try to test other conditions that formed preliminary crystals. The other conditions could be explored and optimized, potentially leading to better crystals. The most apparent issue throughout our project was the inconsistency of the purifications of the thioesterase domains. Occasionally, the purifications would work and elute pure protein, but often there were unforeseen variables that prevented this from occurring. If we were able to yield more pure protein, we could have tested many more conditions. Ultimately, we want crystals that diffract so that the thioesterase domain crystal structure can be solved.

Once the crystal structure for Them1 is solved, inhibitor and drugs screens can be done. This would include developing inhibitors and crystallizing Them1 with the inhibitor. With this crystal structure, the inhibitor can be improved as we can visualize the active site. We could

continue to find ways to manipulate the effect that Them1 and other type II proteins have on thermogenesis in brown adipose tissue. If we could safely determine a mechanism to increase the energy expenditure in these tissues, we could be one step closer to battling the obesity epidemic.

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