Spring 5-1-2013

Investigation of ghrelin acylation by ghrelin O-acyltransferase

Edward P. Prybolsky

Investigation of ghrelin acylation by ghrelin *O*-acyltransferase

A Capstone Project Submitted in Partial Fulfillment of the

Requirements of the Renée Crown University Honors Program at

Syracuse University

Edward P. Prybolsky

Candidate for BS Degree

and Renée Crown University Honors

May 2013

Honors Capstone Project in Biochemistry

Capstone Project Advisor: _____
Dr. James Hougland

Capstone Project Reader: _____
Dr. James Dabrowiak

Honors Director: _____
Stephen Kuusisto, Director

Date: 4/24/13

Abstract:

Ghrelin is a peptide hormone involved in hunger signaling and other physiological processes. To become active, ghrelin first must be acylated by ghrelin *O*-acyltransferase (GOAT). This enzyme carries out a post-translational modification that attaches an octanoyl group to the side chain of the serine 3 (S3) residue of ghrelin. With ghrelin linked to hunger signaling, memory, and other physiological processes, GOAT has been identified as a potential drug target. However, inhibitor design is difficult because not much is known regarding the active site structure and catalytic mechanism of GOAT. This study reports the design of a novel fluorescence-based assay that allows the use of ghrelin substrate mimics and derivatives to interrogate ghrelin recognition by GOAT. The results of this research can directly contribute to helping in the development of novel GOAT inhibitors.

Acknowledgements:

I would like to thank Dr. Hougland, Joe Darling, and the rest of the Hougland lab, in addition to Syracuse University, the Departments of Chemistry and Biology, and the Renée Crown University Honors Program for allowing me to learn and grow as a scientist by participating in this program and following their guidance.

Table of Contents

I. Introduction:

Ghrelin is a secreted peptide hormone initially discovered in 1999.[1,2] While first found to stimulate appetite, it has since been proposed to play a role in other functions such as uterine signaling, memory, and glucose metabolism.[2-5] In order to become biologically active, ghrelin must undergo a series of maturation steps after expression. One of these steps is acylation of the ghrelin precursor proghrelin (which shall be referred to only as ghrelin from this point forward) by the enzyme ghrelin *O*-acyltransferase (GOAT). This acylation results in addition of an octanoyl group to the side chain hydroxyl of serine 3 (S3) of ghrelin (Figure 1). This octanoylation is required for ghrelin to bind and activate the growth hormone secretatogue receptor GHSR-1a.[2]

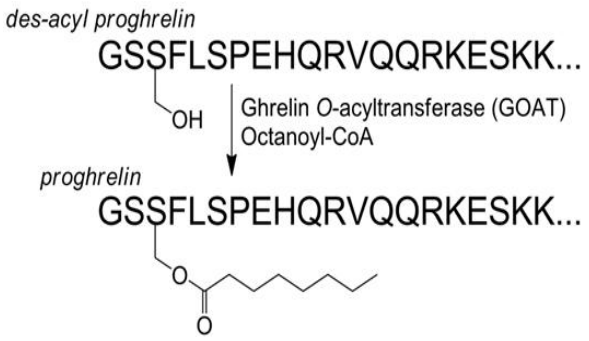

Figure 1: Acylation of the ghrelin hormone by GOAT.

GOAT was first identified in 2008 as an integral membrane protein member of the MBOAT (membrane-bound *O*-acyltransferase) enzyme family.[6,7] However, the structure, active site, and catalytic mechanism of GOAT remain largely undefined, due to the difficulty of purifying properly folded GOAT from the cell membrane. Hydropathy plots suggest that GOAT has 7 or 8 transmembrane helicies, and two conserved residues (Asn307 and His338) have been shown to be important for enzyme activity, with alanine mutations at these positions resulting in a loss of enzyme activity.[6,8] Regarding GOAT substrate

recognition, previous work has shown that the G1 and F4 residues of ghrelin are involved in recognition by GOAT and subsequent catalysis.[8]

With these previous results and limitations in mind, the purpose of my research in the Hougland lab during my tenure at Syracuse University has been dedicated to the development of tools for studying GOAT activity and substrate selectivity. This research is significant because it opens the doors for the rational design of GOAT inhibitors by determining the interactions that occur on the molecular level between the active site of GOAT and its substrate, of which the only known is ghrelin. Such inhibitors could be made for the purpose of becoming drugs that combat obesity through the prevention of hunger signaling, as well as diseases that could be affiliated with ghrelin's other biological roles such as Prader-Willi Syndrome and diabetes.

Towards these goals, I have pursued two complementary projects. The first project was construction of several affinity-tagged versions of the human isoform of GOAT (hGOAT) to allow for expression and purification in Sf9 insect cells. My second project involved the development of a fluorescence-based assay for hGOAT activity. This assay utilizes a fluorescently labeled peptide substrate that permits structure-reactivity analysis of hGOAT activity. Changes in substrate reactivity with hGOAT upon substrate mutation or chemical modification can reveal interactions within the GOAT active site involved in ghrelin recognition and octanoylation.

II. hGOAT/tag Gene Construction- Overview

In the Hougland lab, hGOAT is expressed in Sf9 insect cells from an hGOAT DNA construct containing a C-terminal His_{10}-tag, using a previously reported protocol.[8] However, confirmation of hGOAT expression using Western blotting against the C-terminal His tag has been difficult due to cross-reactivity between the anti-His antibody and endogenous proteins in the GOAT-containing membrane fractions and negative control fractions (Figure 2). The negative control in lane 8 is protein expressed from the pFastBacDual/GusCat plasmid which contains no GOAT-His_{10} gene, while lane 1 contains TEV Protease with a His_6 tag.

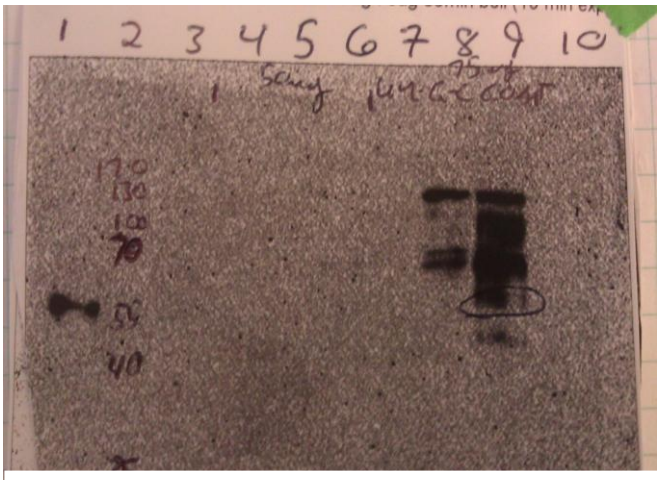

Figure 2: Anti-His antibody Western Blot showing cross-reactivity. A TEV Protease-His_6 positive control (lane 1), pFastBacDual/GusCat negative control (lane 8), and Sf9 membrane fractions (lane 9) all react with the anti-His antibody. The molecular weight of GOAT is 55kDa (circled in sample).

To resolve this issue, I have constructed new hGOAT constructs wherein the His-tag is replaced with one of three affinity tags: FLAG (amino acid sequence DYKDDDK), HA (YPYDVPDYA), and Myc (EQKLISEEDL). These constructs may allow for more facile confirmation of hGOAT expression by Western blot. The new hGOAT constructs may also allow for the purification of

the GOAT enzyme using affinity columns, which has proved unsuccessful with the current His_{10} tag.

II. hGOAT/tag Gene Construction - Results and Discussion

Sf9 Expression: C-terminal tag replacement

The final desired pFastBacDual/MBOAT4-X vector (where X is either the Myc, FLAG, or HA tag) was constructed through a series of restriction enzyme digests and ligations. The pUC57/MBOAT4-His_{10} and pFastBacDual/MBOAT4-His_{10} vectors used in the lab containthree important restriction enzyme sites regarding this cloning project: an EcoRI site at the beginning of the MBOAT4 gene, a XhoI site in the linker region between the MBOAT4 gene and His_{10} sequence, and a XbaI site immediately after the His_{10} sequence (Figure 3).

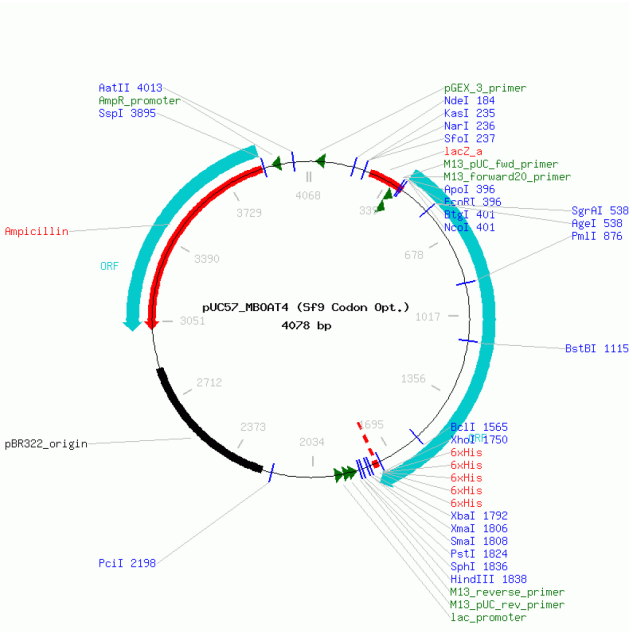

Figure 3: pUC57/MBOAT4i-His vector. The restriction sites used in my work are *EcoR*I (396), *Xho*I (1750), and *Xba*I (1792).

First, the His_{10} tag attached to the 3'-end of theMBOAT4 gene in pUC57must be replaced with DNA coding for either the myc, FLAG, or HA tag. This DNA sequence inserts for these tags was formed by annealing two

complementary oligonucleotide primers to generate the DNA sequence for the desired affinity tag with *Xho*I and *Xba*I restriction site overhangs at the 5' and 3' ends. After successful ligation of the new affinity tag sequence, the MBOAT4-X gene is excised from the pUC57 vector and ligated into the pFastBacDual vector using the *EcoR*I and *Xba*I restriction sites. The pFastBacDual (pFBD) vector allows for the recombination of the MBOAT4-X gene into the baculoviral genome present in the baculovirus shuttle vector (bacmid) within DH10Bac strain of *E. coli*. This bacmid is then transfected into the Sf9 insect cells, leading to baculovirus production and hGOAT expression.

At each of the ligation steps described above, the resulting plasmid was analyzed via gel electrophoresis and gene sequencing (Genscript) to confirm successful ligation. To analyze a ligation to determine success, a single restriction enzyme digest is run in addition to a double restriction digest. The single digest linearizes the entire (plasmid)/MBOAT4-X gene, which removes plasmid supercoiling to allow accurate determination of plasmid size/length on an agarose gel. The double digest excises the gene completely, allowing for the observation and size determination of the ligated insert and parent vector.

The gels in Figure 4 show the confirmation of the pFastBacDual/MBOAT4-X ligations, the last step in creating the recombinant hGOAT gene with FLAG, Myc, and HA tags. Figure 4a is an agarose gel for analysis of *EcoR*I and *Xba*I double digests of multiple preparations of pFastBacDual/MBOAT4-FLAG plasmids (lanes 5, 6, and 7). Following *EcoR*I –

*Xba*I double digestion of the pFastBacDual/MBOAT4-FLAG vector, two bands are observed upon analysis by agarose gel electrophoresis, approximately 5000-

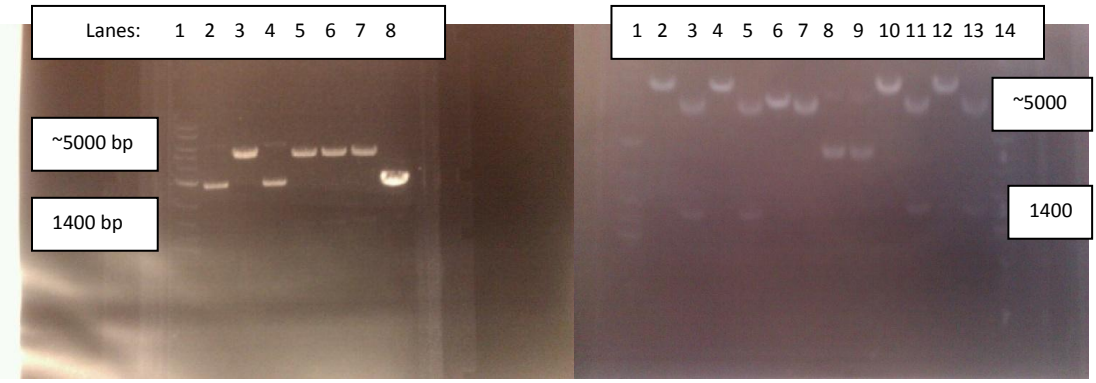

Figure 4: Gel analysis of digests confirming the presence of pFBD/MBOAT4-X.
Left: *EcoR*I and *Xba*I double digests of pFBD/MBOAT4i-FLAG plasmids (5, 6, 7) and pFBD/MBOAT4-Myc/HA plasmids (lanes 2,4, 8). 2-log DNA ladder is run in lane 1.
Right: *EcoRI and XbaI* single and double digests of pFBD/MBOAT4i- Myc (lanes 2-5) and HA (lanes 10-13). 2-log DNA ladder is run in lanes 1, 14. Lanes 6-9 were failed pFBD/MBOAT4-Myc/HA digests.

5500bp and 1400bp, corresponding to parent pFBD vector and MBOAT4-FLAG insert respectively. Following confirmation of ligation by gel electrophoresis, successful construction of the pFBD/MBOAT4-FLAG plasmid was confirmed by DNA sequencing.

Ligations of the pFastBacDual/MBOAT4-Myc and pFastBacDual/MBOAT4-HA plasmids were similarly analyzed by agarose gel electrophoresis (Figure 4b; Myc (lanes 2-7), HA (lanes 8-13)). Following *EcoR*I – *Xba*I double digestion, pFBD/MBOAT4-Myc plasmid ligation was confirmed for the plasmid samples in lanes 3 and 5, based on the cut DNA running at the appropriate band size. Lane 7 DNA is a failed EcoRI/XbaI double digest,

suggesting a failed ligation. These results were confirmed later by DNA sequencing.

Identical digest and analysis is applied to lanes 9, 11, and 13, which contain preps of separate pFastBacDual/MBOAT4-HA ligation products. Lanes 11 and 13 show bands at the appropriate 5000-5500bp range and 1400bp, indicating successful ligation. Multiple bands are shown in lane 9, characteristic of uncut DNA and a failed ligation. DNA sequencing confirmed the successful construction of the MBOAT4-HA sequence for the plasmids in lanes 11 and 13.

To confirm the size of the ligated plasmids, the pFBD/MBOAT4-Myc (lanes 2, 4, 6 in Figure 4b) and pFBD/MBOAT4-HA (lanes 8, 10, 12 in Figure 4b) plasmids were digested with *EcoR*I alone to yield linearized plasmid (pFBD/MBOAT4-Myc, lanes 3, 5, 7; pFBD/MBOAT4-HA, lanes 9, 11, and 13). These single digests confirm the presence of the MBOAT4i-X insert in the pFastBacDual vector by the linearized plasmids exhibiting a shift of +1400bp relative to the pFastBacDual parent vector. The single digests also indicate a failure of ligation for several plasmids (lanes 6/7 and 8/9).

In conclusion, I have synthesized hGOAT expression constructs with C-terminal FLAG, Myc, and HA affinity tags. These constructs are currently being investigated as options for expression and purification of hGOAT.

III. GOAT Assay Design and Utilization- Overview

The main focus of my Capstone research has been the development and employment of a novel fluorescence-based assay to analyze the substrate

specificity of GOAT. This assay involves the attachment of acrylodan, a small fluorophore, to the cysteine residue on a short synthetic peptide that will serve as a ghrelin substrate mimic. The resulting fluorescent peptide is incubated with recombinantly-expressed hGOAT, leading to peptide octanoylation. The extent of peptide acylation is then determined using reverse-phase HPLC to separate the substrate and octanoylated product. Octanoylation of the product peptide is also confirmed using mass spectrometry. The development of this fluorescent substrate was recently published in *Analytical Biochemistry*.[9]

The use of acrylodan also provides the opportunity for development of a continuous assay for hGOAT-catalyzed acylation. Acrylodan is an environmentally sensitive fluorophore whose fluorescence increases in response to changes in the polarity/hydrophobicity of its local environment.[10] When GOAT acylates the peptide conjugated to acrylodan, the octanoyl group added to the side chain of serine 3 will increase local hydrophobicity. This reduction in local polarity will lead to an increase in acrylodan fluorescence, thereby providing a signal that reflects the extent of ghrelin peptide acylation.

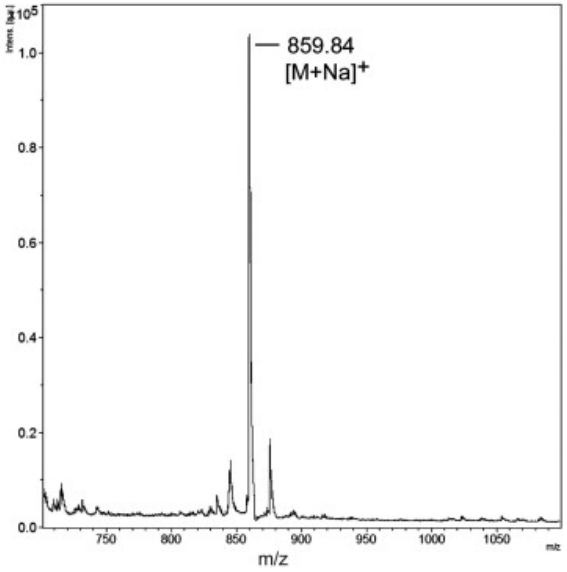

Figure 5: Mass spectra of GSSFLC-AcDan $(M+Na)^+$ = 859.84. Na^+ adducts are frequently observed during mass spectrometry of substrates.

III. GOAT Assay Design and Utilization- Results and Discussion

Fluorescent hGOAT substrate Development

For substrate development, we used the N-terminal sequence of ghrelin (GSSFLS) as a template, and incorporated cysteine residues at several positions to allow for acrylodan attachment. Short peptides mimicking the N-terminal sequence of ghrelin have been shown to serve as GOAT substrates.[8] One of first substrates is the 6-mer GSSFLC-AcDan. The formation of the substrate upon acrylodan labeling of the GSSFLC peptide was confirmed by mass spectrometry (Figure 5).

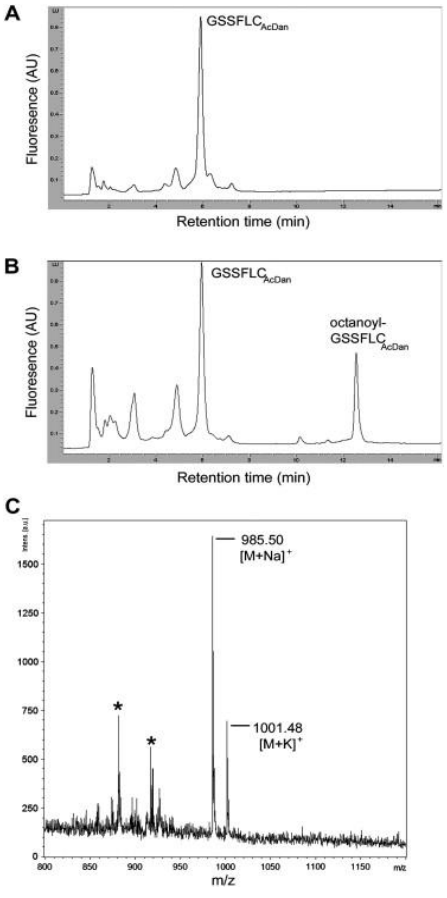

Figure 6: hGOAT octanoylation analysis. GOAT assays using the (-) membrane fractions (A) lacks the peak at 12.5min present in the (+) fraction assay (B). This peak was isolated and analyzed via mass spectrometry (C), which shows the octanoylated peptide with Na^+ and K^+ adducts.

GOAT Octanoylation of GSSFLC-AcDan

GSSFLC-AcDan was tested as a substrate for expressed GOAT by incubation in the presence of membranes that expressed GOAT as described in Experimental Details; membranes from Sf9 cells expressing β-glucuronidase serve as a negative control. As seen in Figure 6, incubation with the (-) membrane fractions did not alter the GSSFLC-AcDan peptide. However, incubation with hGOAT-containing

membranes produces a new peak with a longer retention time. This shift in retention time in reverse-phase HPLC is consistent with the increase in substrate peptide hydrophobicity upon octanoylation. Mass spectrometry of the new peak with longer retention time confirmed it to be octanoylated GSSFLC-AcDan.

Inhibition with Metal Chelators

In addition to being inhibited by octanoyl-[Dap3]-ghrelin, a known GOAT inhibitor, GOAT is also inhibited by the addition of metal chelators to the reaction. Assays have been performed with EGTA (ethylene glycol tetraacetic acid) and 1,10-phenanthroline. EGTA was found to partially inhibit GOAT activity, while 1,10-phenanthroline efficiently and completely inhibits GOAT activity. These data suggest that a metal ion may be required for GOAT- catalyzed ghrelin acylation.

Structure-activity Studies of ghrelin recognition by hGOAT

Following the development of our fluorescent peptide substrate for hGOAT, this substrate was used to investigate hGOAT recognition of ghrelin focusing on interactions with the glycine 1 (G1) and serine 2 (S2) residues of the GSSFL-sequence. In these assays, a reaction with

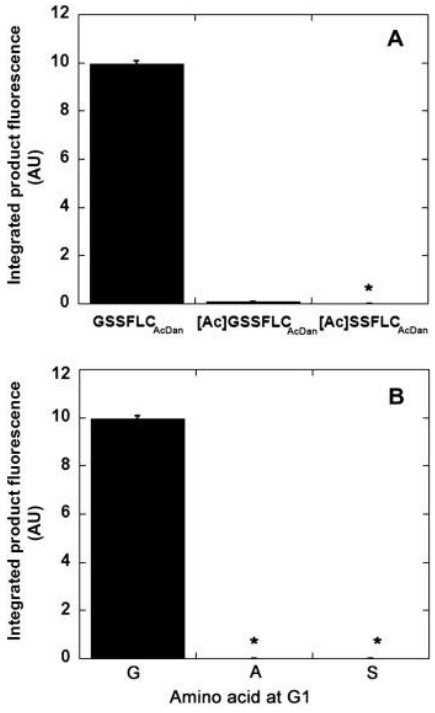

Figure 7: Acetylation of GSSFLC-AcDan and SSFLC-AcDan inhibit GOAT acylation. These results cumulatively suggest the importance of the free N-terminal amine and lack of an α-sidechain as key recognition factors for GOAT.

unmodified GSSFLC-AcDan was always run in parallel to control for changes in enzyme activity, substrate degradation, and other potential factors.

Previous studies of mouse GOAT have indicated that the N-terminal glycine of ghrelin is necessary for GOAT activity.[8] To identify the specific interactions involved in recognition of this glycine, three potential recognition elements of this glycine residue were analyzed: the N-terminal amine, the carbonyl, and the lack of steric bulk on the side chain. These recognition elements were separately tested by assaying GOAT activity with two acetylated substrates, [Ac]GSSFLC-AcDan and [Ac]SSFLC-AcDan. The former removes the N-terminal amine by forming an amide and adds steric bulk, while the latter only removes the N-terminal amine. To test the effect of steric bulk at this position, substrates SSSFLC-AcDan and ASSFLC-AcDan were also analyzed, which add methyl or hydroxymethyl side chains at the alpha carbon as compared to glycine. As shown in Figure 7, a severe loss of activity was observed with all modifications, resulting in either no activity or extremely low activity in the case of [Ac]GSSFLC-AcDan. This indicates that the N-terminal amine, in addition to a lack of steric bulk on the first amino acid, is important for substrate recognition by hGOAT.

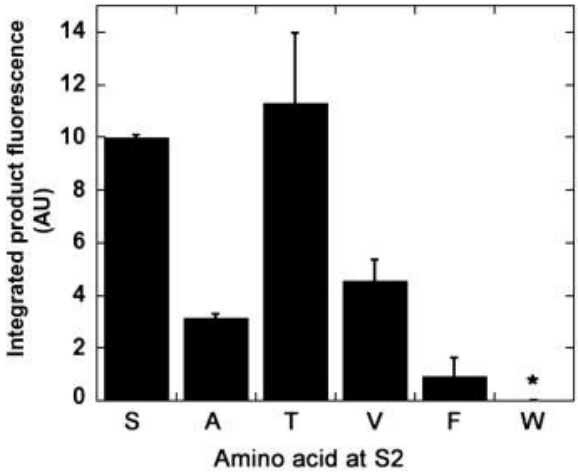

Figure 8: Mutating the S2 residue results in levels of activity that vary with steric interactions and the presence of a hydroxyl on the X2 sidechain.

GOAT recognition at the S2 residue was tested by mutating this serine to an alanine, threonine, valine, phenylalanine, and tryptophan. Alanine removes the polar hydroxyl group while slightly decreasing steric bulk, threonine maintains the polar hydroxyl while increasing sterics, and valine removes the hydroxyl while increasing sterics. Greater steric changes were also tested through phenylalanine and tryptophan. The results of these experiments are shown in Figure 8, which indicates that the presence of a hydroxyl group at the second position of the GSSFL- sequence is important for GOAT recognition. Activity also decreases with increasing side chain size, suggesting the possibility of steric crowding the serine 2 binding pocket. The loss of activity upon increase in steric bulk at serine 2 is consistent with the lack of activity observed for a substrate with acrylodan at this position (GC(AcDan)SFLS).

These results, in addition to data from previous studies reporting the importance of the F4 residue, allow for us to begin to construct a basic model of

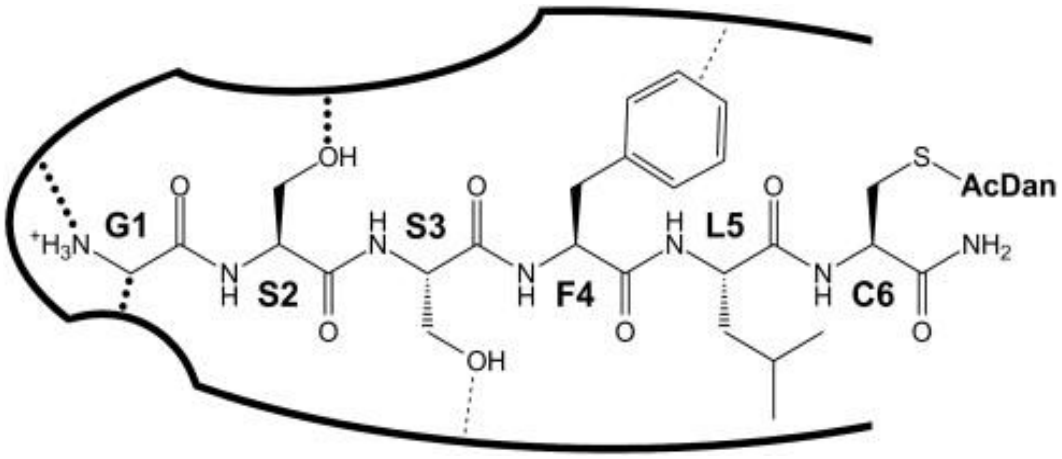

Figure 9: A schematic model of the GOAT-substrate complex can be established based on the findings of this research. The knowledge of these interactions can help in the design of specific novel inhibitors of GOAT to treat disease.[5]

the interactions that occur between the GOAT active site and GOAT substrates, as shown in Figure 9.[8,11,12] The N-terminal glycine is recognized based on at least two properties/groups: the free amine at the N-terminus, and the lack of steric bulk at the alpha carbon. At the second amino acid position, the presence of a hydroxyl group is aids in ghrelin recognition and modification by hGOAT. There is also a steric constraint at the S2 position, but that this constraint is less severe than at the N-terminal glycine.

IV. Experimental Details

Sf9 Expression: Annealing of FLAG, Myc, HA oligonucleotides for ligation

The sense and antisense oligonucleotides for each tag gene insert (Myc, HA, FLAG) were annealed in 10μL total volume, with the forward (sense) and reverse (antisense) oligonucleotides (50 pmol in 1μL each) added to ultra pure water (8μL). Each mixture was mixed by vortex, spun down, and annealed using a BioRad MyCycler thermocycler with the following program: 94˚C, 5 min; two cycles of 94˚C, 30s; 80˚C, 5 minutes; 72˚C, 7 min; 66˚C, 7 min; 60˚C, 3 min; 52˚C, 3 min; 46˚C, 3 min; 37˚C , 20 min; final hold at 4˚C. This process anneals the corresponding primers together to form the DNA sequence for the desired affinity tag with *Xho*I and *Xba*I cut sticky ends on the 5’ and 3’ ends of the annealed duplex. These annealed products can then be ligated directly into the cut pUC57/MBOAT4i vector, and are frozen at -20°C until needed.

Restriction Enzyme Digestion

20μL digests are set up by combining 10X New England Biolabs Buffer 4 (2μL), 10X Bovine Serum Albumin (2μL), DNA (500-1500ng), and the appropriate restriction enzymes (1μL, 20 units), then voluming up to 20μL with ultrapure water. To cut out MBOAT4-X EcoRI and XbaI are used, while XhoI and XbaI are use to only cut out the X-tag. The range in amount of DNA and restriction enzyme is due to the purpose of the digest: if only for analytical purposes 500ng is used, but if DNA will be extracted and used in a ligation more is digested. All digests are run for 2h at 37°C, followed by analysis and purification of the vector digestion by agarose gel electrophoresis (0.8% agarose, 1X TAE buffer); the size of the inserts and linearized vector were verified by comparison to a DNA standards ladder. Double digested DNA was purified using the EZ-10 Column PCR Purification Kit (Bio Basic Inc.) per the manufacturer's instructions.

DNA Ligation and DH5α Transformation

Ligations were performed using a 1:3 molar ratio of vector to insert. Ligation reactions were performed under the following conditions: for pUC57/MBOAT4 and the X-tag (20μL total volume), *Xho*I-*Xba*I double digested pUC57/MBOAT4 (50ng), annealed X-tag oligomer reaction (50pmol), 2x Quick Ligase buffer (10μL), T4 Quick Ligase (New England Biolabs, 1μL), and ultra pure H_2O; for pFBD and MBOAT4-X (20 μL total volume), *Eco*RI-*Xba*I double digested pFBD (50ng), *Eco*RI-*Xba*I double digested MBOAT4-X (50ng), 2x Quick Ligase buffer (10 μL), T4 Quick Ligase (New England Biolabs, 1 μL), and

ultra pure H_2O. Following addition of Quick Ligase, reactions were incubated at room temperature for 5 minutes. Ligation mixtures (4μL) were then transformed into a 50 μL aliquot of chemically competent Z-competent DH5α cells (Zymo Research) followed by incubation on ice for 10 minutes. Transformed bacteria were spread on LB-ampicillin plates (100μg/mL) and incubated at 37 °C overnight.

Following overnight incubation at 37 ^{o}C, single colonies were inoculated into LB media (5 mL) containing ampicillin (100μg/mL) in sterile culture tubes. These cultures were incubated overnight at 37 °C with shaking (225 RPM). Following overnight growth, plasmids were purified from the saturated cultures using EZ-10 Spin Column Plasmid DNA kit (Bio Basic Inc.) per manufacturer's instructions. Inserts were verified by double digestion with *EcoR*I and *Xba*I to excise the ligated insert followed by agarose gel electrophoresis and DNA sequencing (Genscript).

Transformation into DH10Bac Cells, Blue-White Screening

To generate bacmid for transfecting into Sf9 insect cells, pFastBacDual vectors were transformed into DH10Bac *E. coli* cells by adding chilled plasmid (400ng) to an aliquot of Z-competent DH10Bac cells (100μL), followed by incubation on ice for 5 minutes. The transformed bacteria were then spread onto LB agar plates containing kanamycin (50μg/mL), tetracycline (10μg/mL), gentamicin (7μg/mL), IPTG (40μg/mL), and XGal (100μg/mL). Plates were incubated at 37 °C for 48 hours, at which point bacteria with successful bacmid

recombination were identified by blue/white screening. White colonies were re-streaked onto LB agar plates containing kanamycin, tetracycline, gentamicin, IPTG, and XGal and incubated for 48 hours at 37 °C to verify bacmid recombination by blue/white screening.

The presence of X-gal in the plate media enables determination of successful transformation by blue-white screening, where colonies possessing the recombinant DNA are clear while those without are blue. This difference is due to a gene in DH10Bac bacmid genome that codes for β-galactosidase, of which X-gal is a substrate. If transformation is successful, the MBOAT4i-X gene is inserted in the middle of the gene coding for β-galactosidase, preventing synthesis of the enzyme and keeping all X-gal in the colony intact. If transformation is unsuccessful, β-galactosidase will cleave X-gal, forming a blue chromophore as a side-product (Figure 10). This allows one to easily distinguish and select for transformed plasmid.

Clear colonies are picked and re-streaked on identical plates and grown up for another 2 days, to ensure there are

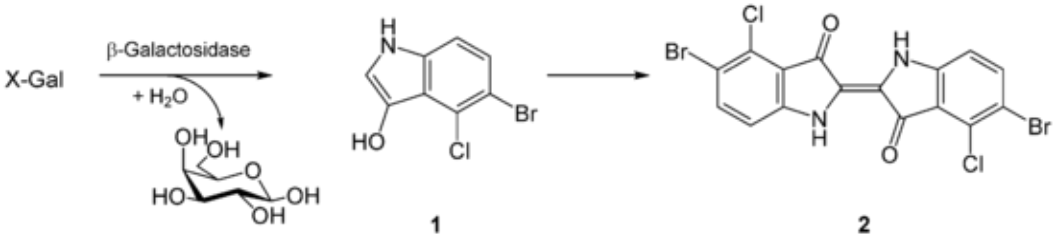

Figure 10: X-gal cleavage leads to chromophore production. (1) is an indole derivative, with (2) being a dimerized product between two cleaved indole molecules. (2) is the chromophore that produces the blue coloration.

no non-recombinant bacmid colonies. The clear colonies from these plates are picked and grown up in, then their recombinant bacmid is purified using a Macherey-Nagel Nucleobond Bac100 Kit. This purified DNA is then ready to be transfected into Sf9 insect cells for expression.

GOAT Assay Fluorescent Substrate Development

To synthesize the acrylodan-labeled substrate, the short synthetic peptide and acrylodan are combined in 1mL of 1:1 50mM N-(2-hydroxyethyl)piperazine-N'-(20hydroxypropanesulfonic acid) buffer (pH 7.8)/acetonitrile, with final concentrations being 50μM peptide and 500μM acrylodan. This reaction mixture is allowed to incubate overnight at room temperature covered in aluminum foil to prevent photobleaching of the acrylodan. During this process, the acrylodan conjugates to the free thiol of a singular cysteine residue on the peptide through the Michael Addition reaction, as shown in Figure 11.

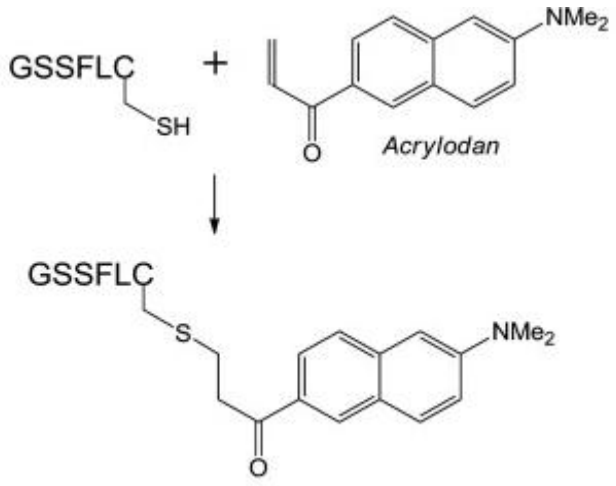

Figure 11: Substrate formation. The α,β-unsaturated ketone of acrylodan reacts with the free thiol on the ghrelin mimic GSSFLC. At pH = 7.8 this thiol is close to 50% deprotonated, helping to push the reaction further.

Acrylodan-labeled peptides are then purified by reverse phase HPLC on a 9.4mm x 250mm column using a mobile phase of water containing

0.05% trifluoroacetic acid (TFA) (65%) and acetonitrile (35%) flowing at 4.2 ml/min over 21 min. Labeled peptides eluting at approximately 6 to 10 min are easily detected by UV absorbance at 360nm and fluorescence at 485nm. As well, fractions of the initial peptide-acrylodan labeling reactions were analyzed by MALDI–TOF mass spectrometry to verify the presence of the product at the appropriate retention time. This was done using a matrix of saturated sinapinic acid in 0.1% TFA and 50mM ammonium phosphate, mixed in a 1:1 volume with the fraction sample.

GOAT Activity, Inhibition, Substrate Selectivity

The assay for GOAT activity is carried out by combining octanoyl-CoA, the fluorescent peptide substrate of choice, palmitoyl-CoA, and membrane fractions of Sf9 cells that have expressed GOAT and allowing the enzyme to carry out the acylation reaction using octanoyl-CoA. During inhibition experiments, the desired inhibitor is also added, with the fluorescent substrate always being GSSFLC-acrylodan, the sequence that mimics natural ghrelin.

Membrane fractions from Sf9 cells expressing hGOAT are thawed on ice and passed through an 18-gauge needle 10 times. Membranes are then centrifuged (1000*g*, 1 min), with the supernatant collected and added to hGOAT reactions. Assays are performed with 50μg of membrane protein, 1.5μM labeled peptide substrate, 500μM octanoyl-CoA, 100μM palmitoyl-CoA, and 50mM HEPES (pH 7.0) in a total volume of 50μl, with the substrate being added last to initiate the acylation reaction. Assays comparing the reactivity of different peptide substrates

were performed in parallel using the same stock of membrane protein and other reaction components, with peptide substrates added last. All assays are incubated at room temperature and stopped by the addition of 50µl of 20% acetic acid/isopropanol to halt enzymatic activity. Addition of the stop solution prior to substrate addition resulted in no activity.

Assays are analyzed by reverse phase HPLC with a 4.6 × 150mm column, using a gradient from 30% acetonitrile in water containing 0.05% TFA to 63% acetonitrile in water containing 0.05% TFA flowing at 1 ml/min over 14 min, followed by 100% acetonitrile for 5 min. Acrylodanylated peptides were detected by UV absorbance at 360 nm and fluorescence (λ_{ex} = 360 nm, λ_{em} = 485 nm). Peptide substrates typically elute with a retention time of 5 to 7 min (approximately 42–46% acetonitrile), with the octanoylated peptide product eluting at approximately 12 min (~58% acetonitrile). MALDI-TOF mass spectrometry was also used to initially determine presence of the acylated substrate in the 12min fraction, with matrix conditions identical to those used in the peptide labeling reaction analysis.

To determine the percent conversion of the acylation reaction, peaks on the HPLC Fluorescence Chromatogram corresponding to unacylated peptide substrate and acylated peptide substrate are integrated using Chemstation for LC (Agilent Technologies). The percent of total substrate reacted is determined by dividing the area of the emission peak at 485nm of the 12min peak by the total area of the 5-7min peak and the 12min peak, as shown below:

$$\%\ conversion = \frac{area\ of 12\ \text{min}\ peak}{(area\ of\ 5-7\ \text{min}\ peak + area\ of 12\ \text{min}\ peak)}$$

Fluorescence is used instead of UV absorbance due to the lower detection limit of the lab fluorometer compared to the lab UV spectrophotometer. For reactions involving GOAT inhibitors, the degree of enzyme activity when comparing GOAT with inhibitor to GOAT without inhibitor is calculated as follows:

$$\%\ activity = \frac{\%\ conversion\ with\ inhibitor}{\%\ conversion\ without\ inhibitor}$$

V. Conclusion:

I have worked toward developing the tools that will aid future researchers in the Hougland Lab in determining the structure and catalytic mechanism of the GOAT active site. The fluorescence-based assay I have developed along with my colleagues allows for structure-activity studies of hGOAT-catalyzed ghrelin acylation to investigate GOAT substrate specificity and catalytic mechanism. Our assay also provides the capability for rapid screening of potential hGOAT inhibitors. These tools developed in my research will aid in hGOAT inhibitor design, with these inhibitors potentially serving as therapeutics for diseases such as obesity, Prader-Willi Syndrome and diabetes.

References

1. Kojima M., Hosoda H., Date Y., Nakazato M., Matsuo H., and Kangawa K. **Ghrelin is a growth-hormone-releasing acylated peptide from stomach**. Nature, 402 (1999), pp. 656–660
2. Kojima M., and Kangawa K. **Structure and function of ghrelin**. Results Probl. Cell Differ., 46 (2008), pp. 89–115
3. Heppner K.M., Tong J., Kirchner H., Nass R., and Tschop M.H. **The ghrelin *O*-acyltransferase–ghrelin system: a novel regulator of glucose metabolism**. Curr. Opin. Endocrinol. Diabetes Obes., 18 (2011), pp. 50–55
4. O'Brien M., Earley P., Morrison J.J., and Smith T.J. **Ghrelin in the human myometrium**. Reprod. Biol. Endocrinol., 8 (2010).
5. Gahete M.D., Cordoba-Chacon J., Kineman R.D., Luque R.M., and Castano J.P. **Role of ghrelin system in neuroprotection and cognitive functions: implications in Alzheimer's disease**. Peptides, 32 (2011), pp. 2225–2228
6. Gutierrez J.A., Solenberg P.J., Perkins D.R., Willency J.A., Knierman M.D., Jin Z., Witcher D.R., Luo S., Onyia J.E., and Hale J.E. **Ghrelin octanoylation mediated by an orphan lipid transferase**. Proc. Natl. Acad. Sci. USA, 105 (2008), pp. 6320–6325
7. Yang J., Brown M.S., Liang G., Grishin N.V., and Goldstein J.L. **Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone**. Cell, 132 (2008), pp. 287-296
8. Yang J., Zhao T.J., Goldstein J.L., and Brown M.S. **Inhibition of ghrelin *O*-acyltransferase (GOAT) by octanoylated pentapeptides**. Proc. Natl. Acad. Sci. USA, 105 (2008), pp. 10750–10755
9. Darling J.E., Prybolsky E.P., Sieburg M., and Hougland J.L. **A fluorescent peptide substrate facilitates investigation of ghrelin recognition and acylation by ghrelin *O*-acyltransferase**. Anal Biochem, 2013. *In Press.*
10. Hibbs R.E., Talley T.T., and Taylor P. **Acrylodan-conjugated cysteine side chains reveal conformational state and ligand site locations of acetylcholine-binding protein**. J Biol Chem, 279 (2004), pp. 28483-91
11. Ohgusu H., Shirouzu K., Nakamura Y., Nakashima Y., Ida T., Sato T., and Kojima M. **Ghrelin *O*-acyltransferase (GOAT) has a preference for *n*-hexanoyl-CoA over *n*-octanoyl-CoA as an acyl donor**. Biochem. Biophys. Res. Commun., 386 (2009), pp. 153–158
12. Barnett B.P., Hwang Y., Taylor M.S., Kirchner H., Pfluger P.T., Bernard V., Lin Y.Y., Bowers E.M., Mukherjee C., Song W.J., Longo P.A., Leahy D.J., Hussain M.A., Tschop M.H., Boeke J.D., and Cole P.A. **Glucose and weight control in mice with a designed ghrelin *O*-acyltransferase inhibitor**. Science, 330 (2010), pp. 1689–1692

Capstone Summary

After the human genome was sequenced in 2003, it was revealed that human DNA only coded for approximately 30,000 genes. This was surprising to many in the scientific community, as the human proteome was predicted to contain 10- to 100-fold higher diversity than that predicted within the genome. One potential explanation for the difference between the number of genes and the number of chemically distinct protein forms encountered in humans (and other complex organisms) is posttranslational modification (PTM).

PTMs are chemical modifications to proteins after they are synthesized by the ribosome. These modifications can introduce new functionality and alter protein structure, leading to new reactivity. Post-translational modifications play a central role in biology and have been linked to human diseases like cancer, cardiovascular disease, and obesity. In Dr. Hougland's lab, my research focuses on developing a better understanding of the enzymes (proteins that perform biological reactions) that carry out these modifications. My project focuses on acylation of the hormone ghrelin by the enzyme ghrelin *O*-acyltransferase (GOAT).

Ghrelin is a small protein hormone that stimulates hunger. One key step in activating ghrelin is its acylation by GOAT, a reaction where the enzyme reacts with a fatty acid group (called octanoyl-CoA) and attaches it onto the hormone. This PTM allows the now-activated ghrelin molecule to bind to its receptor, GHSR-1a, and activate hunger signaling.[2] There is also evidence suggesting ghrelin is involved in regulating glucose metabolism, uterine cell signaling, and

memory.[3-5] With this wide range of potential roles for ghrelin in biology, our studies of ghrelin acylation by GOAT have the potential to impact multiple research areas and diseases through aiding in the development of drugs that stop GOAT activity. Drugs that block GOAT-catalyzed ghrelin acylation could be used as potential anti-obesity treatments. Inhibiting ghrelin acylation could also be involved in treating other diseases affiliated with ghrelin such as diabetes and Prader-Willi Syndrome.

The enzyme that catalyzes ghrelin octanoylation, ghrelin *O*-acyltransferase (GOAT), was discovered in 2008.[6,7] GOAT is an integral membrane protein, with the folded protein associating with cell membranes. Studying integral membrane proteins introduces many complications in enzyme expression and characterization, as it is very difficult to purify these types of proteins from the cell membrane. Because of this, not much is known about the structure of GOAT or its chemical mechanism of acylation.

For the past two years, my research in the Hougland lab has focused on developing tools that can be used in the basic characterization of GOAT-catalyzed ghrelin acylation. The first set of tools I have constructed are several affinity-tagged versions of human GOAT (hGOAT), which allows for expression and purification in Sf9 insect cells. The second is the development of a novel fluorescence-based assay (or test) for hGOAT activity. This assay utilizes a fluorescently labeled peptide substrate that permits structure-reactivity analysis of hGOAT activity. Using a series of synthetic fluorescent ghrelin mimics, we have been able to identify specific groups on ghrelin that potentially contribute to its

recognition by GOAT. This data can be used in future studies to help elucidate the GOAT active site structure and design drugs to inhibit GOAT activity.

GOAT Expression

The lab chose to express hGOAT in *S. frugiperda* insect cells mainly due to the fact that there have been previous examples of this cell type being used for GOAT expression in the literature.[8] My contribution to this project involved the separate engineering of three affinity tags onto the end of GOAT: FLAG, Myc, and HA. Each of these tags is a unique sequence of amino acids that can be selectively recognized by antibodies. Antibody recognition is a key tool in protein expression studies, as it can be used to isolate the desired protein from all the other proteins made in the cell. This would allow researchers in the Hougland lab to easily identify that GOAT has been expressed, something that has been difficult in the past.

This project was carried out by taking artificial DNA that codes for the different Myc, HA, and FLAG protein tags and attaching them separately to the end of the DNA encoding hGOAT. When this gene is expressed by the Sf9 cells, a GOAT-X protein conjugate (where X is either the Myc, FLAG, or HA) will be synthesized. This conjugate can then be bound by antibodies designed to recognize the specific tags. This project was successfully completed, with my synthesis of the GOAT-X DNA constructs being confirmed through gel electrophoresis and DNA sequencing.

Fluorescence Assay

While working on expressing GOAT, I also worked to develop a new assay to analyze GOAT reactivity. The designed assay uses a shortened mimic of ghrelin with a fluorescent molecule called acrylodan attached to the end. When the ghrelin mimic (called a substrate) is acylated by GOAT, a fatty acid chain is added to it. This fatty acid makes the peptide less polar, which allows for separation from the unacylated substrate and also increases the fluorescence of the fluorescent tag.[10] These changes in substrate properties upon acylation can be measured to determine how much ghrelin mimic has reacted, how quickly this reaction occurred, and properties of GOAT-catalyzed acylation.

The first step in developing this assay was designing a synthetic scheme to create the fluorescent ghrelin mimic substrate. The synthesis is done in one step, where the fluorescent acrylodan group is attached to a specific region of the ghrelin mimic. The new fluorescent substrate is then purified using Reverse Phase High Performance Liquid Chromatography (RP-HPLC), a technique that can separate different molecules based on their polarity. The acrylodan group makes the substrate less polar than the unreacted ghrelin mimic, so the produced fluorescent ghrelin mimic can be easily separated and purified. We also use a fluorescence detector during this process, which allows us to observe fluorescence emission as molecules pass through it. This allows us to quickly determine when to purify our fluorescent substrate.

After successfully synthesizing the fluorescent substrate and expressing hGOAT, I was then able to test the validity of the assay by incubating the substrate with hGOAT and octanoyl-CoA and injecting the reaction on the HPLC. A new fluorescence peak not associated with any of the reactants was observed, later confirmed to be the acylated ghrelin mimic. Knowing that the assay worked, I was then able to move forward designing and carrying out experiments that tested GOAT's substrate specificity and inhibition of activity by small molecules.

I have been able to test GOAT substrate specificity by changing the amino acid sequence of the fluorescent ghrelin mimic so that it does not correspond with the natural sequence of ghrelin. These substrates are reacted with GOAT and analyzed to see if GOAT would acylate a molecule with different molecular structure and characteristics than natural ghrelin. These experiments indicate new natural amino acid sequences GOAT could recognize, which leads to the conclusion that there are other proteins in the body GOAT may modify. In addition, my work sheds light on the kinds of molecular interactions that are occurring between GOAT and its substrates that allows for the acylation reaction to occur.

GOAT inhibition is tested by incubating the traditional fluorescent ghrelin mimic with GOAT, octanoyl-CoA, and the compound being tested for inhibitive properties. If the peak corresponding to the acylated substrate does not appear when the reaction mixture is injected on the HPLC, then the compound is indicated to be an inhibitor of GOAT. This information is useful when considering the design of specific GOAT inhibitors for potential drug candidates.

Two metal chelators (molecules that bind metal ions) I have analyzed using this method have both successfully inhibited GOAT. These results imply that a metal ion may be needed either for GOAT reactivity or to maintain GOAT structure.

Conclusion

The experiments I have completed over the past two years in the Hougland lab lay the foundation for future studies of GOAT-catalyzed ghrelin acylation. In addition to constructing protein conjugates of GOAT to assist in purification, I have also developed a novel fluorescent assay for GOAT activity. As well, I have successfully used this assay to determine characteristics of GOAT including substrate specificity as well as molecules that are capable of inhibiting it. These results shed light on GOAT's structure and catalytic mechanism, key information that is needed to design inhibitors that can decrease hunger and potentially cure life-threatening diseases such as obesity, diabetes, and Prader-Willi Syndrome, in the near future.