Methods to Study Tubulin S-glutathionylation

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Methods to Study Tubulin S-glutathionylation

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelors of Science in Chemistry from the College of William and Mary

By Anna Jean Wirth

Accepted for: ______________________
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Abstract

The effects of oxidative damage and the mechanisms through which it is repaired have been a topic of intense research for several years. Research has shown that small changes in the redox state of cells can lead to protein modification which, in turn, can result in changes in protein function and activity. This thesis discusses the development of a method to study tubulin S-glutathionylation that avoids several of the inherent technical difficulties in studying oxidative modifications of tubulin. Biotinylated GSSG (oxidized glutathione) was synthesized, purified, and incorporated into tubulin via thiol-disulfide exchange. S-glutathionylated tubulin was isolated on an avidin-agarose resin by utilization of biotin’s affinity for avidin. This biotin affinity capture method allows for future quantification of S-glutathionylation in tubulin as well as exploration of its role in polymerization regulation.
Introduction

Protein Oxidation

Aerobic respiration, the process through which humans and other higher organisms produce ATP, is achieved through electron transfer to oxygen and subsequent reduction of O₂ to water. The transfer of electrons to oxygen is highly efficient. However, 1-2% of electrons are leaked during this process, and reactive oxygen species are inadvertently produced\(^1\). Superoxide (\(\text{O}_2^-\)), hydrogen peroxide (\(\text{H}_2\text{O}_2\)), and the hydroxyl radical (\(\text{OH}^-\)), are the most common of these and can harmfully oxidize biomolecules\(^1\). Many other biological processes in addition to aerobic respiration also produce reactive oxygen species. For example, oxidative damage is often detected in human disease, and the proteins NADPH oxidase, lipoxygenase, and cyclooxygenase have been reported to produce radical byproducts\(^1\).

Reactive oxygen species can cause considerable damage to cells via protein oxidation. Generally, protein oxidation can lead to defects in conformation or tertiary structure that result in a decrease or complete loss of protein function\(^2\). Additionally, some oxidized proteins are targeted heavily for degradation by proteinases and thus have a shorter lifespan than their undamaged counterparts\(^2\). Alternatively, oxidation of cysteine residues, in particular, can lead to the formation of disulfide bonds between two or more proteins. This creation of higher order species can result in pathological protein aggregates that are resistant to proteinase degradation\(^2\). Not surprisingly, increasing or decreasing the natural lifespan of any protein by any mechanism can have a deleterious effect on the cell. In Alzheimer’s disease this is the case, and it is protein aggregates that...
lead to the diagnostic symptoms of amyloid plaques and neurofibrillary tangles found in the brain.

Protein Oxidation in Alzheimer’s Disease

Protein deposits in the brains of Alzheimer’s patients were first detected by scientists in the mid nineteenth century and correlated to dementia, although their structure and protein composition was not understood until the 1970s. Neurofibrillary tangles are one of two types of protein deposits found throughout the brains of Alzheimer’s patients. Tangles have a distinct morphology of paired helical filaments that associate into a dense protein mass via non-covalent interactions. While their protein composition varies, much of the aggregated protein consists of Tau, a microtubule associated protein. Indeed, many inherited forms of Alzheimer’s disease are traced back to mutations in this protein, and a hyperphosphorylated form is thought to aggregate more readily.

Amyloid plaques, with their beta sheet structure, are the most well studied protein aggregate associated with Alzheimer’s disease. They are similar to neurofibrillary tangles in their defined structure and insolubility, but their primary component is the incorrectly cleaved amyloid precursor protein (APP). Interestingly, several sources have linked this pathological form of APP, also known as the Abeta peptide, to the oxidative damage seen in the brains of Alzheimer’s patients. However, the exact relationship between Alzheimer’s disease pathology and oxidative damage is still incompletely understood.
Alzheimer’s patients generally show symptoms indicative of the presence of damaging reactive oxygen species in the brain, but it is not apparent whether these are the root of neurodegeneration, a step in the pathway leading towards the loss of neurons, or merely a side effect of the widespread neuronal cell death. One study, which used carbonyl formation as a marker of oxidative damage, found that proteins associated with the cytoskeleton were more likely to have oxidative damage in Alzheimer’s brains\(^2\). Interestingly, they found oxidative damage in both degenerative and healthy neurons, suggesting that oxidative damage might be a causative agent in neural degeneration rather than just a byproduct of the process\(^2\). Similarly, a comprehensive review of the role of oxidative damage in Alzheimer’s disease suggested that the formation of amyloid plaques and neurofibrillary tangles may be driven by intense oxidative stress\(^7\). They postulated that these aggregates may merely be an oxidative species “sponge”—a hypothesis that challenges a body of research that implicates protein aggregates as the primary neurophysiological factor leading to widespread neurodegeneration. They went further to identify several possible sources of reactive oxygen species in Alzheimer’s patients including mitochondrial malfunction, improper lipid peroxidation, and issues with cell cycle arrest\(^7\).

However, numerous other sources have suggested that oxidative damage might be merely a destructive byproduct of Alzheimer’s disease. For example, several studies have found that mice and C. elegans over expressing the Abeta peptide show increased levels of protein oxidation\(^5\). Furthermore, it has been demonstrated that one particular residue in the Abeta peptide, a methionine, is essential for the increased levels of protein oxidation seen in Abeta peptide knock up model organisms. This suggests that there may
be a distinct molecular mechanism for Abeta peptide mediated oxidative damage\textsuperscript{5}.

Regardless of which point in the pathology of Alzheimer’s disease that oxidative species play a part, it is abundantly clear that their study is relevant and necessary. Accordingly, this thesis will focus on oxidative modification of cysteines, how these changes can lead to alterations in the neuronal cytoskeleton, and ultimately how this understanding can impact the field of Alzheimer’s disease research.

The Glutaredoxin System

Cells throughout the body have pathways for returning oxidized cysteines to their native, reduced state. The well studied superoxide dismutase and catalase are examples of general anti-oxidant enzymes that convert reactive oxygen species to less harmful derivatives before they cause damage\textsuperscript{1}. However, there are several systems that are devoted exclusively to preventing or repairing damage to cysteine residues. The most important of these are the thioredoxin reductase system (TRS) and the glutaredoxin reductase system (GRS).

The GRS is a system that protects and rescues sensitive cysteine residues in proteins from harmful oxidation, and consists of glutathione (GSH), glutaredoxin (Grx), glutathione reductase, and NADPH\textsuperscript{7,8}. GSH, is a tripeptide that exists in a redox couple with its oxidized form, GSSG\textsuperscript{8,9}. Because GSH can be oxidized to GSSG during times of oxidative stress, the cellular concentration of these species serves as a buffer that can regulate the oxidative state of the cell\textsuperscript{8}. That is, the reduced form of glutathione is an easy target that can oxidized by reactive oxygen species in place of proteins. In the brain, this buffer system is particularly important because the brain has a very high
concentration of reactive oxygen species. Despite being only 2% of the overall body weight, the brain uses 20% of the body’s oxygen, and this prolific use of oxygen leads to high concentrations of reactive oxygen species\(^{10}\). Even further adding to the load of reactive oxygen species in the brain are high concentrations of polyunsaturated fatty acids and metal ions that can undergo radical reactions readily, hence facilitating the process of reactive oxygen species propagation\(^5\). Because there are few antioxidant enzymes in the brain, the glutathione redox couple is the primary way through which the brain guards itself against oxidative damage\(^5\).

Rounding out the GRS are several key enzymes. Glutathione reductase reduces GSSG back to GSH thus maintaining the buffered state of the cell\(^8\). This reduction uses NADPH as an electron donor\(^9\):

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

Glutaredoxin is the other important enzyme in this system. It has a disulfide bond in its active site which undergoes thiol/disulfide exchange to catalyze the reduction of substrate disulfide bonds\(^{11}\).

Grx can reduce oxidized cysteine residues that are in disulfide bonds with glutathione groups and can add glutathione groups to cysteine residues\(^9\). One mechanism of disulfide bond reduction is thiol/disulfide exchange:

\[
P(\text{SS}) + \text{GSH} \rightarrow P(\text{SH})_2 + \text{GSSG}
\]

While this process can occur directly in the cytoplasm, the more common mechanism occurs through catalysis in the active site of Grx\(^{11}\). The step by step mechanism for this process as it is catalyzed by Grx is as follows\(^{11}\):

\[
P(\text{SS}) + \text{Grx(red)} \rightarrow P(\text{SH})_2 + \text{Grx(ox)}
\]
Grx(ox)+2GSH=>Grx(red)+GSSG

Generally, any disulfide bond can be reduced directly by the TRS\textsuperscript{12, 13}. The GRS, however, requires a mixed disulfide as the substrate. Mixed disulfides are formed by several mechanisms, but the most straightforward is thiol/disulfide exchange\textsuperscript{12, 13}:

\[
PSSP + GSH \rightarrow PSSG + PSH
\]

This initial step in the reduction of a disulfide, the addition of glutathione to a cysteine residue, is also referred to as S-glutathionylation. Because S-glutathionylation, often a signaling mechanism, can be both catalyzed and reversed by Grx, this enzyme has implications in both reduction of oxidative damage and in mediating signal transduction\textsuperscript{8}.

There are several mechanisms through which S-glutathionylation can occur, although the dominant form in vivo is not yet known\textsuperscript{8}. As mentioned previously, thiol/disulfide exchange can result in S-glutathionylation\textsuperscript{11}:

\[
PSH + GSSG \rightarrow PSSG + GSH
\]

Other mechanisms through which S-glutathionylation can occur include direct oxidation:

\[
GSH + PSH \rightarrow PSSG + 2H
\]

and nitric oxide induction\textsuperscript{8}:

\[
PSNO + GSH \rightarrow PSSG + HNO
\]

Which mechanism dominates likely depends on cellular conditions. For example, thiol/disulfide exchange can only occur when high concentrations of GSSG are present such as during times of oxidative stress where the GSH/GSSG ratio is low\textsuperscript{8}.

Protein Regulation by S-glutathionylation

Somewhat analogously to phosphorylation of serine, threonine, and tyrosine residues, S-glutathionylation of cysteine residues has been associated with protein
S-glutathionylation (like phosphorylation) is reversible, which makes it a viable mechanism for cell signaling and regulation. This type of regulation is observed in many transcription factors. One of the first observed examples of regulation via S-glutathionylation was in the E. coli bacterium transcription factor OxyR. One cysteine residue in OxyR undergoes a variety of post-translational modifications—oxidation to a disulfide, nitrosylation, or S-glutathionylation—resulting in several different active forms of the transcription factor. These different forms bind DNA either at different strengths or at different sequences. Thus, post-translational modification of OxyR leads to different transcriptional responses that depend on the level and type of oxidative stress in the cell.

Another early example of protein regulation via S-glutathionylation are the enzymes associated with glycolysis, also known as the glycolytic enzymes. As early as 1984, it was noted the level of thiol-disulfide exchange effected the activity level of a few glycolytic enzymes. Later study led to the discovery that five of the glycolytic enzymes undergo S-glutathionylation including aldolase, GAPDH, phosphoglycerate kinase, enolase, and pyruvate kinase. Several of these show a reduction of activity in response to higher levels of S-glutathionylation. Because thiol-disulfide exchange leading to S-glutathionylation occurs primarily in cells with significant oxidative stress, this regulation suggests that the enzymes of glycolysis are responsive to the oxidative state of the cell. Indeed, it would be to a cell’s benefit to lower the amount of oxidative respiration, which can inadvertently produce reactive oxygen species, in circumstances where oxidative stress is high.

Recently, there have been many new examples of protein regulation via S-glutathionylation. In humans, these include protein tyrosine phosphatase, creatine kinase,
caspase-3, and actin. The role of S-glutathionylation in actin dynamics has been particularly well studied. In growth factor stimulated cells where actin polymerization activity is high, actin has been shown to be deglutathionylated\textsuperscript{16}. This suggests that actin polymerization correlates inversely with the amount of S-glutathionylation\textsuperscript{16}. Other cytoskeletal proteins also seem to be affected by S-glutathionylation. A recent study of S-glutathionylation in the central nervous system found that S-glutathionylated proteins are present throughout the cerebral cortex and the gray matter of the spinal cord\textsuperscript{10}. In particular, they found that two of the cytoskeletal proteins necessary for neuron viability—β-tubulin and actin—showed the highest amount of s-glutathionylation\textsuperscript{10}. This is consistent with results from the Landino lab that show that tubulin’s ability to polymerize into microtubules is sensitive to the oxidative state of its cysteine residues\textsuperscript{11}.

\textit{Microtubules}

Microtubules consist of α-β-tubulin dimers arranged in a head-to-tail polymerization pattern called a protofilament. The hollow microtubule structure is formed by the association of 13 of these protofilaments\textsuperscript{17}. Because the microtubule monomer is a dimer consisting of two different protein subunits, microtubules have polarity. This gives them the ability to grow and shrink from either end at different rates. The cap of the microtubule, called the “plus” end, has an exchangeable GTP molecule associated with β-tubulin\textsuperscript{18}. During the process of polymerization, this GTP is hydrolyzed to GDP and, upon association with the α-tubulin of another monomer, the nucleotide binding site is no longer exchangeable\textsuperscript{18}.

Microtubule dynamics are such that microtubules can grow and shrink quite readily. In a situation referred to as dynamic instability, microtubules depolymerize
rapidly from the plus end. There are several factors that can lead to dynamic instability such as the loss of the GTP-cap\textsuperscript{18}. There are also a number of microtubule binding proteins that can effect the rate of assembly and disassembly of tubulin subunits. Some of the most crucial microtubule associated proteins are Tau, MAP2, and MAP4, which comprise the tau protein family\textsuperscript{17}. Tau contributes to the stability of microtubules and can bind to their surface in order to facilitate formation of complex cellular structures. Similarly, MAP2 helps cross-link microtubules to other microtubules and to intermediate filaments. The microtubule binding ability of microtubule associated proteins is mediated by their reversible phosphorylation, and several kinases are responsible for regulating the assembly, disassembly, and maintenance of microtubules. In processes that require extensive microtubule mobility, such as mitotic cell division, MAP kinases are activated to initiate rapid microtubule dynamic changes\textsuperscript{17}. However, there are a number of non-MAP related regulators of tubulin polymerization besides the microtubule associated proteins, including alterations to individual protein residues.

\textit{S-glutathionylation and Tubulin}

Research suggests that S-glutathionylation is involved in microtubule polymerization regulation. The Landino lab has demonstrated that tubulin shows decreased polymerization activity following peroxynitrite damage. Tubulin’s polymerization ability was restored by addition of the GRS components\textsuperscript{7,11}. This demonstrates that tubulin’s cysteines are substrates for the GRS. Furthermore, following oxidative damage by ONOO\textsuperscript{−} and treatment with glutathione, tubulin shows S-glutathionylation of cysteine residues\textsuperscript{11}. As this S-glutathionylation was correlated with a decrease in tubulin’s polymerization ability, this suggests that S-glutathionylation is a
player in the regulation process of tubulin polymerization and, as follows, also microtubule polymerization.

From a structural and biochemical standpoint, it is plausible that tubulin polymerization could be affected by S-glutathionylation. Since the early 1990’s, tubulin’s sulfur based residues have been suggested as targets for anti-cancer and antimicrobial agents that would halt microtubule polymerization\textsuperscript{19}. Furthermore, tubulin’s polymerization abilities are tied to its three dimensional structure\textsuperscript{20}. Ravelli et al. found that a common inhibitor of tubulin polymerization (a chemotherapeutic molecule), Colchicine, functions by preventing tubulin dimers from forming a straight conformation. They demonstrated with crystal structures that a curved conformation of tubulin cannot polymerize\textsuperscript{20}. Thus, the three dimensional shape of the dimer is absolutely critical for proper microtubule formation. As post-translational modifications of proteins, such as S-glutathionylation, can alter protein conformation it is logical that they could have an effect on the ability of tubulin to polymerize. Indeed, there are examples of cytoskeletal proteins that have altered polymerization abilities upon reaction with glutathione\textsuperscript{16}.

\textit{Studying Tubulin S-glutathionylation}

While S-glutathionylation has been observed in tubulin, it has not yet been quantified\textsuperscript{11}. There are 20 cysteine residues present in tubulin, and understanding the specificity of their S-glutathionylation in response to oxidative stress is important to further elucidate the mechanism through which microtubule polymerization is regulated\textsuperscript{11}. Furthermore, little is known about the selectivity of S-glutathionylation in any system, thus quantifying this process in tubulin would be beneficial to scientific
knowledge in general\textsuperscript{15}. Ultimately, understanding the interplay between microtubule polymerization and oxidative stress will further benefit research in neurodegenerative disorders.

Studying tubulin S-glutathionylation from a biochemical perspective has several inherent challenges. Tubulin, due to the reactivity of its cysteines, tends to form disulfide bonds with other tubulin subunits under oxidative conditions. If a sample of tubulin is run on SDS-PAGE under non-reducing conditions, the result is a difficult to interpret smear. Biochemists avoid this issue by adding a reducing agent to a tubulin sample prior to running on SDS-PAGE which eliminates these higher order species. However, this is not possible when studying S-glutathionylation as addition of reducing agent would remove the attached glutathione groups. Thus, if S-glutathionylated tubulin is run under reducing conditions any tag that could be used for detection of S-glutathionylation would be lost prior to running the gel. One way to avoid this problem is through use of biotinylated glutathione.

Biotin, also known as vitamin H, has an extremely high affinity for the protein avidin\textsuperscript{21}. Because of this affinity and because biotin can be easily attached to biomolecules without disrupting their reactivity, biotin is typically used as a tag that allows for later separation of tagged species\textsuperscript{21}. Attaching biotin to a molecule is known as biotinylation, and modifications of biotin’s valeric acid side chain can facilitate the biotinylation process\textsuperscript{21}. A common alteration of the valeric acid side chain is to add an NHS group, a highly efficient leaving group. This addition makes the biotinylation of primary amines, a schematic of which is shown in Figure 1, highly efficient. Because
oxidized and reduced glutathione has a free primary amine, this process can be used to biotinylate the primary amines of oxidized glutathione.

Reduced glutathione has one primary amine that can be biotinylated. GSSG, the oxidized form of GSH, is composed of two GSH sub-units attached by a disulfide bond and thus has two primary amines that are open to biotinylation. The following summarizes the biotinylation reaction between GSSG and NHS-biotin

\[
\text{GSSG} + 2\text{NHS-biotin} \rightarrow \text{biotin-GSSG-biotin} + \text{NHS}
\]

This reaction can also yield a singly tagged product in addition to the doubly tagged oxidized glutathione\textsuperscript{22}. One can also synthesize BGSH by this method, but because BGSH air oxidizes readily to BGSSGB, it is more efficient to produce BGSSGB directly.

Biotinylated GSSG, abbreviated BGSSGB, can easily be incorporated via thiol disulfide exchange into tubulin. Because of biotin’s high affinity for avidin, the S-glutathionylated tubulin can be isolated from unmodified tubulin by incubation with an immobilized avidin resin. Treatment with DTT will reduce the disulfide bond between the GSH and tubulin and thus release the tubulin from the immobilized avidin. The tubulin can then be analyzed further by SDS-PAGE or isoelectric focusing. This method essentially “fishes” for S-glutathionylated protein thus avoiding issues with tubulin smearing and loss of label following reduction. The following thesis presents a developed and rigorously examined protocol to carry out this experimental approach.
Materials and Methods

Reagents

Oxidized glutathione, monoclonal anti-β-tubulin antibody, HABA dye, avidin protein, and anti-mouse IgG peroxidase were purchased from Sigma. NHS-biotin, Hypersep C18 solid phase extraction columns, and Supersignal West Pico chemiluminescent substrate were purchased from Thermo Scientific. Rapid fixer and Industrex manual developer were purchased from Kodak. The C8 solid phase extraction columns were purchased from Alltech. The avidin-HRP conjugate, the immobilized Neutravidin biotin binding resin, and the BCA assay were purchased from Pierce. All other reagents were from Fisher.

Purification of Tubulin

Tubulin was purified from porcine brains (Smithfield Packing Company) by Lisa Landino as outlined in Landino et. al23.

BGSSGB Synthesis

BGSSGB was synthesized by reaction of 125 µL of 100 mM NHS-biotin (DMF as solvent) with 312.5 µL 20mM GSSG (dH2O as solvent) in 812.5 µL 50 mM phosphate buffer pH 8.0. Final reactant concentrations were 10 mM NHS-biotin and 5 mM GSSG. In this scheme, there are two moles of NHS-biotin per mole of GSSG, thus both reactants will be completely consumed if the reaction goes to completion. Reaction was complete after 18 hours incubation at 37°C. Reaction completion was verified via thin layer chromatography. TLC solvent composition was acetonitrile | H2O | acetic acid (80:20:1). The free amines of unreacted GSSG were visualized on the TLC plate via ninhydrin
spray (1% ninhydrin in 90% methanol) followed by heating. The disappearance of the GSSG spots was indicative of reaction completion as the addition of biotin blocks the free amine.

**BGSSGB Purification**

BGSSGB was purified via solid phase extraction columns. All columns were conditioned by washing with 2 mL methanol followed by 2 mL H₂O. After collecting the load eluent as fraction 1, each column was washed with 1 mL H₂O twice (fractions 2 and 3) and 1 mL MeOH twice (fractions 4 and 5). To purify BGSSGB from the unreacted NHS-biotin and the singly tagged BGSSGB product, the reaction mixture was run through a C8 column and fraction 2 saved. This fraction was then run through a C18 column. Fraction 2 from this second separation contained pure BGSSGB and was saved. HABA assays were used to determine which fractions contained biotin or biotinylated species. Thin layer chromatography was used as described previously to detect presence of GSSG or the singly tagged product, BGSSG, in fractions.

**Measurement of BGSSGB Concentration via Reduction to BGSH**

Aliquots of the BGSSGB containing fractions were reduced to BGSH by addition of DTT to a final concentration of 100 mM. A C18 column was used to separate BGSH from DTT. The reduced reaction mixture was acidified to a concentration of 0.1% acetic acid. The H₂O and MeOH used for fractionation were acidified to the same concentration. After column conditioning, the acidified reaction mixture was fractionated over a C18 column. Water washes were continued until fractions were clear of DTT. DTT presence in fractions was assayed by the BCA protein reagent. Generally, at least
20 washes of 1 mL each were required for complete removal of reducing agent. BGSH elutes in the first methanol wash, which was dried via Speed Vac, and resuspended in desired volume of 0.1M phosphate buffer pH 8.0. After resuspension, BGSH requires storage at -20°C to prevent oxidation to BGSSGB.

A DTNB assay was used to determine the concentration of BGSH in the sample. A GSH standard curve (absorbance measured on 96 well plate reader) was generated and used to convert sample absorbance values to concentrations of free thiol. This determined concentration of BGSH was converted to moles, and is approximately two times the amount of BGSSGB in the fraction prior to reduction. Thus, BGSH concentration can be used to estimate the concentration of BGSSGB in the original sample and the percent yield of the reaction after purification.

_HABA Assay to Determine Presence of Biotin in Column Fractions_

To test for the presence of biotin or biotinylated species in the solid phase extraction column fractions, fractions were diluted 1:20 with 0.1 M phosphate buffer pH 7.4. 90 µL of avidin/HABA solution (0.5 mg/mL avidin and 0.3 mM HABA dye in 50 mM phosphate buffer pH 7.4) was combined with 10 µL of the diluted fraction. Color change from orange to bright yellow indicated the presence of biotinylated species or pure unreacted biotin. Fraction dilution factor was adjusted if necessary to achieve sufficient contrast between fractions.

_Reaction of Protein (Model or Tubulin) with BGSSGB_

To S-glutathionylate protein, 20 µL 4 mg/mL tubulin or 2 mg/mL model protein (either GAPDH, CK, ADH, LDH, or Papain) was combined with 10 µL of ~1.5 mM
BGSSGB or 10 µL 0.1 M phosphate buffer pH 8.0 for controls. Samples were incubated for 20 minutes at 37°C.

Ethanol Precipitation

200 µL cold (-20°C) ethanol was added to protein samples that had completed their incubation with BGSSGB. Samples were incubated for 20 minutes at -20 °C, and the precipitated protein was pelleted via centrifugation (14,000 RPM on Eppendorf centrifuge 5415C for 5 minutes). Supernatant was pipetted off, and 1 µL of 10% SDS was pipetted directly on to the pellet to facilitate resuspension. The pellets were resuspended in 30 µL 0.1 M phosphate buffer pH 8.0 and allowed to incubate overnight at 37°C to ensure that all protein was completely solvated.

Isolation of S-glutathionylated Tubulin with Avidin-agarose Resin

The samples were increased in volume by the addition of 30 µL of 0.1 M phosphate buffer pH 8.0. The avidin-agarose resin was suspended by inversion 10 times. Prior to its addition to each sample, the resin was inverted 3 times to ensure uniform resin density in the slurry. 20 µL of resin slurry was added to each sample, and the samples mixed on a rotator for 24 hours at room temperature. Following incubation, each sample was centrifuged for 5 minutes at 14,000 rpm to pellet the resin. The supernatant was carefully pipetted off without disturbing the pellet. The pellet was then rinsed twice (400 µL of 0.1 M phosphate buffer pH 8.0 was added to each sample and mixed via vortex. The resin was then again pelleted and the supernatant carefully pipetted off). After rinsing, the resin pellet was suspended in 30 µL of 50 mM DTT with 0.1% SDS and
incubated for 1 hour at 37°C. The entire sample, including resin, was then loaded onto a gel for SDS-PAGE.

**SDS-PAGE and Western blots of Tubulin and Other Model Proteins**

Samples were analyzed by SDS-PAGE on 10% polyacrylamide gels. Gels containing tubulin were run under reducing conditions whereas model protein sample containing gels were run under non-reducing conditions. Tubulin gels were visualized either via Western blot or via Coomassie blue staining. For the western blots, proteins were transferred to PVDF membranes via the Panther semi-dry transfer apparatus. For tubulin visualization, a mouse anti β-tubulin antibody was the primary antibody followed by goat anti-mouse HRP-conjugated secondary antibody. For model proteins, bands were transferred to a PVDF membrane as described above, and S-glutathionylation was detected directly via avidin-HRP conjugate (which binds to the biotin labeled glutathione via the avidin-biotin interaction). In both cases, a chemiluminescent substrate was ultimately used for visualization.

**Dot Blots**

Dot blots were used as a method to quickly detect the presence of protein or S-glutathionylation. 1.5 µL of sample was dotted onto nitrocellulose membrane followed by blocking with 3% milk. Spots were assayed for presence of β-tubulin or biotinylated glutathione via the detection methods outlined above.
Results and Analysis

Synthesis and Purification of BGSSGB

We synthesized biotinylated oxidized glutathione (BGSSGB) and purified it from other reaction species via a solid phase column chromatography approach. Synthesis of BGSSGB was attempted using the protocol found in Sullivan et al., although this protocol was ultimately abandoned\textsuperscript{22}. There were several challenges in generating a sufficient yield of BGSSGB. NHS-biotin is insoluble in water and thus must be first dissolved in DMF before it can be incorporated into the reaction mixture. Because DMF contains a carbonyl, it is a potential target of the nucleophilic amine in GSSG and can compete with NHS-biotin. Because this secondary product is undesirable, the amount of total DMF in solution had to be carefully controlled. Excess DMF can also alter the polarity of the reaction mixture, which can effect the later purification process.

Furthermore, the reaction will only proceed in 0.1 M phosphate buffer. The use of other buffers, such as ammonium bicarbonate, lead to extremely low yields. As of present, it is not clear why buffer choice is so critical.

Figure 2 shows a TLC plate visualized with ninhydrin solution that demonstrates the successful completion of the reaction. In the control lane, which consists of a GSSG only solution, unmodified GSSG is seen as a bright spot that migrates only slightly. Lane 2 shows the reaction mixture which lacks unreacted GSSG and in which a singly tagged product, BGSSG, has migrated halfway up the plate. This presence of an intermediate product and absence of unreacted GSSG shows that the reaction has gone to completion. One would not expect to see any spot corresponding to the doubly tagged product.
(BGSSGB) as it lacks free amines. Figure 3 summarizes the various species present in the reaction mixture and their appearance on a TLC plate.

We separated the reaction species via solid phase column chromatography. The initial separation was achieved with a C8 column, which elutes species in order from most polar to least polar. Thus, according to theory, any unreacted GSSG should elute first, the singly tagged product (BGSSG) second, the doubly tagged product (BGSSGB) third, and the unreacted NHS-biotin last. The TLC plate results (Figure 2) demonstrate this separation which occurs according to these theoretical predictions. As the entire reaction TLC plate shows only singly tagged product and no unreacted GSSG, we expected to only see BGSSG on the TLC plates of various fractions.

Fractions 3 (water), 4 (methanol), and 5 (methanol) show no free amine containing species on the TLC plate, indicating that those fractions could only potentially contain either unreacted NHS-biotin or the doubly tagged product (BGSSGB). However, the HABA assay (Figure 4) of these fractions shows that biotin was present only in fraction 2 (water). Thus fractions 3 (water), 4 (methanol), and 5 (methanol) contained neither unreacted biotin nor BGSSGB. Generally, unreacted NHS-biotin elutes in fraction 4 (methanol), and its absence from that fraction gives further evidence for the completion of the reaction. Figure 5 shows the HABA assay controls for comparison of intensity.

In Figure 2, the TLC plate of fraction 1 (load eluent) shows a large amount of BGSSG while fraction 2 (water) shows a significantly smaller amount of BGSSG. The HABA assay of these fractions (figure 4) shows that biotin is present in fraction 2 (water) and very slightly present in fraction 1 (load eluent). Because the HABA assay indicated
that the bulk of the biotin labeled species were present in fraction 2 (water), we concluded that fraction 2 (water) contained the desired doubly tagged product and a small amount of the singly tagged product. Fraction 1 (load eluent) contains most of the BGSSG, which explains its slight positive in the HABA assay. Figure 6 is a tabulation of the TLC and HABA results for the C8 column separation for the whole reaction, assorted controls, and each fraction from the C8 column separation.

A C18 column was used to separate the components of the C8 column fraction 2 (water) in order to separate the BGSSGB from the contaminant singly tagged product. Like the C8 column, the C18 column stationary phase retains non-polar species longer than polar species. However, the C8 column stationary phase is more polar than the C18 stationary phase, thus retention times differ across the two columns. Thus, according to this theory, the singly tagged product should elute before the doubly tagged product, and the change in stationary phase polarity will allow the two compounds to elute in different fractions.

Figure 7 shows the TLC plate of the C18 column fractions. There are no species visible in any fraction, which suggests that the amount of singly tagged product present in this fraction was negligible. HABA assay (figure 8) shows that biotin is present only in fraction 2 (water). As the TLC plates demonstrate that this cannot correspond to the singly tagged product, it was concluded that this fraction contained the desired product, BGSSGB. Figure 9 summarizes the HABA assay and TLC plate results for the C18 separation. Figure 10 is a theoretical schematic showing the likely distribution of species by fraction in each separation.
Quantification of BGSSGB

The amount of BGSSGB in the pure fraction was quantified via DTNB assay. The DTNB reagent reacts with free thiols to generate a measureable change in absorbance. In order to use this method BGSSGB was reduced to BGSH with DTT. Because DTT contains a free thiol and could interfere with the results of the DTNB assay, the reducing agent was separated from the BGSH via a solid-phase column extraction.

This separation was unexpectedly challenging. The Landino lab routinely separates fluorescein tagged glutathione, or FGSH, from DTT via a C18 column extraction, but this protocol was unsuccessful when applied to BGSH. While FGSH elutes in the methanol fractions and the reducing agent in the water fractions, the BGSH eluted with the reducing agent in the water fractions. This difference in elution is probably due the polarity difference between fluorescein and biotin. With several secondary amines, biotin is more polar than fluorescein, and biotinylated species are thus more likely to be only weakly retained by the non-polar C18 column used in the separation. This issue was addressed by acidification which decreased the polarity of BGSH via protonation. After this change, the BGSH eluted in the methanol fractions allowing for the separation of BGSH from DTT.

The concentration of BGSH in the assayed sample was found to be 1.2 mM. After accounting for dilution factors and various conversions, the amount of BGSSGB in the purified fraction was found to be $1.44 \times 10^{-6}$ moles or 1.44 mM. Based on the concentrations of GSSG and NHS-biotin in the original reaction, the theoretical yield of
BGSSGB is $5.0 \times 10^{-6}$ moles or 5.0 mM in the 1 mL fraction. This gives a percent yield for the BGSSGB synthesis reaction after purification of 71%.

The calculated concentrations of BGSSGB via DTNB assay are meant only to be approximations of overall concentration. As the DTNB assay measures free thiol concentration, it has no way of distinguishing between GSH and BGSH. If there were any remnants of BGSSG in the purified fraction, reduction would generate a BGSH molecule and a GSH molecule. In this situation, the DTNB assay would indicate a higher concentration of BGSSGB than was actually present in the original purified fraction. Furthermore, DTT contains a free thiol, and if any DTT contamination would introduce error. The complexity of the reduction and purification procedure also likely contributes significantly to error. However, despite these limitations, the results of this assay considered together with the TLC plate and HABA assay data) indicate that the BGSSGB synthesis reaction went to completion and was separated to reasonable purity via solid phase chromatography.

*Development of Biotin Affinity Capture Method*

As was discussed in the introduction, it is difficult to study S-glutathionylation in tubulin directly via Western blot. Figure 11 demonstrates the issues inherent in running tubulin on SDS-PAGE under non-reducing conditions. While the intensity of the bands shows that BGSH has been incorporated into tubulin via thiol/disulfide exchange with BGSSGB (i.e. S-glutathionylation has occurred), it does not give any sort of information as to which sub-unit of tubulin was modified. Indeed, the tubulin runs as a large smear in which the $\alpha$ and $\beta$ subunits are impossible to distinguish. Thus, this indicates the necessity for development of an alternative protocol to explore S-glutathionylation.
The developed protocol can be carried out over several days. Tubulin is incubated with BGSSGB for 30 minutes followed by an ethanol precipitation to separate protein from excess reagent. Resuspension of S-glutathionylated tubulin in phosphate buffer requires the addition of a small amount of SDS and incubation over night. Resuspended protein is then incubated on a turner for 24 hours with 20 μL of avidin-agarose resin in addition to 30 μL of phosphate buffer to increase the overall reaction volume. After incubation, the resin is washed with phosphate buffer twice (the pellet containing the resin and modified tubulin is retained) and then reduced by DTT for 2 hours. This reduced sample can be run, still containing the resin, on an SDS-PAGE gel under reducing conditions.

There were several inherent challenges in developing this affinity capture of biotin protocol as it is presented above. First, S-glutathionylated tubulin is difficult to resuspend following ethanol precipitation. Without the addition of a small amount of SDS and incubation at 37ºC, tubulin can take several days to resuspend. Indeed, even with these conditions designed to promote solvation of protein, resuspension requires at least twelve hours. Second, because reaction volumes are so small and surface tension is significant, sufficient sample mixing was an issue. Incubating a 30 μL sample on a rotating mixer for 48 hours did not result in sufficient mixing for biotinylated tubulin to be retained by the avidin-agarose resin. Due to economic concerns, the amount of tubulin and resin per sample could not be increased, and a wide variety of unsuccessful approaches to circumvent this limitation and increase mixing were attempted. Ultimately, increasing reaction volume with the addition of buffer enough to facilitate
mixing, but not so much as to over-dilute reaction components, was sufficient for biotin/avidin binding.

Figure 12 represents the successful incorporation of biotinylated glutathione into tubulin and the isolation of the modified tubulin from the bulk reaction mixture via avidin-agarose resin. The two bands present in each sample lane (which have identical composition) represent \( \alpha \) and \( \beta \)-tubulin, and the amount of protein retained by the resin is comparable across both samples. Because \( \alpha \)- and \( \beta \)-tubulin are associated until denaturing, these two species will always elute together from the avidin-agarose column.

Figure 13 summarizes the assorted controls. The “no resin control”, to which BGSSGB but not the avidin-agarose resin was added, shows only a very light band. This demonstrates that the amount of protein clinging to the side of the microfuge tube is negligible and that the protein retained by the resin in the sample lanes is significant. The “no BGSSGB control”, to which the avidin-agarose resin but not BGSSGB was added, similarly shows only a light band. This demonstrates that the resin retains only that protein which is modified by a biotinylated glutathione molecule. The “tubulin only control” is representative of the initial concentration of tubulin added to the reaction mixture and serves as a guide to estimate the percentage of tubulin that is ultimately retained by the avidin-agarose resin.

In order to verify the success of the protocol, a series of dot blots were performed at key steps. Figure 14 shows dot blots taken before and after the ethanol precipitation. Biotin is visualized, thus the reduction in the size of the spot from incubation to post precipitation demonstrates the success of the ethanol precipitation in removing excess BGSSGB. Worth note, however, is the aberrant spot in the “no BGSSGB control” after
resuspension. This false positive can likely be attributed to avidin-HRP’s tendency to sometimes loosely bind to non-biotinylated species. Figure 15 shows the same dot blots again, although in these β-tubulin is visualized. While there is some reduction in spot intensity from reaction to post incubation, the presence of protein following ethanol precipitation demonstrates that resuspension was successful and did not result in a significant loss of tubulin.

Figure 16 shows dot blots, with biotin visualized, from the reaction taken after incubation with the avidin-agarose resin and from the first wash supernatant. After incubation with the resin, the “no resin control” has a larger spot size than either of the experimental samples. This shows that the presence of resin leads to a reduction in the amount of biotinylated species present, indicating that in the experimental samples a portion of the biotinylated tubulin has been bound by the resin. The blots of the first wash show a significant reduction in the amount of biotinylated tubulin present, which is expected given the wash’s dilution compared to sample size.

*Estimation of Amount of Tubulin S-glutathionylation*

Estimation of band intensities between the two tubulin sample lanes and the tubulin only control lanes (Figures 12 and 13) suggests that perhaps 10-20 percent of the total tubulin is retained by the avidin-agarose resin. However, it is likely that somewhat more than 10-20 percent of the tubulin is S-glutathionylated as Figure 16 shows that some biotinylated species are not bound by the avidin-agarose resin.

It is expected that total S-glutathionylation of tubulin would be low. Tubulin has 20 cysteines, but not all are displayed on the surface and, as will be discussed in detail in the next section, it is possible that some of these cysteines could be blocked by
participation in disulfide bonds between protein subunits. Furthermore, as S-glutathionylation is reversible and dynamic, there is no guarantee that residues that are initially S-glutathionylated remain modified. Thus, the low yield of glutathionylated tubulin is expected given the circumstances of the reaction.
Discussion and Future directions

Evaluation of Affinity Capture Method

There are a number of strengths and weaknesses with the developed affinity capture method. Its most obvious advantage is the ease in which it can be carried out. Tubulin does not need to be in its native state in order to carry out this analysis, which allows for considerable freedom in reaction conditions and sample work up. Furthermore, the amount of time required, relative to other methods, is quite low. One particularly apparent time advantage occurs in the capability of using Coomassie blue staining over a Western Blot to visualize SDS-PAGE results. The biotin affinity capture method is also relatively cheap and requires little in the way of instrumentation. Other methods of studying structural changes to a protein, such as mass spectrometry or x-ray crystallography, require advanced instrumentation that is not always available to every researcher. This method, on the other hand, requires instrumentation that nearly every biochemistry lab possesses and requires only basic biochemical technical skills.

However, there are a several disadvantages to this method. First, it is difficult to know how much of the protein is actually modified. Certainly, one can gain understanding of relative amounts of S-glutathionylation across a variety of reaction conditions with this method. However, given the number of steps and complexity of the procedure, it is likely that a considerable amount of modified protein is lost throughout the process. Lane 1 in figure 13 is unmodified, reduced tubulin at the same concentration as the initial tubulin concentrations of the sample lanes. It is clear that the magnitude of tubulin present after the biotin affinity capture method is dramatically lower than when tubulin is run without modification. Whether this reduction in the amount of tubulin
present is due to protein loss during the procedure or simply low levels of BGSH incorporation is difficult to distinguish. It would be very useful to corroborate this experimental method with another, such as mass spectrometry, to get a sense of how much of the modified protein is actually retained and visualized.

Another issue is the undesirable types of tubulin oxidation that occur in the reaction with BGSSGB. The addition of BGSSGB, an oxidant, to tubulin leads to the formation of higher order protein structures. This was demonstrated in figure 11, which shows the smearing of tubulin when it is not run under reducing conditions. If so much tubulin aggregation is occurring via disulfide bond formation, many cysteines that may usually be available for post translational modification are trapped in disulfide bonds between protein molecules. This very likely changes the amount of S-glutathionylation that occurs in vitro compared to what occurs in vivo and could lower the yield of S-glutathionylated protein. There are several ways this issue could be addressed, such as alteration of reaction conditions to more closely mimic in vivo environments, that should be pursued in future experiments.

Future Experiments Using Biotin Affinity Capture Method

Despite several weaknesses that should be further explored, this method is first step in carrying out a number of interesting and useful experiments to study S-glutathionylation. Previous work in the Landino lab has quantified tubulin thiol/disulfide exchange with fluorescein labeled glutathione using an HPLC approach, and it would be relevant to expand these results.\textsuperscript{11} A simple experimental approach that utilizes the biotin affinity capture method would be to determine whether the magnitude of S-glutathionylation changes in response to different reaction conditions such as differing
pH or amount of oxidant present. One could also design in vitro experiments that more mimic the cellular environment and thus generate a more realistic predictor of the amount of S-glutathionylation that actually occurs in vivo. An interesting approach would be to determine whether addition of several of the enzymatic components of the GRS, such as glutathione reductase, could yield increased S-glutathionylation.

A more challenging, but possibly more rewarding application of the biotin affinity capture method would be analysis of modified tubulin fragments following protease digestion. If the protease was chosen carefully, digestion post reaction would yield a series of segments of known composition and length. That is, when proteases that cut at only particular sequences were used, one could predict the number and identity of cysteines in a segment of a particular length. Only those segments that possessed a cysteine that was modified by glutathione would be retained by the avidin agarose resin, and SDS-PAGE of the reduced resin mixture would yield a series of protein segments that could be identified based on their molecular weight. These segments that appeared on the gel would correspond to the segments containing a modified cysteine. Thus through a series of different digestions one could piece together which cysteines are modified preferentially. As determining which specific cysteine residues are modified is possible only through three dimensional structural analysis, which is difficult and time consuming, this technique would be an extremely useful angle to pursue.

Take a theoretical example: perhaps one wanted to analyze the cysteine residues in β-tubulin that could be critical for regulating polymerization. Cys 12, cys 129, and cys 131 are three surface cysteines that three dimensional structural analysis show to be relevant. Figure 17 shows these cysteines in the three dimensional structure of tubulin.
As can be seen in this figure, cys 12 is located in very close proximity to the GTP binding site in β-tubulin. Modifications to cys 12 with glutathione could lead to alterations of GTP binding abilities or GTP hydrolysis. As discussed in the introduction, the GTP cap of microtubules is closely related to microtubule dynamics and stability, and any modification that altered this system could have dramatic effects on microtubule polymerization ability.

As is seen in figure 17, cys 129 and 131 are in close proximity to one other. This proximity suggests that they could participate in a disulfide bond, although x-ray crystallography data indicates that in free tubulin no bond forms\(^\text{18}\). As disulfide bond formation can result in dramatic changes in tertiary structure, it is conceivable that S-glutathionylation of one of these residues (which would block disulfide bond formation) could lead to substantial changes in the structure of tubulin.

With these key cysteines in mind, it is relatively simple to use proteolytic digestion to generate peptide chains of known length that contain the cysteines in question. To isolate cys 12, one could use Carboxypeptidase Y from baker’s yeast, which cuts only between two aromatic residues, to cut between tyr 52 and tyr 53. This would yield a 51 amino acid long peptide containing cys 12 that could be identified on a gel with the assistance of a molecular weight ladder. If cys 12 was modified by glutathione, this peptide would appear on SDS-PAGE after the entire digested sample was incubated with avidin-agarose resin.

Similarly, one could generate a peptide containing cys 129 and 131 using Pancreatic Elastase which cuts only after alanine residues. A protease that cuts only at alanine would generate a series of peptides including a 75 amino acid residue containing
cys 129 and 131. While using this approach would not be able to distinguish whether cys
129, cys 131, or both were S-glutathionylated, the presence of any S-glutathionylation
could indicate the possible disruption of a disulfide bond\textsuperscript{25}.

Future Investigations of Tubulin S-glutathionylation

There are a number of future directions this research could lead. As discussed in
the introduction, there are many diseases in which oxidative damage is a primary
symptom. In Alzheimer’s disease, for example, oxidative damage is an area of increasing
focus. Understanding tubulin's activity in response to oxidative changes in the cell is thus
an extremely valid approach to addressing Alzheimer’s treatment. If cysteine residues
key to tubulin polymerization are S-glutathionylated with specificity under conditions of
oxidative stress, this could be a key target for an anti-neurodegeneration drug.

An understanding of how tubulin is S-glutathionylated could also lead to a
number of new investigations in the field of anti-cancer drugs. One especially interesting
field to explore is the use of cysteine residue modification as a way to target actively
dividing cancer cells\textsuperscript{26}. A number of anti-tumor drugs such as Vincristine, Vinblastine,
and Colchicine bind reversibly to tubulin to prevent the formation of mitotic spindles
during mitosis. Because of the effectiveness of these drugs, other methods of modifying
tubulin are being explored—especially those involving cysteine modification. One of
these methods is alkylation of tubulin cysteine residues. This method is an effective way
of inhibiting microtubule polymerization but is so non specific that it is not a feasible
approach for pharmaceuticals\textsuperscript{26}. Thus, other cysteine modifications need to be examined,
and understanding the process of tubulin S-glutathionylation could lead to the discovery
of potential targets for anti-tumor drugs. If tubulin polymerization is indeed partially
regulated by S-glutathionylation, anti-cancer drugs could be developed that either mimic S-glutathionylation or enhance S-glutathionylation in dividing cells.
Conclusion

The study of S-glutathionylation in tubulin is relevant both to improving general knowledge and furthering biomedical research. It falls within the broad field of oxidative signaling that is of great interest currently both in terms of biomedical research and in cell biology. More specifically, its focus on tubulin is very appropriate, as understanding microtubule polymerization is essential to combating the prevalent neurodegenerative disorders that involve the malfunction of this process.

The presented biotin affinity capture method is an effective way of further probing this field. In addition to circumventing several of the inherent challenges in studying oxidative modifications to tubulin, it is inexpensive and simple. Whether it is used as a method to directly study the regioselectivity of S-glutathionylation in tubulin or as a routine screen to determine S-glutathionylation in a specific tubulin segment or derivative, it is a useful technique to add to the biochemical technique library.
Appendix A: Figures

Figure 1: $1^\circ$ amine biotinylation by reaction with sulfo-NHS-biotin
Schematic showing generic amine biotinylation via nucleophilic attack to a carbonyl. The mechanism of reaction with sulfo-NHS-biotin is analogous to that with NHS-biotin$^{27}$.

Figure 2: TLC plate of controls and C8 column separation
Free amines are visualized by ninhydrin spray followed by heating. The GSSG only control contains 5.0 mM GSSG (which is representative of the concentration GSSG initially present in reaction mixture) and the prominent GSSG spot is visible. The whole reaction lane contains a sample of the reaction immediately following incubation. A prominent band corresponding to BGSSG is visible. The fraction lanes contain samples of each fraction from the C8 separation. Fractions 1 (load eluent) and 2 (water) show bands corresponding to BGSSG while fractions 3 (water), 4 (MeOH), and 5 (MeOH) show no bands.
Figure 3: Schematic of species migration on TLC plate
Summarizes positioning of various species and their general appearance (both in spot shape and color).

Figure 4: HABA assay of C8 column fractions
Each well contains 10 µL of 1:10 dilutions of each fraction and 90 µL of HABA/avidin solution. Fraction 2 (water) shows a strong positive for the presence of biotin and fraction 1 (load eluent) shows a slight positive for the presence of biotin.

Figure 5: HABA assay of GSSG only control and reaction
Each well contains 10 µL of 1:10 dilutions of either a 5 mM GSSG solution or the BGSSGB synthesis reaction immediately following incubation and 90 µL of HABA/avidin solution. The bright yellow color in the unseparated reaction is indicative of high concentrations of biotin.
<table>
<thead>
<tr>
<th>Fraction</th>
<th>1 (load eluent)</th>
<th>2 (water)</th>
<th>3 (water)</th>
<th>4 (water)</th>
<th>5 (water)</th>
<th>GSSG only control</th>
<th>Whole reaction</th>
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<tbody>
<tr>
<td>TLC (GSSG)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+ (faint)</td>
</tr>
<tr>
<td>TLC (BGSSG)</td>
<td>+ (faint)</td>
<td>+ (faint)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HABA</td>
<td>+ (very faint)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
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Figure 6: Summary of TLC and HABA results for the C8 column extraction
A “+” indicates either that a band was present on the TLC plate for that particular species or that the HABA assay indicated the presence of biotin. A “-” indicates either that a band was absent on the TLC plate for that particular species or that the HABA assay was negative for the presence of biotin.

Figure 7: TLC plate of C18 column separation
Each lane contains a sample of each fraction, and the plate is visualized by ninhydrin spray. All fractions do not contain any species with a free amine.
Figure 8: HABA assay of C8 column fractions
Each well contains 10 µL of 1:10 dilutions each fraction and 90 µL of HABA/avidin solution. Fraction 2 (water) shows a strong positive for the presence of biotin.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>1 (load eluent)</th>
<th>2 (water)</th>
<th>3 (water)</th>
<th>4 (MeOH)</th>
<th>5 (MeOH)</th>
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<tr>
<td>TLC (BGSSGB)</td>
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<tr>
<td>HABA</td>
<td>+ (very faint)</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</tr>
</tbody>
</table>

Figure 9: Summary of TLC and HABA results for the C18 column extraction
A “+” indicates either that a band was present on the TLC plate for that particular species or that the HABA assay indicated the presence of biotin. A “-” indicates either that a band was absent on the TLC plate for that particular species or that the HABA assay was negative for the presence of biotin.
Figure 10: Solid-phase extraction summary
Schematic showing the theoretical partitioning of species over the column chromatography procedure.

Figure 11: SDS-PAGE of tubulin with biotin-labeled glutathione visualized
Samples were run under non-reducing conditions in lanes 2 and 4 and contained 10 µL PB and 20 µL 4 mg/mL tubulin. Transferred bands were visualized on PVDF membrane via avidin-HRP conjugate. Smear demonstrates need to run tubulin under reducing conditions.
Figure 12: SDS-PAGE of tubulin eluted from resin (Coomassie Blue staining)
Both lanes are identical and consisted of 20 µL 4 mg/mL tubulin and 10 µL ~5 mM BGSSGB to which 20 µL avidin-agarose resin and 30 µL PB were added. Protein was visualized via Coomassie Blue staining. Gel was 10% acrylamide and run under reducing conditions.

Figure 13: SDS-PAGE of controls (Coomassie Blue staining)
The tubulin only control contained 20 µL 4 mg/mL tubulin, 10 µL PB, and no resin was added. The no resin control contained 20 µL 4 mg/mL tubulin, 10 µL ~5 mM BGSSGB, and no resin was added. The no BGSSGB control contained 20 µL 4 mg/mL tubulin, 10 µL PB, and 20 µL resin was added. Protein was visualized via Coomassie Blue staining. Gel was 10% acrylamide and run under reducing conditions.
Figure 14: Dot blots after incubation and resuspension with biotin visualized
1.5 µL of reaction was spotted onto a nitrocellulose membrane after the reactants were assembled and initially incubated and then again following ethanol precipitation and resuspension. All samples contained 20 µL of 4 mg/mL tubulin. The no resin control contained tubulin, 10 µL of ~5 mM BGSSGB but ultimately no avidin-agarose resin was added. The no BGSSGB control contained tubulin, 10 µL of PBS in the place of BGSSGB, and eventually 20 µL avidin-agarose resin. Samples 1 and 2 were identical and contained tubulin, 10 µL ~5 mM BGSSGB, and eventually 20 µL avidin-agarose resin. Protein was visualized via avidin-HRP conjugate.
Figure 15: Dot blots after incubation and resuspension with β-tubulin visualized

1.5 µL of reaction was spotted onto a nitrocellulose membrane after the reactants were assembled and initially incubated and then again following ethanol precipitation and resuspension. All samples contained 20 µL of 4 mg/mL tubulin. The no resin control contained tubulin, 10 µL of ~5 mM BGSSGB, but ultimately no avidin-agarose resin was added. The no BGSSGB control contained tubulin, 10 µL of PB in the place of BGSSGB, and eventually 20 µL avidin-agarose resin. Samples 1 and 2 were identical and contained tubulin, 10 µL ~5 mM BGSSGB, and eventually 20 µL avidin-agarose resin. Protein was visualized via B-tubulin primary antibody and Goat-anti mouse secondary antibody.
After incubation with resin

First wash

<table>
<thead>
<tr>
<th></th>
<th>No resin control</th>
<th>No BGSSGB control</th>
<th>Sample 1</th>
<th>Sample 2</th>
</tr>
</thead>
</table>

Figure 16: Dot blots after resin incubation and first wash with biotin visualized

1.5 µL of solution was spotted onto a nitrocellulose membrane from the reaction after incubation with the avidin-agarose resin and from the first wash. All samples contained 20 µL of 4 mg/mL tubulin. The no resin control contained tubulin, 10 µL of ~5 mM BGSSGB, but ultimately no avidin-agarose resin was added. The no BGSSGB control contained tubulin, 10 µL of PB in the place of BGSSGB, and eventually 20 µL avidin-agarose resin. Samples 1 and 2 were identical and contained tubulin, 10 µL ~5 mM BGSSGB, and eventually 20 µL avidin-agarose resin. Protein was visualized via avidin-HRP conjugate.
Figure 10: Rasmol image of tubulin. Protein in the foreground is β-tubulin and rear subunit is α-tubulin. Cys12 is highlighted in blue and is seen in close proximity to bound GDP which is highlighted in purple. Cys129 is highlighted in red and is in close proximity to Cys131 which is highlighted in orange. Tubulin crystal structure is from Lowe et. al\textsuperscript{18}. 
Appendix B: Abbrevations

ADH: Alcohol dehydrogenase
APP: Amyloid precursor protein
BCA: Bicinchoninic acid
BGSH: Biotin tagged reduced glutathione
BGSSG: Singly biotin tagged oxidized glutathione
BGSSGB: Doubly biotin tagged oxidized glutathione
CK: Creatine kinase
Cys: Cysteine
DMF: Dimethylformamide (a solvent)
DTNB: Ellman’s reagent
DTT: Dithiothreitol (a reducing agent)
FGSH: Flourescein tagged reduced glutathione
GAPDH: Glyceraldehyde 3-phosphate dehydrogenase
GDP: Guanosine diphosphate
GRS: Glutaredoxin reductase system
Grx: Glutaredoxin
GSH: Reduced glutathione
GTP: Guanosine triphosphate
HABA: 4’-hydroxyazobenzene-2-carboxylic acid
HPLC: High pressure liquid chromatography
HRP: Horseradish peroxidase
LDH: Lactate dehydrogenase
MAP: Microtubule associated protein
MeOH: Methanol
**NADPH**: Nicotinamide adenine dinucleotide phosphate

**NHS**: N-hydroxysuccinimide

**PBS**: Phosphate buffered saline

**PVDF**: Polyvinylidene fluoride

**SDS**: Sodium dodecyl sulfate (a detergent)

**SDS-PAGE**: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

**TLC**: Thin layer chromatography

**Tyr**: Tyrosine
Appendix C: Chemical Structures Index

GSH:

\[
\text{HOOC-CH}_2-\text{CH}_2-\text{NH}_2-\text{CH}_2-\text{COO}^-. \quad \text{NH}_3^+ \\
\text{HS} \\
\text{HS}
\]

BGSH

\[
\text{HOOC-CH}_2-\text{CH}_2-\text{NH}_2-\text{CH}_2-\text{COO}^-. \quad \text{biotin} \\
\text{HS} \\
\text{HS}
\]
GSSG

BGSSGB
NHS-Biotin

\[
\begin{align*}
\text{NHS-Biotin} & \\
\text{Biotin} & \\
\end{align*}
\]
Appendix D: Biochemical Techniques Index

The following are brief descriptions of some of the more important techniques mentioned throughout the thesis.

**HABA Assay**

The HABA assay is a convenient way to assess the amount of biotinylated species in a sample\(^2\). HABA has an affinity for avidin and can bind to the protein at four sites\(^2\). When HABA is bound to avidin, the complex absorbs strongly at 500 nm\(^2\). However, with the addition of a biotinylated molecule, the biotin displaces the HABA due to its high affinity for avidin. The HABA dye molecule without bound avidin does not absorb at 500 nm, thus the decrease in absorption at 500 nm can be correlated to biotin concentration\(^2\). Qualitatively, the addition of biotin to the HABA/avidin solution leads to a color change from orange to yellow. Thus, this assay can determine whether a sample contains a biotinylated species.

**BCA Assay**

The BCA assay is traditionally used to measure total protein concentration\(^3\). The assay is sensitive to a number of amino acids, including cysteine residues. Because DTT contains a thiol (which is the functional group of a cysteine), the BCA assay indicates positive for DTT\(^3\). Thus, this technique can also assay for DTT in a solution and has the advantage of being quick and simple. A color change from light green to a dark purple indicates presence of DTT.

**DTNB Assay**

The DTNB assay uses 5,5'-Dithio-bis(2-nitrobenzoic acid), also known as Ellman’s reagent, to quantify cysteine residues in a sample\(^4\). Reaction of DTNB with thiols leads to a decrease in absorbance at 412 nm, which can be correlated to the concentration of thiol-containing species in a sample\(^4\).

**SDS-PAGE**

SDS-PAGE is a form of polyacrylamide gel electrophoresis that has smaller pore sizes than gel electrophoresis and thus has better resolving power\(^5\). In the process, proteins are denatured by SDS, a detergent, and are uniformly coated with negative charge\(^5\). Proteins migrate across a charge gradient and are separated according to size. Because SDS provides a uniform charge coating for the denatured proteins, size alone is the factor by which they are separated. This
technique allows for identification of the types and relative amounts of proteins present in a particular sample.

Gels can be run under reducing or non-reducing conditions. Running a gel under reducing conditions entails addition of a reducing agent to the sample just prior to loading\textsuperscript{31}. Some proteins, such as tubulin, readily form higher order structures from air oxidation and require reduction before they are loaded onto a gel.

\textit{Western Blot}

A Western blot is a way to analyze proteins after they have been separated on a polyacrylamide gel. Proteins are transferred to a membrane, generally PVDF, via either gravity or a charge gradient\textsuperscript{32}. Antibodies can then be applied which bind to their protein target. To prevent antibodies from uniformly binding the membrane, which is absorbent to all protein, milk (which is very protein rich) is used to coat the membrane prior to antibody addition\textsuperscript{32}. This technique facilitates identification of specific proteins, protein subunits, or even post-translational modifications.

\textit{Coomassie Blue Staining}

Coomassie blue staining is a way to visualize proteins in a gel. After proteins have fully migrated, polyacrylamide gels are placed in a solution containing Coomassie Brilliant Blue G-250\textsuperscript{29}. The dye stains protein by binding non-covalently to amino and carboxyl groups\textsuperscript{29}. Following dye binding, a destaining solution consisting of methanol and acetic acid is used to remove excess dye from the gel. Proteins are visible as blue bands. Gels can be then be archived through a gel drying procedure.

\textit{Dot Blots}

Dot blots are a method in which one can very quickly assess the presence of a protein in a sample. Very small sample volumes are spotted onto a nitrocellulose membrane. Washing the membrane with PBS leads to the dissociation of all non-protein molecules while the proteins are retained by the membrane. Similarly to the Western technique, the membrane is blocked and probed with the desired antibody.
Appendix E: Sources Cited


25) All proteases were identified using the Sigma Protease Finder tool. http://www.sigmaaldrich.com/life-science/metabolomics/enzyme-explorer/learning-center/protease-finder.html


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