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Reactive Sulfur: Redox Reactions of Cysteines and Methionines in the Cytoskeletal Protein Tubulin

Tara Dawn Hagedorn
College of William & Mary - Arts & Sciences

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Reactive sulfur: redox reactions of cysteines and methionines in the cytoskeletal protein tubulin

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A Thesis presented to the Graduate Faculty of the College of William and Mary in Candidacy for the Degree of Master of Science

Department of Chemistry

The College of William and Mary
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This Thesis is submitted in partial fulfillment of the requirements for the degree of

Master of Science

Tara Dawn Hagedorn

Approved by the Committee, July 2011

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The College of William and Mary

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The College of William and Mary

Professor Gary W. Rice, Chemistry
The College of William and Mary
Microtubules are cytoskeletal fibers formed by the polymerization of the protein tubulin, coupled to the hydrolysis of GTP. These highly dynamic structures have been associated with a variety of cellular functions, such as cellular movement, maintenance of the cell shape, and transport of neuronal vesicles. It is therefore reasonable to suggest that a large number of proteins exist which are capable of interacting with microtubules. It is also likely that microtubules may be the basis for the spatial organization of some proteins within the cell. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is known to interact with microtubules and is of particular interest in neurodegenerative disease research as more studies reveal that it’s a multifunctional protein whose non-glycolytic functions are regulated by reactive oxygen species. The interaction between tubulin and GAPDH is partially electrostatic based on tubulin subunits being slightly acidic, with an isoelectric point (pl) of ~5.4, while GAPDH is slightly basic with a pl of 8.5.

Previous studies in our lab have shown that the cysteine residues of several proteins, including tubulin and GAPDH, are susceptible to oxidation by a variety of biologically relevant oxidants. However, the possibility of electrostatic and/or redox interactions of GAPDH with tubulin have not been previously investigated. We assessed tubulin cysteine oxidation in the presence of GAPDH by detecting interchain tubulin disulfides using immunoblotting techniques under nonreducing conditions and report a novel redox interaction between tubulin and GAPDH. The cysteine residues of one protein are becoming oxidized by H$_2$O$_2$ and then undergoing thiol/disulfide exchange with an available cysteine residue on the other protein. The original sulfhydryl that was oxidized, presumably the most reactive, is then available to get re-oxidized and undergo subsequent exchange. We have also confirmed the presence of GAPDH in MAPs isolated from porcine brains using SDS-polyacrylamide gel electrophoresis and immunoblotting techniques. GAPDH enzymatic activity has been observed in these isolates and suggests that GAPDH is stable even after exposure to the elevated temperatures used in the purification process.

In addition, our lab has assessed the effect of oxidants on methionine residues of tubulin and GAPDH using proteolysis and immunoblotting techniques. CNBr is a fairly common method for proteolysis, but it was still necessary to create a procedure specific for our experimental purposes and institution. While the previous method was able to detect methionine sulfoxide, improvements were made to the procedure by eliminating the sodium formate by-product.
Acknowledgements

I would like to express my deepest appreciation to Dr. Lisa Landino, who served as my faculty advisor, providing valuable resources and insight. This thesis would not have been possible without her help and encouragement. I would also like to show my gratitude to Dr. Gary Rice, Dr. Christopher Abelt for serving on the committee to read and critique this manuscript. These professors have also played a huge role during my time here at the College of William and Mary, and I most likely would not be attending UCSD in the fall if it were not for them. My return to school has been quite the challenge, this last year in particular, and their dedication to the success of their students has made it so much easier and is greatly appreciated.

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It is important that I also take this time to thank my family and friends. All of my accomplishments would have never been achieved without their sacrifices and reassurances. To Hillary Clark, Stephanie Brooks, and even Andy Palomo, thanks for making the hours I spent in lab quite interesting, and I wish you continued success. To Dr. D., I cherish our friendship more than you know and I am so grateful for all the advice and support you have given me. To my children, Dakota Harich and Makayla Hagedorn, you have given me strength when I have needed it most, and without you both, none of this would have been possible.
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<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ADH</td>
<td>alcohol dehydrogenase</td>
</tr>
<tr>
<td>PC-MAPs</td>
<td>all MAPs eluted from phosphocellulose column</td>
</tr>
<tr>
<td>CNBr</td>
<td>cyanogen bromide</td>
</tr>
<tr>
<td>cys</td>
<td>cysteine</td>
</tr>
<tr>
<td>GAP</td>
<td>D-glyceraldehyde-3-phosphate</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>ETC</td>
<td>electron transport chain</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>MP³⁺</td>
<td>ferric myeloperoxidase enzyme</td>
</tr>
<tr>
<td>FADH</td>
<td>flavin adenine dinucleotide</td>
</tr>
<tr>
<td>Grx</td>
<td>glutaredoxin</td>
</tr>
<tr>
<td>GSH</td>
<td>glutathione</td>
</tr>
<tr>
<td>GR</td>
<td>glutathione reductase</td>
</tr>
<tr>
<td>GRS</td>
<td>glutathione/glutaredoxin repair system</td>
</tr>
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<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>CTAB</td>
<td>hexadecylmethylammonium bromide</td>
</tr>
<tr>
<td>HRP</td>
<td>horse radish peroxidase</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>‘OH</td>
<td>hydroxyl radical</td>
</tr>
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<td>HOCl</td>
<td>hypochlorous acid</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
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<td>met</td>
<td>methionine</td>
</tr>
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<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>MAPs</td>
<td>microtubule-associated proteins</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NADH</td>
<td>nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>GSSG</td>
<td>oxidized form of glutathione</td>
</tr>
<tr>
<td>ONOO⁻</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>PIPES</td>
<td>piperazine-N,N’-bis(2-ethanesulfonic acid)</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>NaOCl</td>
<td>sodium hypochlorite</td>
</tr>
<tr>
<td>SODs</td>
<td>superoxide dismutases</td>
</tr>
<tr>
<td>O₂⁻</td>
<td>superoxide radical</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>Trx</td>
<td>thioredoxin</td>
</tr>
<tr>
<td>TrxR</td>
<td>thioredoxin reductase</td>
</tr>
<tr>
<td>TRS</td>
<td>thioredoxin reduction system</td>
</tr>
<tr>
<td>1x MTP</td>
<td>tubulin and MAPs after 1 cycle of polymerization</td>
</tr>
<tr>
<td>2x MTP</td>
<td>tubulin and MAPs after 2 cycles of polymerization</td>
</tr>
</tbody>
</table>
1. Introduction

Oxidative stress represents an imbalance between the generation of reactive oxygen species (ROS) and the biological system’s antioxidant defenses. Free radicals are a natural byproduct of cellular respiration, and play an important role in many biochemical processes, such as the intracellular killing of bacteria by activated neutrophils and in certain cellular signaling pathways. However, oxidative stressors such as infection, inflammation, metabolic abnormalities, and environmental contaminants may overwhelm the body’s defense mechanisms, allowing cellular concentrations of ROS to increase. These excessive amounts of ROS and their high reactivity often lead to unwanted side reactions and inevitably damage to biological macromolecules. An accumulation of this oxidative damage over a long period of time plays a significant role in the aging process, inflammatory diseases (arthritis, vasculitis), heart disease, cancer and several neurodegenerative diseases such as Alzheimer’s and Parkinson’s diseases.

ROS Formation

Redox reactions involve the transfer of electrons from one reactant (reductant) to another (oxidant), and are an essential part of aerobic metabolism in eukaryotes. Eukaryotic aerobic metabolism occurs within the mitochondrion. Acetyl-CoA, the product of pyruvate, fatty acids and certain amino acid degradation, is oxidized by the reactions of the citric acid cycle, producing the reduced coenzymes, NADH and FADH$_2$. The electrons of NADH and FADH$_2$ are subsequently donated to the electron transport chain (ETC), consisting of a series of electron carriers in the inner membrane, with oxygen as the terminal electron acceptor. The energy derived from the ETC drives ATP synthesis by creating a proton ($H^+$) gradient across the inner membrane.
A single enzyme, cytochrome oxidase, located in a cell’s mitochondria, handles approximately 98% of the oxygen metabolized. This enzyme functions as a catalyst in the ETC. Cytochrome oxidase contains four redox centers, two hemes and two copper ions, each with the ability to accept a single electron. When all centers are reduced, cytochrome oxidase transfers the four electrons to oxygen with no detectable intermediates, producing two water molecules.

There are advantages for organisms to use oxygen to extract the energy from organic molecules. Oxygen is readily available and can diffuse easily through cell membranes. Furthermore, oxygen is a highly reactive molecule and can easily accept electrons, releasing a substantial amount of energy which can then be used. However, an inescapable consequence of electron transport through the mitochondrial membrane is occasional leakage of electrons, leading to the formation of ROS. Estimates of the magnitude of ROS formation in the mitochondria vary from −0.2% to 2% of the total oxygen consumption. While a number of cellular processes contribute to ROS formation, the ETC is considered the major source of ROS, accounting for ~90% of the total production. Additional sources of ROS and reactive nitrogen species (RNS) include pollutants in the atmosphere and irradiation (Table 1).

<table>
<thead>
<tr>
<th>Source</th>
<th>ROS/RNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>atmosphere pollution</td>
<td>CO, Ozone, NO₂, N₂O₂</td>
</tr>
<tr>
<td>electron transport by-products</td>
<td>O₂⁻⁻</td>
</tr>
<tr>
<td>irradiation (X-, γ-, UV)</td>
<td>O₂⁻, 'OH, O₂</td>
</tr>
<tr>
<td>metal-catalyzed oxidation</td>
<td>'OH, H₂O₂, ferryl ion</td>
</tr>
<tr>
<td>inflammation (neutrophils, macrophages)</td>
<td>OCl⁻, H₂O₂, O₂⁻⁻, NO, ONOO⁻</td>
</tr>
<tr>
<td>oxidases</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>arginine metabolism</td>
<td>NO</td>
</tr>
</tbody>
</table>
Molecular oxygen ($O_2$) is a triplet diradical containing twelve valence shell electrons. As seen in Figure 1, instead of its discrete molecular orbital containing only pairs of electrons with opposite spins, oxygen contains two electrons that are not spin-paired, each residing in its own orbital.$^3$ Unequivocally, unpaired electrons increase the chemical reactivity of an atom or molecule.$^1$ Even though it is thermodynamically favorable for oxygen to take on additional electrons, this distribution of electrons makes it impossible for oxygen to accept a spin-matched pair of electrons. The unpaired electrons in the oxygen molecule must first undergo a spontaneous spin reversal to make the pairing possible, which is highly unlikely at ordinary collision frequency because the period of contact is too brief. This results in a kinetic barrier for many oxidative reactions. In order to compensate, oxygen accepts electrons one at a time and results in the formation of free radicals.

![Figure 1. Oxygen electron configuration](image-url)
Singlet oxygen ($^1$O$_2$) is the lowest excited state of molecular oxygen. Even though it is not a radical, it is extremely reactive. Singlet oxygen violates Hund's rule of electron filling in that it has all spin-paired electrons, leaving one orbital of the same energy level empty. This distribution of electrons makes it possible for oxygen to accept spin-matched electrons, thereby increasing the oxidizing ability of oxygen.$^5$

As illustrated in Figure 2, the transfer of one electron to O$_2$ produces the superoxide radical, O$_2^-$, a weak oxidizing agent.$^1$ Superoxide is the initial oxidant that escapes from the mitochondrial ETC and is an important source of other radicals. Hydrogen peroxide, H$_2$O$_2$, is produced when the second electron is transferred.$^3$ Even though hydrogen peroxide is not a radical, it is still a cytotoxic oxidant because it is eager to accept two electrons. H$_2$O$_2$ is a weak oxidant and is relatively stable.$^1$ However, unlike superoxide, hydrogen peroxide can rapidly diffuse across cell membranes. Certain chelates of ferrous iron or cuprous copper are capable of transferring an electron to hydrogen peroxide, breaking the O-O bond and producing a water molecule and a hydroxyl radical, *OH, the most potent ROS.

Other oxidants derived from molecular oxygen include hypochlorous acid, HOCl (produced from oxygen by NAPDH oxidase and myeloperoxidase)$^3$, and peroxynitrite, ONOO$^-$$^-$; which is produced from superoxide and nitric oxide, NO.$^1$ HOCl is highly reactive with a large range of bio-molecules, thiols being the most reactive, however, the reactivity of HOCl with thiols is too rapid to monitor experimentally.$^6$ The conversion of amines to chloramines by HOCl is much slower than the reaction of HOCl with thiols. Considering the availability of
amino groups, this reaction is physiologically likely, and therefore using chloramines is an excellent alternative to HOCl because they are not only less reactive oxidants, but also retain a preference for thiols.

**Antioxidants**

Normal cellular function relies heavily on the tightly controlled intracellular redox environment. The generation of ROS is usually minimized by antioxidants that buffer the redox environment. An antioxidant is any molecule found in low concentrations compared to that of an oxidizable substrate, that can easily react with ROS, thereby delaying or inhibiting the oxidation of substrate (Table 2). Antioxidants can be divided into categories based on their functions.
Table 2. Antioxidants

<table>
<thead>
<tr>
<th>Proteins</th>
<th>Metabolites/Vitamins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide Dismutase</td>
<td>Vitamins A, E, C</td>
</tr>
<tr>
<td>Catalase</td>
<td>Bilirubin</td>
</tr>
<tr>
<td>Glutathione peroxidase</td>
<td>GSH/GSSG</td>
</tr>
<tr>
<td>Glutathione transferase</td>
<td>Ubiquinol</td>
</tr>
<tr>
<td>Glutathione reductase</td>
<td>NADP/NADPH</td>
</tr>
<tr>
<td>Methionine sulfoxide reductase</td>
<td>Mn²⁺, Mg²⁺, Zn²⁺</td>
</tr>
<tr>
<td>Ferritin</td>
<td></td>
</tr>
<tr>
<td>Thioredoxin</td>
<td></td>
</tr>
<tr>
<td>Thioredoxin reductase</td>
<td></td>
</tr>
<tr>
<td>Peroxidoxins</td>
<td></td>
</tr>
</tbody>
</table>

(enzymatic or non-enzymatic), physical properties (water-soluble or lipid soluble), and their sources (endogenous or exogenous). Antioxidants not only quench chain reactions by scavenging ROS, but also influence cellular function by initiating signal transduction, activating or inhibiting enzymatic function, and regulating gene expression.

Some small molecule antioxidants (Figure 3), such as vitamin E, vitamin C, and glutathione may be produced endogenously in animals, or acquired through diet. Vitamin E (α-tocopherol) is lipid-soluble and is synthesized exclusively by plants. The hydrophobic side chain anchors it to the cell membrane where it can react with a variety of free radicals. It forms a stable radical species by delocalizing an unpaired electron through resonance with the aromatic moiety of its chromanol ring. The tocopheroxyl radical formed can then be reduced by other antioxidants to regenerate α-tocopherol. The prevalence of α-tocopherol, as well as other lipid-soluble antioxidant molecules, demonstrates the importance of protecting cell membranes from lipid peroxidation under oxidative stress conditions.

Vitamin C (ascorbate) is a water soluble antioxidant that readily reacts with a variety of reactive species, including superoxide, the hydroxyl radical, and singlet oxygen. The resulting
Figure 3. Small antioxidant molecules

radical, monodehydroascorbate, stabilizes an unpaired electron by delocalization throughout the conjugated structure of the five-membered lactone. An important function of ascorbate is its ability to regenerate the reduced form of vitamin E from the tocopheroxy radical. Ascorbate plays a crucial role in defense against reactive free radicals. It is the terminal antioxidant in many biological systems, and consequently several enzymes exist that are capable of regenerating ascorbate from monodehydroascorbate.

One of the most important antioxidants that occurs in almost all organisms is glutathione (GSH), which is a tripeptide comprised of glutamate, cysteine, and glycine. Approximately 90% of the intracellular GSH is located in the cytoplasm, with the remainder in the organelles. In most cell types, the estimated GSH concentrations vary from 0.5 to 10 mM, with the vast
The oxidized form, GSSG, results from the linking of two GSH molecules via a disulfide bond. The redox status of a cell is usually expressed by the ratio of GSH to GSSG. Typically, in the cytosol and mitochondria, the GSH:GSSG ratio is >10:1, and in the endoplasmic reticulum, it can be as low as 1:1. It is this lower GSH:GSSG ratio that facilitates the folding of secretory and membrane proteins in the endoplasmic reticulum. GSH is involved in many biological processes including: (1) maintenance of protein structure and function, (2) regulation of enzyme activity by glutathionylation, (the addition of GSH to a protein cysteine via a disulfide) or through the reduction of disulfide bonds, and (3) the maintenance of intracellular redox homeostasis.

Protection against ROS is largely provided by the combined efforts of several enzymatic systems. These families of antioxidant enzymes localize in the mitochondria, cytosol, and the extracellular space and have evolved to catalyze reactions that eliminate ROS. Superoxide dismutases (SODs) catalyze the conversion of two superoxide radicals, $O_2^•−$, into hydrogen peroxide and oxygen (Eq. (1)). Hydrogen peroxide can then be converted to water by hydrogen peroxide dismutases (catalases), or glutathione peroxidases (Eqs. (2) and (3)). Under normal cellular conditions, the GSSG generated by glutathione peroxidases is rapidly reduced by the NADPH-dependent glutathione reductase in order to sustain a high GSH:GSSG ratio. When concentrations of GSSG increase, mixed disulfides between GSH and protein thiols (glutathionylation) can form, resulting in lower GSSG levels while protecting the free sulphydryls (protein thiol).

\[
O_2^•− + O_2^•− + 2 H^+ \rightarrow H_2O_2 + O_2 \quad (1)
\]

\[
H_2O_2 + H_2O_2 \rightarrow 2 H_2O + O_2 \quad (2)
\]
There are two known enzyme systems capable of reducing disulfide bonds in vivo. The first is the thioredoxin reduction system (TRS), which is made up of thioredoxin reductase (TrxR), thioredoxin (Trx), and NADPH and is present in both unicellular prokaryotes and multicellular eukaryotes. Thioredoxin (Trx) is a small redox protein, approximately 12 kDa, and exists in either the reduced dithiol form, or in the oxidized disulfide form. TrxR uses NADPH to catalyze the reduction of Trx\textsubscript{ox} to the dithiol, which can then undergo thiol/disulfide exchange with most protein disulfides. While this enzyme system facilitates a low redox potential within the cell, additional functions include the regeneration of the reduced form of methionine sulfoxide reductases (Msr) and the regulation of enzymes and transcription factors via its protein disulfide reductase activity.

The second is the glutathione/glutaredoxin repair system (GRS), which is made up of glutathione (GSH), glutaredoxin (Grx), glutathione reductase (GR) and NADPH. This repair system often works in parallel with the TRS in regulating the redox homeostasis within the cell. Grx is a small heat stable protein, approximately 10-24 kDa, and catalyzes the reduction of proteins that are thiolated by GSH (Eq. (4)), or reduction of protein -S-S-glutathione (Eq. (5)).

\begin{align}
RSS'R' + GSH & \rightarrow RSH + GSSR' \\
GSS'R' + GSH & \rightarrow GSSG + R'SH
\end{align}

\textit{Biological importance of sulfur}

Nearly all amino acids are susceptible to oxidative modification by one or more forms of ROS. However, only the modifications involving the sulfur containing amino acids, cysteine
(cys) and methionine (met), can be repaired. Additionally, sulfur-containing amino acids are readily oxidized in the presence of low concentrations of ROS. Sulfur exists in multiple, stable oxidation states, that makes it a useful component in biological systems. The most highly active form of sulfur in biomolecules is the sulfhydryl (-SH), present in the amino acid cysteine. Cys is found in the active site of many proteins and in protein motifs that function in enzyme regulation, protein trafficking, control of gene expression, and receptor signaling. The sulfur in met is present as a thioether (-CH₂-S-CH₃), which is inherently less reactive but nonetheless critical for many protein functions and subject to oxidation.

Figure 4. Pathways for the oxidation of protein thiols.
The oxidation of protein thiols can occur by either one or two electron oxidation reactions. Figure 4 illustrates a thiol’s utilization of both pathways with one electron oxidation creating a thiyl radical and two-electron oxidation producing sulfinic acid (-SOH). The sulfinic acid undergoes additional reactions producing sulfinic acid (-SO2H), sulfonic acid (-SO3H), sulfinamide (RSONR2), sulfonamide (RSO2NHR'), and disulfide bond (-S-S-) formation. The thiyl radical undergoes a distinct set of reactions, the most favorable being with the thiolate anion resulting in disulfide formation and a superoxide radical, which can ultimately amplify the oxidant response.

**Chemistry of disulfide bond formation**

A disulfide bond (RS-SR) is a covalent bond formed between two thiols (RSH) as a result of a two-electron chemical oxidation (Eq. (6)). The thiols forming the bond may reside in the same polypeptide chain (intramolecular disulfide) or in the different polypeptide chains (intermolecular disulfide). For an intramolecular disulfide, the cysteine residues may be close to each other in the primary sequence or far away. However, to form a disulfide, the two residues must approach each other closely enough to form a covalent bond, and an electron acceptor must be able to remove electrons.

\[
2 \text{RSH} \rightarrow \text{RS-SR} + 2\text{H}^+ + 2\text{e}^- \tag{6}
\]

Isomerization of disulfide bonds within a protein or between two proteins can occur when free sulphydryl groups react with another reactive sulphydryl group or disulfides that are in close proximity. Entropy plays a central role in determining the rate of exchange within proteins. If the two are in close proximity within a rigid region of the protein, the rate of disulfide bond
formation will be enhanced. On the other hand, if the region is more flexible, then the rate will be decreased.

**Stability of disulfide bonds**

Disulfide bond formation is used in such diverse biological processes as enzyme catalysis and regulation, extracellular protein stabilization, and protection against oxidative damage. The stability of disulfide bonds often plays an important role in their biological utility because of the reversible nature of thiol-disulfide exchange reactions. Disulfide bonds in small molecules and proteins are not equally stable, and their stabilities span a range corresponding to a redox potential difference of 0.33V. Therefore, the ability to form and break a specific disulfide bond depends on the redox state of the environment in which the reaction occurs, the nature of the oxidant or reductant, and the kinetics of the forward and reverse reactions.

**Thiol-disulfide exchange reactions**

Thiol/disulfide exchange reactions alter the oxidation states of cysteine residues within a protein, and these different oxidative states alter the protein’s conformation and biological activity. Reversible thiol-disulfide exchange reactions occur by the nucleophilic attack of a thiolate anion (RS') on one of the two sulfurs of the disulfide. This results in the

![Figure 5. Thiol-disulfide exchange](image-url)
expulsion of a thiolate anion from the original disulfide and the formation of a new disulfide. The ionization of the reacting sulfhydryl group is very important since the initiation of the thiol/disulfide exchange reaction involves the nucleophilic attack by the thiolate ion.\textsuperscript{12} Thiols typically have a pK\textsubscript{a} of \textasciitilde8.3, and therefore the reaction is favored in an alkaline pH. Any factor that alters the pK\textsubscript{a} of a protein thiol, such as microenvironment, will also change the equilibrium position and inevitably the rate of the reaction.

**Microtubules**

The molecular architecture inherent to cell morphology is provided by tubulin, a cytoskeletal protein present in all mammalian cells, with higher concentrations in brain than in any other tissue.\textsuperscript{13,14} Tubulin is a dimer under physiological conditions, composed of two similar polypeptides, $\alpha$ and $\beta$, at least one of which is phosphorylated.\textsuperscript{15} $\alpha\beta$ heterodimers possess binding domains for other tubulin dimers, allowing them to assemble into a polar, helical lattice. The polymerization of tubulin into microtubules is necessary for basic cell function, and therefore regulation of microtubule assembly and disassembly has significant consequences in vivo.

Microtubule assembly originates from defined nucleation sites and is dependent on energy found in guanine nucleotides. $\beta$-tubulin binds to GTP which is hydrolyzed to GDP during microtubule assembly.\textsuperscript{16} The different polymerization rates of the two ends of the microtubule are a result of the polarity; the faster growing end is referred to as the plus end and the slower growing end as the minus end. At steady state, a treadmilling polymer has constant assembly of subunits at one end, with a balanced loss of subunits at the opposite end. Microtubule
polymerization is dependent on free tubulin concentration, whereas microtubule depolymerization is independent of the free tubulin concentration.

Both α- and β-tubulin is expressed in multiple isotypes in higher organisms and the roles of the different isotypes continue to be investigated. In adult mammalian brain tissue, tubulin is a mixture of all gene products with a concentration range from 12-14% of total cellular protein and only 2-4% in non-neuronal tissue. These high cellular concentrations make tubulin a likely target for modification by oxidants. When tubulin is treated with peroxynitrite anion (ONOO⁻) in vitro, cysteine thiols rather than other amino acids are more susceptible to oxidation. It is unclear whether all of the twenty reduced cysteines of the tubulin dimer are oxidized, but the disulfide bonds formed are at least partially responsible for inhibition of microtubule polymerization. A significant portion of the polymerization activity can be restored when the disulfides are exposed to the thioredoxin reductase systems or to small molecule reductants like dithiothreitol (DTT).

Figure 6. Microtubule structure
Microtubule-associated proteins (MAPs)

The microtubule lattice associates with a variety of proteins, however there are only certain proteins that co-purify with tubulin through repetitive cycles of temperature-dependent assembly and disassembly. These proteins are collectively referred to as microtubule-associated proteins (MAPs) and co-purify in approximately constant stoichiometry. MAPs are defined on the basis of their binding interactions with microtubules and are known to promote microtubule assembly while lowering the critical concentration for polymerization. These binding interactions are predominantly electrostatic based upon the negatively charged tubulin and the positively charged binding domains of MAPs. Most of the identified MAPs are regulated by phosphorylation, which inhibits MAP function by reducing the affinity of the MAP for the microtubule lattice, presumably by weakening this electrostatic interaction.

Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH)

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is an essential enzyme in the glycolytic pathway. An abundant cellular protein, GAPDH has been used as a model for investigations examining basic mechanisms of enzyme action, as well as the relationship between amino acid sequence and protein structure. Recent studies have identified several diverse biological properties which appear to be distinct from its glycolytic function. These include roles in vesicular transport, protein phosphotransferase/kinase reactions, translational control of gene expression, and apoptosis. Similarly, GAPDH was identified as a tubulin binding protein, catalyzing tubulin polymerization. GAPDH has also become of interest to neurodegenerative disease research as more studies reveal that its non-glycolytic functions are regulated by ROS. It is well-known that oxidative damage to proteins is a major
characteristic of Alzheimer disease pathology. GAPDH specifically binds to the carboxyl terminal end of the β-amyloid precursor protein (βAPP), a critical feature in Alzheimer’s disease.28

Myeloperoxidase

Neutrophils are a key component in the body’s immune response.29 Invading microbes are ingested by neutrophils and exposed to a potent antimicrobial system involving myeloperoxidase (MPO). When stimulated, neutrophils release large quantities of superoxide, which is then dismutated by NADPH oxidase forming hydrogen peroxide (Eq. (7)).30

\[ 2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \] (7)

Myeloperoxidase is a heme protein expressed in neutrophils31 and possesses several catalytic activities including the oxidation of chloride to hypochlorous acid (HOCl).30 This reaction is thought to proceed via a classical peroxidase mechanism in which H\textsubscript{2}O\textsubscript{2} reacts with ferric myeloperoxidase enzyme (MP\textsuperscript{3+}) to give the MP\textsuperscript{3+}•H\textsubscript{2}O\textsubscript{2} complex (Eq. (8)). This enzyme complex will react with Cl\textsuperscript{-} producing hypochlorous acid and a hydroxyl anion.

\[ \text{MP}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{MP}^{3+}\cdot\text{H}_2\text{O}_2 \] (8)

\[ \text{MP}^{3+}\cdot\text{H}_2\text{O}_2 + \text{Cl}^- \rightarrow \text{MP}^{3+} + \text{OH}^- + \text{HOCl} \] (9)

Hydroxyl radicals are not efficient at killing bacteria because their high reactivity enables them to react with other targets prior to reaching the bacterium. On the other hand, hypochlorous acid is a strong nonradical oxidant that is much more selective than the hydroxyl radical. HOCl is membrane permeable, its preferred substrates are thiols and thioethers, and it converts amines to chloramines (Eq. (10)).

\[ \text{RNH}_2 + \text{HOCl} \rightarrow \text{RNHCl} + \text{H}_2\text{O} \] (10)
It has also been shown that HOCl converts methionine residues of ingested *Escherichia coli* proteins to methionine sulfoxide in high yield.\(^9\) As the oxidation of microbial methionine residues increased, the viability of the exposed organisms decreased. Additionally, there was increased sensitivity to HOCl in the bacterial strains that lacked the methionine sulfoxide reductase repair system and greater resistance in strains that over-expressed this repair enzyme. This suggests that methionine oxidation by the myeloperoxidase system does contribute to its bactericidal effects.

There has been strong evidence implicating neuron-specific oxidative damage to proteins as well as cytoskeleton abnormalities in the pathogenesis of several neurodegenerative diseases including Alzheimer disease.\(^{18,32,33}\) The *in vivo* mechanisms for oxidative damage are not well understood, however, one possibility is the over-expression of MPO.\(^{31}\) It has been observed that MPO is expressed and enzymatically active in normal brain tissues, which raises the possibility that MPO has a physiological function in the central nervous system. The levels of enzymatic expression in neurons were low when compared with neutrophils and monocytes where it makes up 1-3% percent of total cell protein. Nevertheless, immunohistochemistry revealed the expression of MPO to be increased in brain tissues showing Alzheimer pathology, with localization in amyloid plaques and neurofibrillary tangles.\(^{31}\)
2. Interactions between microtubules and binding proteins

Microtubules are cytoskeletal fibers formed by the endothermic polymerization of the protein tubulin, coupled to the hydrolysis of GTP. These highly dynamic structures have been associated with a variety of cellular functions, such as cellular movement and the maintenance of the cell shape, transport of neuronal vesicles and of chromosomes during cell division. It is therefore reasonable to suggest that a large number of proteins exist which are capable of interacting with microtubules. Microtubules may be the basis for the spatial organization of other proteins and their functions.

Based on electrostatic interactions, endogenous biomolecules such as basic proteins or polyamines bind to tubulin, while acidic proteins or polynucleotides bind to MAPs. Brain microtubules are associated with several enzymatic activities, and it is difficult to distinguish whether these enzymes are intrinsically involved with microtubules. Some enzymes that are known to interact with microtubules are alkaline phosphatase, lactoperoxidase, tyrosine hydroxylase, adenylate cyclase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This latter glycolytic enzyme is of particular interest in neurodegenerative disease research as more studies reveal that it’s a multifunctional protein whose non-glycolytic functions are regulated by ROS.

Kumagai et al. first identified GAPDH as a protein that copurified with microtubules and formed microtubule networks they called bundles. More recently, Somers et al. revealed the mechanism of GAPDH-tubulin bundle formation and a linkage effect suggesting the existence of high and low affinity binding sites on the enzyme, both sites capable of interacting with microtubules and showing positive cooperativity. Durrieu et al. have studied the interaction of
GAPDH with microtubules by measuring the amount of enzyme which co-assembles with in vitro reconstituted microtubules.\textsuperscript{26} These studies indicated that microtubules possess a limited number of binding sites for GAPDH, and there was enhanced polymerization of microtubules in the presence of GAPDH similar to that seen with MAPs.

Studies from our lab confirm the presence of GAPDH in MAPs isolated from porcine brains using SDS-polyacrylamide gel electrophoresis and immunoblotting techniques. Additionally, GAPDH enzymatic activity has been observed in these isolates and suggests that GAPDH is stable even after exposure to the elevated temperatures used in the purification process. While microtubule-associated proteins (MAPs) are defined on the basis of their binding interactions with microtubules, the exact nature of the interactions between tubulin and GAPDH is not well understood. As part of our investigation of GAPDH as a potential MAP, \textit{in vitro} experiments uncovered a novel redox interaction between tubulin and binding proteins. We hypothesize that the redox interaction may be a result of inter-molecular disulfide bond formation between GAPDH and tubulin.

\textit{Materials}

Porcine brains were obtained from Smithfield Packing Company (Smithfield, VA). Mouse antibodies against \(\alpha\)-tubulin (monoclonal, clone B-5-1-2; 1:1 w/ glycerol), \(\beta\)-tubulin (monoclonal, clone tub 2.1; 1:1 w/ glycerol) and GAPDH (monoclonal, produced in mouse; 1:1 w/glycerol), a horseradish peroxidase-conjugated goat anti-mouse secondary antibody, GAPDH (from rabbit muscle), Lactic Dehydrogenase (from rabbit muscle), Alcohol Dehydrogenase (from \textit{Saccharomyces cerevisiae}), Lysozyme (from chicken egg white), Trypsin inhibitor (from \textit{Glycine}}
Max), D-glyceraldehyde-3-phosphate (GAP), β-Nicotinamide adenine dinucleotide hydrate (NAD\(^+\)), Hexadecylmethylammonium bromide (CTAB), and 3,3',5,5'-tetramethylbenzidine (TMB) were obtained from Sigma Chemicals (St. Louis, MO). Whole bovine blood was from Pel-Freez Biologicals (Rogers, AR). Cyanogen Bromide was from MP Biomedical (Solon, CA). X-ray film, SuperSignal West Pico chemiluminescent substrate, and bicinchoninic acid protein assay reagent were from Pierce (Rockford, IL). Dithiothreitol (DTT) was from Fisher Scientific (Pittsburg, PA). DG10 desalting column and Macro-prep CM cation exchange support was obtained from Bio-Rad Laboratories (Hercules, CA).

**Methods**

*Purification of brain tubulin and heat-stable MAPs*

Tubulin and heat-stable MAPs were purified from porcine brains by 2 cycles of temperature-dependent polymerization and depolymerization and phosphocellulose chromatography as described\textsuperscript{37}. Tubulin (typically 3–4 μg/μl) in PME buffer (0.1 M PIPES, pH 6.9, 1 mM MgSO\(_4\), 2 mM EGTA) was aliquoted and stored at -80 °C. Total MAPs were eluted from the phosphocellulose column in PME buffer containing 500 mM NaCl. Heat-stable MAPs were obtained as described by Vallee\textsuperscript{38}, desalted, and stored at -80 °C. Tubulin and MAP concentrations were determined by the bicinchoninic acid protein assay.

*Purification of myeloperoxidase*

Native myeloperoxidase was isolated from 1 L of bovine whole blood, which was stored at 4°C. Buffy layers were collected by centrifugation of the whole blood at 3500 rpm for 15
minutes at 5°C, and contaminating erythrocytes were removed by the addition of 3 parts de-
ionized water (2 minutes) followed by the addition of 1 part 0.6 M NaCl to restore isotonicity. Leukocytes were sedimented by centrifugation for 10 minutes at 3500 rpm and 5°C. The washing cycle was repeated until the pellet became off-white.

The leukocyte pellet was then resuspended in 10 mM sodium phosphate buffer (pH 7.4) containing 0.5% CTAB (hexadecylmethylammonium bromide), incubated at room temperature for 15 minutes and centrifuged for 15 min at 11000 rpm. This step was repeated once again prior to treating the supernatant with solid ammonium sulfate for a final concentration of 50% saturation. The supernatant was placed on ice for 30 min, followed by centrifugation at 11000 rpm for 15 min at 5°C. The supernatant was again treated with ammonium sulfate to yield final concentration of 65% saturation, placed on ice for 30 minutes, and again centrifuged at 11000 rpm for 15 min at 5°C. The resulting pellet, containing the bulk of MPO, was resuspended in 10 mM sodium phosphate buffer (pH 7.4). The resuspended MPO (10 mL) was loaded onto a 0.5 mL (packed volume) carboxymethyl cation exchange column and allowed to equilibrate for 10 minutes. Using increasing NaCl concentrations, the column was eluted and the resulting fractions tested for MPO activity using 3,3′,5,5′-tetramethylbenzidine (TMB) and H$_2$O$_2$. Fractions that resulted in a visible color change were combined, desalted and stored at 0°C. MPO concentration (5 ng/μL) was determined by the bicinchoninic acid protein assay.

**Desalting of GAPDH**

GAPDH (8.1 mg) was suspended in 3 mL of 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM DTT in order to restore cysteine residues to their reduced form. GAPDH
suspension was then desalted using a Bio-rad DG10 column eluting in 4 mL buffer, free of DTT. GAPDH (2 µg/µL) was aliquoted (typically 200 µL fractions) and stored at -80 °C.

**GAPDH activity assay**

Enzymatic assays were performed using an ELx800 Absorbance Microplate Reader (BioTek) and following the reduction of NAD⁺ to NADH. The molar concentration of NADH was calculated using a molar extinction coefficient of ε = 6.22 x 10³ M⁻¹cm⁻¹.³⁹ Assays contained 0.5 mM NAD⁺, 1.0 mM D-glyceraldehyde-3-phosphate, and 2.7 µM GAPDH in 0.1 M sodium phosphate buffer, pH 8.5 for a total volume of 200 µL. Absorbance was measured at 340 nm at 60 second intervals for a total of 10 data points.

**MPO activity assay**

The enzymatic activity of myeloperoxidase was determined using 3,3′,5,5′-tetramethylbenzidine (TMB) as described by Marquez and Dunford.⁴⁰ The assay solution contained 0.25 mM H₂O₂, 0.17 mM TMB, and 0.25-36 nM MPO in 0.1 M acetate buffer, pH 5.4 for a total volume of 1 mL. The reaction was initiated by the addition of the enzyme, and was followed by measuring the rate of formation of the oxidation product of TMB (ε = 3.9 x 10⁴ M⁻¹cm⁻¹) at 653 nm using a LAMBDA 35 UV/Vis Spectrophotometer (PerkinElmer).

**Tubulin oxidation reactions**

Oxidation reactions of tubulin were performed with and without one of the following proteins: GAPDH, LDH, ADH, lysozyme, trypsin inhibitor, MPO, PC-MAPs, or heat stable
MAPs. Reactions were conducted at 25 °C for 10–30 minutes, using either 500 μM H₂O₂ or 100 μM NaOCl as the oxidant in 0.1 M sodium phosphate, pH 7.4, for a total volume of 20 μL. Protein samples were separated by SDS–PAGE on 7.5% gels under nonreducing conditions. Following SDS–PAGE, proteins were transferred electrophoretically at 30 V overnight to PDVF membranes and β- tubulin or GAPDH were detected using the appropriate antibodies. Antibody complexes were visualized by chemiluminescence using HRP-conjugated secondary antibodies.

Detection of GAPDH in MAPs

Protein samples were separated by SDS–PAGE on 7.5% gels under reducing conditions. Following SDS–PAGE, proteins were transferred electrophoretically at 30 V overnight to PDVF membranes and GAPDH was detected using the appropriate antibodies. Antibody complexes were visualized by chemiluminescence using HRP-conjugated secondary antibodies.

Detection of GAPDH and MPO enzyme activity in MAPs

Enzymatic assays to detect GAPDH activity in MAPs at different stages of the purification process were performed using an ELx800 Absorbance Microplate Reader (BioTek). The molar concentration of NADH was calculated using a molar extinction coefficient of ε = 6.22 x 10^3 M⁻¹cm⁻¹. Assays contained 0.5 mM NAD⁺, 1.0 mM D-glyceraldehyde-3-phosphate, and 10 μL of MAP samples in 0.1 M sodium phosphate buffer, pH 8.5 for a total volume of 200 μL. Absorbance was measured at 340 nm at 60 second intervals for a total of 10 data points.

The enzymatic assays to detect MPO activity in MAPs before and after treatment with heat were performed using a LAMBDA 35 UV/Vis Spectrophotometer (PerkinElmer). The assay
solution contained 0.25 mM H₂O₂, 0.17 mM TMB, and 30 μL of MAP samples in 0.1 M acetate buffer, pH 5.4 for a total volume of 1 mL. The reaction was initiated by the addition of the enzyme, and was followed by measuring the rate of the oxidation product of TMB (ε = 3.9 x 10⁴ M⁻¹cm⁻¹) at 653 nm.

**Results and Discussion**

Previous studies in our lab have shown that the cysteine residues of several proteins, including tubulin and GAPDH, are susceptible to oxidation by a variety of biologically relevant oxidants. Recently, competition studies were performed in which tubulin and GAPDH were combined and then treated with an oxidant in an attempt to determine whether the cysteine residues of tubulin or those of GAPH were more likely to be oxidized. However, an unexpected redox interaction was observed between the two proteins. In Figure 7, higher molecular weight disulfide-linked protein species are observed, with the arrows marking the dimers and tetramers that are routinely observed when tubulin cysteines are oxidized. There are multiple dimers and tetramers of slightly different molecular weights because of the diversity of α- and β-tubulin gene products. The amount of tubulin or GAPDH in each lane of the gel is identical, but the amount of oxidant target is twice that in the lanes containing both proteins. Therefore, one would expect to see less oxidation by H₂O₂ in the lanes containing both, not more.

![Figure 7. Detection of tubulin oxidation in competition studies](image)

**Figure 7. Detection of tubulin oxidation in competition studies**
Detection of β-tubulin (1.5 μM tubulin, 30 μM cys) following treatment with 500 μM H₂O₂ or 100 μM HOCl in the presence or absence of GAPDH (6.9 μM, 28 μM). Lanes 1, 3 and 5 contain tubulin only whereas lanes 2, 4 and 6 contain both tubulin and GAPDH. The β-tubulin antibody complex was detected with a 2° antibody-HRP conjugate and a chemiluminescent substrate.
There was greatly enhanced disulfide formation in tubulin by H$_2$O$_2$ when both proteins were present (lane 4) relative to when tubulin alone was oxidized (lane 3).

These observations were preliminary and the possibility of electrostatic and/or redox interactions of GAPDH with tubulin had not been previously reported. Several studies have shown that GAPDH co-purifies with tubulin and enhances microtubule polymerization; however, the exact nature of the interactions between these two proteins has not been investigated until now. Our hypothesis is that the cysteines of one protein are becoming oxidized by H$_2$O$_2$ and then undergoing thiol/disulfide exchange with an available cysteine residue on the other protein. The original sulfhydryl that was oxidized, presumably the most reactive, would then be available to get re-oxidized and undergo subsequent exchange.

The interactions between tubulin and GAPDH are likely electrostatic based on tubulin subunits being slightly acidic, with an isoelectric point (pI) of ~5.4, while GAPDH is slightly basic with a pI of 8.5. To determine the overall effect of electrostatics on the observed redox interaction, protein samples containing 1.5 μM (30 μM cysteine) tubulin and 7.5 μM (30 μM cysteine) GAPDH were exposed to 500 μM H$_2$O$_2$ in the presence of 0.1-1.0 M NaCl. Oxidized samples were subjected to SDS-PAGE under nonreducing conditions and higher molecular weight species with interchain tubulin disulfides were detected by immunoblotting techniques using an anti-β-tubulin antibody. Unless otherwise stated, immunoblot figures are representative of at least 2 experiments. As illustrated in Figure 8, the control (lane 1) contains some tubulin dimers which could be the result of either air oxidation of tubulin or the redox interaction with GAPDH assuming some of the protein in the sample was previously air oxidized.
Figure 8. Detection of interchain tubulin disulfides in varying concentrations of NaCl

Oxidized tubulin was separated by SDS–PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. Monomeric β-tubulin (50 kDa) is labeled as well as dimers and tetramers. Samples (1.5 μM tubulin, 30 μM cysteines; 7.5 μM GAPDH, 30 μM cysteines) were treated with 500 μM H₂O₂ for 20 minutes at 25°C in the presence of 0.1, 0.3, 0.5, 0.6, 0.7, 0.8, 0.9, and 1 M NaCl.

The oxidized control (lane 2) contains dimers and tetramers formed by oxidation of tubulin with GAPDH present. At the lower concentrations of NaCl (lanes 3-4), there was not a significant decrease in dimers and tetramers produced by the oxidation reaction. It appears that only at the highest concentrations, 0.9 and 1.0 M NaCl (lanes 9-10), is there a considerable decrease in the tetramer band intensity. It can be concluded that the electrostatic interaction permits tubulin and GAPDH to come into close proximity to allow for disulfide bond formation.

Another aspect of the redox reaction that needed to be considered was the rate of disulfide formation. Control samples (1.5 μM tubulin), and protein samples (1.5 μM tubulin, 7.6 μM GAPDH) with and without 0.7 M NaCl were treated with 500 μM H₂O₂ for 10, 20, or 30 minutes. Figure 9 shows that there is a negligible increase in the intensity of the dimer and tetramer bands of the oxidized control (lanes 1-3) when the reaction time varied.
Figure 9. Detection of interchain tubulin disulfides varying reaction time using hydrogen peroxide

Oxidized tubulin was separated by SDS-PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. Monomeric β-tubulin (50 kDa) is labeled as well as dimers and tetramers. Control samples (1.5 μM protein, 30 μM cysteines), and samples containing 7.6 μM GAPDH with and without the presence of 0.7 M NaCl were treated with 500 μM H$_2$O$_2$ for 10, 20, or 30 minutes at 25°C.

However, in the oxidized protein sample (lanes 4-6) the dimer band clearly disappears after the 10 minute reaction time, and the monomer β-tubulin band continues to decrease in intensity over the entire time range. When the ionic strength of the sample is increased by the addition of 0.7 M NaCl (lanes 7-9), there is a definite decrease in the rate of dimer and tetramer formation demonstrating again that electrostatics plays a role in the binding interaction of tubulin and GAPDH, though it appears that there is still a significant increase in formation of dimers and tetramers when compared to the control.

Our lab is also interested in the strong oxidant hypochlorous acid (HOCI) because recent reports show that myeloperoxidase, the enzyme that catalyzes the oxidation of chloride ion to HOCI, is abnormally expressed in Alzheimer diseased brain. Therefore the previous experiment was repeated using 100 μM NaOCl as the oxidant. The oxidation results seen in Figure 10 are not significantly different from those seen in Figure 9. There is still increased oxidation of tubulin when GAPDH is present (lanes 4-6) when compared to the control (lanes
1-3), which is slightly decreased with the addition of salt (lanes 7-9). On the other hand, there is no significant change in the intensities of the monomer, dimer, and tetramer bands over the entire time range, indicating that the oxidation reaction was essentially complete by 10 minutes. This is not surprising since HOCl is much more reactive than H$_2$O$_2$ and was in lower concentration (100 µM HOCl vs 500 µM H$_2$O$_2$). Furthermore, the HOCl would be consumed by protein methionines or tyrosines as well as cysteines. The higher concentration of H$_2$O$_2$ and its slower reactivity are consistent with the thiol/disulfide exchange hypothesis.

As an overall comparison of the interchain tubulin disulfides produced, protein samples were oxidized using 500 µM H$_2$O$_2$ for 20 minutes, separated by SDS–PAGE under nonreducing conditions and higher molecular weight species were detected by immunoblotting techniques using either anti-β-tubulin (a) or anti-GAPDH (b).

![Figure 10. Detection of interchain tubulin disulfides varying reaction time using NaOCl](image)

Oxidized tubulin was separated by SDS–PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. Monomeric β-tubulin (50 kDa) is labeled as well as dimers and tetramers. Control samples (1.5 µM protein, 30 µM cysteines), and samples containing 7.6 µM GAPDH with and without the presence of 0.7 M NaCl were treated with 100 µM NaOCl for 10, 20, or 30 minutes at 25°C.
Figure 11. Comparison of interchain tubulin disulfides
Oxidized tubulin was separated by SDS-PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with either anti-β-tubulin (a) or anti-GAPDH (b). The oxidized control sample (1.5 μM tubulin), samples containing 7.6 μM GAPDH with and without the presence of 0.7 M NaCl were treated with 500 μM H₂O₂ for 20 minutes at 25°C.

In Figure 11(a), the tubulin control (lane 1) contains some dimers, indicative of air oxidation when tubulin is stored in the absence of a reducing agent. The oxidized tubulin control (lane 2) contains dimers and tetramers, and there was only a slight decrease in the monomer β-tubulin band, demonstrating that when tubulin is oxidized, higher molecular weight species are formed. When tubulin is oxidized in the presence of GAPDH (lane 3), there was a significant decrease in the monomer β-tubulin band, and the tubulin dimer band disappears. The tetramer band shows a slight decrease in intensity, which is most likely the result of larger disulfide-linked species (>250 kDa) being formed and therefore not entering the separating gel. In addition, the disappearance of the dimer band prior to the monomer band suggests that there may be a difference in the accessibility of the remaining oxidizable cysteine residues of these protein subunits. In the tubulin/GAPDH protein sample containing 0.7 M NaCl (lane 4) there is the
reappearance of the dimer band which demonstrates again that electrostatics plays a role in the binding interaction and the cysteine accessibility. Lane 5 contains tubulin and GAPDH without the addition of an oxidant. There is an increase in the intensity of the dimer and tetramer bands when compared to the tubulin control, even without the addition of an oxidant.

In Figure 11(b), the same comparison of oxidized GAPDH was performed. Since GAPDH has only 4 available cysteine residues per monomer, it is likely that it does not form interchain disulfides to the same extent as tubulin. The GAPDH control (lane 1) contains some higher molecular weight species. There is an increase in the intensity of the higher molecular weight bands for the samples that also contain tubulin (lanes 3-4). A notable result is that in the sample that only contained GAPDH and tubulin (lane 5) without the addition of an oxidant, the bands for the higher molecular weight species, decrease in intensity. When compared with the same sample in Figure 11a, as tubulin is oxidized, GAPDH is reduced, indicating an intermolecular redox reaction.

Given the reversibility of redox reactions, it was important to see whether oxidized tubulin could be repaired by GAPDH as well. Tubulin (1.5 μM, 30 μM cysteines) was treated with either 500 μM H₂O₂ or 100 μM NaOCl for 20 minutes. This was followed by the addition of catalase or methionine to scavenge remaining H₂O₂, or NaOCl respectively. GAPDH (7.6 μM, 30 μM cysteine) was then added and allowed to react for 10, 20, or 30 minutes. As seen in Figure 12, there is no significant difference between the tubulin control (lane 1) and the control containing catalase or methionine (lane 2), indicating that they have no effect on the oxidation status of tubulin. As previously seen, higher molecular weigh species are formed when tubulin is exposed to either H₂O₂ or NaOCl (lane 3). In both cases, the oxidized tubulin exposed to
GAPDH for only 10 minutes (lane 4) results in a decrease of the β-tubulin monomer and dimer bands. This is most likely due to the GAPDH having been air oxidized during storage. However, by increasing the time that the oxidized tubulin is exposed to GAPDH (lanes 5-6), there is a significant increase in both the monomer and dimer band intensities. This suggests that the oxidized tubulin is in fact being reduced by the presence of GAPDH.

The question then became whether or not this redox reaction is unique to GAPDH. Since electrostatic interactions allow tubulin and GAPDH to come into close proximity to form intermolecular disulfides, and these disulfides require that there be a free sulfhydryl group available, proteins were selected based on their net charge at physiological pH and available cysteine residues (Table 3).

![Image of tubulin bands](image)

**Figure 12. Detection of oxidized tubulin repair by GAPDH**

Oxidized tubulin was separated by SDS-PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. All samples contain 1.5 μM tubulin. The oxidized control and samples in lanes 4-6 were treated with either (a) 500 μM H₂O₂ or (b) 100 μM NaOCl for 20 minutes at 25°C. Either catalase (a) or methionine (b) was added to scavenge remaining oxidant. GAPDH was then added to lanes 4-6 and allowed to react for 10, 20, or 30 minutes at 25°C.
Both lactate dehydrogenase (LDH) and alcohol dehydrogenase (ADH) contain available cysteines but have opposite charges. In studying their reactivity with tubulin, the amount of available cysteines to be added was normalized such that it was equal to the same cysteine concentration used in the previous GAPDH studies. Normalization of cysteine residues was unnecessary for lysozyme and trypsin inhibitor protein concentrations because neither protein contains free cysteine residues.

All samples contained 1.5 μM tubulin, and with the exception of the tubulin control were exposed to 500 μM H₂O₂ for 20 minutes, subjected to SDS–PAGE under nonreducing conditions and higher molecular weight species were detected by immunoblotting techniques using an anti-β-tubulin antibody. As seen in Figure 13, both GAPDH (lane 3) and LDH (lane 4) caused a significant decrease in the monomer band (GAPDH > LDH) when compared to the oxidized control, and the tubulin dimer band is no longer present, indicating that tubulin undergoes increased oxidation when in the presence of either of these two proteins. There does not appear to be a difference in the any of the bands for lysozyme (lane 6) or trypsin inhibitor (lane 7) when compared to the oxidized tubulin control (lane 2). This is to be expected since neither of these proteins have free cysteine residues that are able to form intermolecular disulfide bonds. The monomer band of the sample containing tubulin and ADH (lane 5) does not appear to differ in intensity from the oxidized control either, however a dimer band is not clearly present. Given the results shown in the appendix (Figure 19), it is clear that tubulin oxidation is not increased in the presence of ADH. This is not surprising since the two proteins both have a net negative charge at physiological pH and therefore it would be unlikely that the two proteins would come within the necessary distance of each other to form a covalent bond.
Table 3. Protein Comparison

<table>
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<tr>
<th>Protein</th>
<th>pI</th>
<th>free cys</th>
<th>MW (kDa)</th>
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<td>myeloperoxidase</td>
<td>11.0</td>
<td>0</td>
<td>60</td>
</tr>
<tr>
<td>MAP2</td>
<td>7.2</td>
<td>2</td>
<td>280</td>
</tr>
<tr>
<td>tau</td>
<td>6.5-8.0</td>
<td>2</td>
<td>56</td>
</tr>
</tbody>
</table>

Figure 13. Comparison of interchain tubulin disulfide formation in the presence of various proteins

Oxidized tubulin was separated by SDS–PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. Controls contained 1.5 μM tubulin, and the protein samples contained 1.5 μM tubulin and either 6.6 μM LDH, 3.7 μM ADH, 10.7 μM lysozyme, or 7.5 μM trypsin inhibitor. Oxidized samples were treated with 500 μM H₂O₂ for 20 minutes at 25°C.
Several studies have shown that GAPDH will copurify with tubulin. Protein samples acquired during different steps of the purification of porcine brain tubulin and heat-stable MAPs were screened for the presence of GAPDH (Table 4). These protein samples, as well as commercially available GAPDH were separated by SDS-PAGE followed by immunoblotting techniques using a monoclonal anti-GAPDH antibody. The fact that the control and protein samples were from different mammalian sources (rabbit muscle vs. porcine brain) was of little significance because of the glycolytic enzyme’s high degree of gene and protein sequence conservation across species.\textsuperscript{41,42} As illustrated in Figure 14, GAPDH is present in the protein collected after one cycle of tubulin polymerization during the purification process (lane 2), MAPs eluted from the phosphocellulose column using 0.5 M NaCl (lane 3), and in the heat-stable MAPs (lane 4).

**Table 4. Protein samples collected during purification process**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1x MTP</td>
<td>porcine tubulin and MAPs after 1 cycle of polymerization</td>
</tr>
<tr>
<td>2x MTP</td>
<td>porcine tubulin and MAPs after 2 cycles of polymerization</td>
</tr>
<tr>
<td>PC-MAPs</td>
<td>all MAPs eluted from phosphocellulose column containing 500 mM NaCl</td>
</tr>
<tr>
<td>heat stable MAPs</td>
<td>boiled for 5 minutes, still soluble and functional</td>
</tr>
</tbody>
</table>

**Figure 14. Detection of GAPDH in purified MAPs**

Lane 1 contains 2 μg of commercially available GAPDH. Lane 2 contains 30 μL of protein collected after one cycle of tubulin polymerization during the purification process. Lane 3 contains 30 μL of MAPs eluted from the phosphocellulose column using 0.5 M NaCl. Lane 4 contains 30 μL of heat-stable MAPs (desalted).
While these results are not surprising, they did raise the question of whether or not GAPDH is enzymatically active during the purification process. GAPDH activity assays were performed using a microtiter plate reader and following the reduction of NAD+ to NADH at 340 nm. The molar concentration of NADH was calculated using a molar extinction coefficient of \( \varepsilon = 6.22 \times 10^3 \ \text{M}^{-1}\text{cm}^{-1} \). Assays contained 0.5 mM NAD+, 1.0 mM D-glyceraldehyde-3-phosphate, and 2.7 µM GAPDH in 0.1 M sodium phosphate buffer, pH 8.5 for a total volume of 200 µL. Controls containing only D-glyceraldehyde-3-phosphate and NAD+ in 0.1 M sodium phosphate buffer resulted in no enzymatic activity. The specific activity for samples containing GAPDH was 87.5 units/mg. The manufacturer claims activity >75 units/mg, and previously published specific activities have ranged from 67 units/mg to 164 units/mg, however all the GAPDH enzymatic assays differed slightly from the one used.

Protein samples acquired during different steps of the purification of porcine brain tubulin and heat-stable MAPs were then screened for the presence of GAPDH enzyme activity (Table 4). As seen in Table 5, GAPDH enzymatic activity was detected in all. While the presence of GAPDH in these protein isolates was confirmed by immunoblot, the exact amount of GAPDH present is unknown and therefore specific activity was not able to be calculated. In spite of this, when analyzing the change in absorbance (milliOD/min) of each protein fraction, as the fraction of the microtubule-associated proteins became more “pure”, there was a definite increase in GAPDH enzyme activity. This result is consistent with GAPDH actually co-purifying with MAPs.

What was unexpected was the decreased enzyme activity that was observed in the heat stable MAPs. This suggests that GAPDH may be heat stable to a certain extent, since the
proteins in this fraction were boiled for 5 minutes, remaining soluble and functional. Therefore GAPDH activity assays were performed on GAPDH that was kept at different temperatures for 5 minutes. As seen in Table 6, when GAPDH was exposed to 37 °C and 50 °C, there was only a slight decrease in specific activity when compared to the control that remained at 25 °C. When GAPDH was boiled for 5 minutes, a white precipitant was observed in the sample which was filtered out by centrifugation prior to activity assay. This resulted in an enormous decrease in the enzyme activity, however, not all activity was lost. This indicates that while a majority of the protein became insoluble and was removed by filtration, some GAPDH remained soluble and functional. The calculated specific activity seen in Table 6 for the boiled GAPDH is theoretical based on all the protein placed in the sample prior to boiling. For an accurate specific activity calculation, the mass of the precipitant would need to measured and subtracted from the mass of the initial GAPDH.

Table 5. GAPDH enzyme activity

<table>
<thead>
<tr>
<th>Protein</th>
<th>ΔA (milliOD)/min</th>
<th>c (μM/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>controls (no GAPDH)</td>
<td>0.000*</td>
<td>-</td>
</tr>
<tr>
<td>GAPDH</td>
<td>12.253*</td>
<td>1.970</td>
</tr>
<tr>
<td>1x MTP</td>
<td>0.804*</td>
<td>0.129</td>
</tr>
<tr>
<td>2x MTP</td>
<td>1.604*</td>
<td>0.258</td>
</tr>
<tr>
<td>PC-MAPs</td>
<td>3.683*</td>
<td>0.585</td>
</tr>
<tr>
<td>heat stable MAPs</td>
<td>0.703*</td>
<td>0.113</td>
</tr>
</tbody>
</table>

* average of three assays

Table 6. GAPDH enzyme activity following temperature change

<table>
<thead>
<tr>
<th>Temperature</th>
<th>ΔA (milliOD)/min</th>
<th>μM/min mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 °C</td>
<td>24.13*</td>
<td>87.5</td>
</tr>
<tr>
<td>37 °C</td>
<td>20.70*</td>
<td>83.0</td>
</tr>
<tr>
<td>50 °C</td>
<td>18.60*</td>
<td>74.8</td>
</tr>
<tr>
<td>100 °C</td>
<td>0.286*</td>
<td>1.14</td>
</tr>
</tbody>
</table>

* average of three assays
In view of the fact that GAPDH does co-purify with MAPs and is enzymatically active, we wanted to see if other enzymes do as well. We decided to investigate myeloperoxidase (MPO) based on its high isoelectric point and reports that the expression of this enzyme is increased in brain tissues showing Alzheimer pathology, with localization in amyloid plaques and neurofibrillary tangles\textsuperscript{31}. Unfortunately we could not test for the presence of MPO in the different MAPs fractions using immunoblotting techniques because anti-porcine MPO antibody is not commercially available. Instead, enzymatic assays to detect MPO activity in MAPs before and after treatment with heat were performed. The assay solution contained 0.25 mM H\textsubscript{2}O\textsubscript{2}, 0.17 mM TMB, and 30 \mu L of protein sample in 0.1 M acetate buffer, pH 5.4 for a total volume of 1 mL.

Figure 15 shows the spectral scans of the oxidation of TMB by MPO/H\textsubscript{2}O\textsubscript{2}. The initial scan (1) was taken from a solution of 0.17 mM TMB and 0.25 mM H\textsubscript{2}O\textsubscript{2} in 0.1 M acetate buffer at pH 5.4. The next scan (2) was taken immediately after (a) 36 nM bovine MPO, (b) 14 nM human MPO, (c) 30 \mu L PC-MAPs, or (d) 30 \mu L of heat stable MAPs was added to the reaction mixture. The succeeding scans were taken at 3 min intervals at a scan rate of 20 nm/s and the reaction was followed by measuring the rate of the oxidation product of TMB (\varepsilon = 3.9 \times 10^{4} \text{ M}^{-1}\text{cm}^{-1}) at 653 nm.

These experiments were performed at pH 5.4 which was found to be the optimal pH for measuring MPO activity using TMB\textsuperscript{45}. Moreover, TMB oxidation products are more stable in acidic conditions\textsuperscript{40} and since the pH inside a phagocyte has been reported to be within pH 4-6\textsuperscript{46}, our experiments were carried out under optimal and physiological pH conditions.
Figure 15. Spectral scans of MPO activity
Spectral scans of the oxidation of TMB by MPO/H$_2$O$_2$ under steady-state conditions. The initial scan (1) was taken from a solution of 0.17 mM TMB and 0.25 mM H$_2$O$_2$ in 0.1 M acetate buffer at pH 5.4. The next scan (2) was taken immediately after (a) 36 nM bovine MPO, (b) 14 nM human MPO, (c) 30 µL PC-MAPs, or (d) 30 µL of heat stable MAPs was added to the reaction mixture. The succeeding scans were taken at 3 min intervals at a scan rate of 20 nm/s.

MPO purified from bovine whole blood (Figure 15a) and commercially available human MPO (Figure 15b) spectra shows that upon addition of the enzyme, new peaks appeared at 370 and 653 nm, the absorbance of which increased steadily with time. A shoulder at 450 nm is also apparent but does not contribute significantly to the optical spectra of the oxidation product of TMB. As seen in Figure 15c, the spectrum shows that upon the addition of PC-MAPs, the same peaks at 370 and 653 nm appear and also increase steadily over time, suggestive of MPO activity in purified MAPs.
Conversely, upon the addition of heat stable MAPs (Figure 15d), there are no visible peaks at 370 and 653 nm indicating that MPO is not heat stable. These results are summarized in Figure 16.

Since microtubule-associated proteins binding interactions are predominantly electrostatic and GAPDH is clearly present in purified porcine MAPs, we questioned if well characterized MAPs also undergo a similar redox reaction with tubulin. A single comparison study was performed which included the PC-MAPs, heat-stable MAPs, and myeloperoxidase (MPO). The protein samples were oxidized with 500 µM H₂O₂ for 20 minutes, separated by SDS–PAGE under nonreducing conditions and higher molecular weight species were detected by immunoblotting techniques using anti-β-tubulin.

In Figure 17, the tubulin control (lane 1) contains some dimers and tetramers, indicative of air oxidation when tubulin is stored in the absence of a reducing agent. The oxidized tubulin control (lane 2) contains dimers and tetramers, and there was only a slight decrease in the monomer β-tubulin band, demonstrating that when tubulin is oxidized higher molecular weight species are formed. As previously shown, when tubulin is oxidized in the presence of GAPDH
(lane 3), there was a significant decrease in the monomer β-tubulin band, and the tubulin dimer band disappears. The tetramer band decreases in intensity, which is most likely the result of larger disulfide-linked species (>250 kDa) being formed and therefore not entering the separating gel. A protein sample was intentionally not loaded into lane 4. The tubulin sample containing MPO (lane 5) resulted in disappearance of both the monomer and dimer bands, and the tetramer band intensity was slightly decreased when compared to the oxidized control (lane 2). The presence of MPO greatly enhanced the oxidation of tubulin, generating a larger number of >250 kDa molecular weight species that did not enter the separating gel. This is an interesting result since MPO does not have any available cysteine residues to undergo thiol-disulfide exchange, but as a peroxidase, it contains a heme prosthetic group that can readily undergo redox reactions. The effects of metalloproteins on the creation of interchain tubulin disulfides is beyond the scope of this thesis and will require further investigation.

Figure 17. Comparison of interchain tubulin disulfide formation in the presence of MPO and MAPs
Oxidized tubulin was separated by SDS-PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. Samples contained 1.5 μM tubulin and one of the following: 7.6 μM GAPDH, 1.9 μM human MPO, 0.084 μg/μL PC-MAPs, 0.25 μg/μL of heat stable MAPs.
The PC-MAPs (lane 6) as defined in Table 4 on page 38, does not appear to be considerably different from the oxidized control, with only a negligible increase in the dimer and tetramer band intensities. However there are many variables that need consideration. The PC-MAPs contain all of the microtubule-associated proteins eluted from the phosphocellulose column and contain 500 mM NaCl. As demonstrated previously, increasing the ionic strength of the sample alters the rate of the reaction through electrostatic interference. In addition, the overall protein concentration of the PC-MAPs was ~0.6 µg/µL, and the final concentration of PC-MAPs protein was 0.084 µg/µL. Even assuming that the PC-MAPs fraction was made up of just tau and MAP2, which is unlikely, the final concentration of MAP2 would be 0.15 µM (0.3 µM cysteine) and the final concentration of tau would be 0.75 µM (1.5 µM cysteine), way below the 30 µM cysteine concentrations previously used.

The sample containing tubulin and heat stable MAPs (lane 7) did appear to undergo a similar redox reaction seen by GAPDH (lane 3). There was a decrease in the monomer β-tubulin band intensity, the dimer band disappears, and the tetramer band intensity shows a slight increase in intensity, indicating that the overall oxidation of tubulin is increased when in the presence of the heat stable MAPs consisting primarily of tau and MAP2. When comparing the overall increased oxidation of tubulin by GAPDH to the heat stable MAPs, it appears that GAPDH increased the oxidation of tubulin more than that of the heat stable MAPs, which is not necessarily the case. One must also consider the final concentrations of the available cysteines. The purified heat stable MAPs had a protein concentration of ~1.85 µg/µL, with a final concentration of heat stable MAPs in the sample of 0.25 µg/µL. Since heat stable MAPs are primarily made up of tau and MAP2, and assuming 1:1 ratio of tau and MAP2, then the final
protein concentrations of MAP2 and tau would be 0.45 μM, 2.2 μM respectively for a total available cysteine concentration of 5.3 μM. This is still way below the 30 μM cysteine concentration of the GAPDH sample and therefore, there are less available cysteines to undergo intermolecular thiol-disulfide exchange in the heat stable MAPs compared to GAPDH. While normalization of the cysteine residues must occur prior to this experiment being repeated, the fact that tubulin and the well characterized tau and MAP2 proteins undergo a redox reaction is quite noteworthy. The binding of microtubule-associated proteins to tubulin has been believed to be primarily electrostatic in nature. The ability of MAPs to undergo a redox reaction and potentially bind tubulin through intermolecular disulfide bond formation is a novel concept that needs further exploration.

In summary, tubulin cysteine oxidation in the presence of GAPDH and LDH is enhanced. While electrostatic interactions permit these proteins to come into close proximity, the increase in interchain tubulin disulfides occur via thiol/disulfide exchange. Since a large number of proteins exist which are capable of interacting with microtubules, it is therefore reasonable to suggest that this novel redox interaction may also occur with other basic binding proteins.
3. Detection of Methionine Sulfoxide

A common method for proteolysis uses cyanogen bromide (Br-C≡N) to cleave the peptide bond on the carboxyl-terminus of methionine residues. Methionine is a relatively infrequent amino acid in proteins, therefore cleavage at that residue generates fairly few peptides which can be used in protein analysis.

The reaction mechanism is fairly straightforward. The carbon atom of CNBr is unusually electrophilic because the electron density is shifted away from the carbon atom and towards the more electronegative bromine and nitrogen. A nucleophilic acyl substitution wherein the bromine is replaced by the sulfur in methionine, initiates the cleavage reaction. This is followed by the formation and hydrolysis of the iminolactone, resulting in the two peptide fragments (Figure 18).

This technique had never been performed in the lab, and therefore an experimental procedure was developed and used in the qualitative analysis of methionine oxidation in tubulin and GAPDH. As a result of its oxidation, the sulfur in methionine would no longer act as a nucleophile and cleavage at that particular residue would not occur. To ensure the only nucleophile present during the cleavage reaction is the sulfur in methionine, the reaction requires acid conditions using a solvent such as 0.1M HCl or 70% formic acid. It has been established that using formic acid can result in the esterification of serine and threonine residues, which can complicate protein characterization. However, the oxidation of methionine to methionine sulfoxide occurs more readily in HCl, and therefore 70% formic acid was selected as our solvent.
Another reaction condition that needed consideration was the removal of excess CNBr in solution. Many of the published procedures removed volatile side products and excess CNBr through lyophilization, which was not a viable option. One recommended method of deactivation is the addition of 1 M NaOH and 1 M NaOCl in volumes of ratio 1:1:2 (CNBr solution:NaOH:NaOCl). The sodium hydroxide hydrolyzes CNBr to form sodium cyanide and bromide. The cyanide can then be oxidized by sodium hypochlorite to the less toxic cyanate ion.

The concentration of CNBr in the cleavage reaction (0.2 M) was more than 100 times greater than the target methionine residue concentration (450 μM). CNBr is readily hydrolyzed to HCN and is the reason for using a large excess. Therefore we considered that the excess CNBr would be converted to NaCN upon the addition of NaOH used to remove the acidic conditions and therefore stop the reaction. In the presence of water, NaCN will readily produce
HCN, which is both volatile and toxic at room temperature. However the reaction volumes are so small and performed in the fume hood such that HCN poisoning was highly unlikely.

**Materials**

Porcine brains were obtained from Smithfield Packing Company (Smithfield, VA). Mouse antibodies against β-tubulin (monoclonal, clone tub 2.1; 1:1 w/ glycerol) and GAPDH (monoclonal, 1:1 w/glycerol), and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody were from Sigma Chemicals (St. Louis, MO). Cyanogen Bromide was from MP Biomedical (Solon, CA). X-ray film, SuperSignal West Pico chemiluminescent substrate. Dithiothreitol (DTT) was obtained from Fisher Scientific (Pittsburg, PA).

**Methods**

**Purification of brain tubulin**

Tubulin was purified from porcine brains by 2 cycles of temperature-dependent polymerization and depolymerization and phosphocellulose chromatography as described37. Tubulin (typically 3–4 μg/μl) in PME buffer (0.1 M PIPES, pH 6.9, 1 mM MgSO₄, 2 mM EGTA) was aliquoted and stored at -80 °C. Tubulin concentrations were determined by the bicinchoninic acid protein assay.

**Desalting of GAPDH**

GAPDH (8.1 mg) was suspended in 3mL of 0.1 M sodium phosphate buffer, pH 7.4, containing 1 mM DTT in order to restore cysteine residues to their reduced form.
suspension was then desalted using a Bio-rad DG10 column eluting in 4 mL buffer, free of DTT. GAPDH (2 μg/μL) was aliquoted (typically 200 μL fractions) and stored at -80 °C.

**Methionine oxidation**

Oxidation of the protein was performed in a 0.6 mL Eppendorf tube. Protein samples consisted of either tubulin (~11-17 μM) or GAPDH (~35-56 μM) in 0.1 M sodium phosphate buffer, pH 7.4. Protein samples were then combined with an oxidant, gently mixed and allowed to react for 30 minutes at room temperature. This was followed by the addition of 70% formic acid (~0.5 - 3.0 M). All samples were gently mixed to ensure pH change prior to the addition of cyanogen bromide (0.05 - 0.20 M). The samples were allowed to react at room temperature overnight, after which NaOH (~1.8 - 2.0 M) or NH₄OH (~0.50 M) was added to the samples to neutralize the pH and deactivate the cyanogen bromide. Due to the high toxicity of HCN, both the addition of CNBr and NaOH (or NH₄OH) were performed in a fume hood.

**Results and Discussion**

In the initial experiments, tubulin and GAPDH proteins were exposed to H₂O₂ or NaOCl, and then treated with cyanogen bromide. The protein control was exposed to the pH changes caused by the addition of the 70% formic acid and the NaOH, and the cleavage control differed only by the addition of the CNBr. It was observed during electrophoresis that the dye front did not migrate linearly across the gel, but instead appeared as a wavy line. This occurred only in samples that contained the formic acid, cyanogen bromide, and sodium hydroxide. It was also noted that the dye front would regain linearity approximately three-fourths of the way through
the gel. It was concluded that this observed effect was most likely the result of the alkali salt produced by the neutralization reaction between the NaOH and the formic acid.

Following electrophoresis, the initial gels were stained using Coomassie Blue. It was originally believed that quantitative data could be obtained through extraction of the protein bands from the gel and measuring the absorbance of the stained bands. Although protein cleavage was observed, there was uneven staining observed in the gels and inconsistencies between the stained protein bands of subsequent gels. After many failed attempts to improve staining, and considering that this method of staining occurs through electrostatic interactions, it was decided that the side products of the cleavage reaction must interfere with the dye’s ability to bind the protein samples.

Further experiments used immunoblotting techniques which proved to be a more sensitive analytical method. Initially an additional control was incorporated to assess the effects of the acid/base on the antibodies ability to recognize the target protein. There was no significant difference between the intensities of the two protein controls, however it was observed that there was a difference in the distance migrated. All samples that contained the acid/base did not migrate as far as the protein control, in other words, they appear to have a higher molecular weight than that of the control. The salt produced by the neutralization reaction interferes with molecular weight determination by increasing the ionic strength of the samples and reducing the amount of SDS bound to proteins. As molecular weight determination was not the focus of these experiments and all samples would theoretically have the same salt concentrations and would migrate the same distance, subsequent experiments no longer contained the additional protein control.
Using immunoblotting techniques provided consistency with respect to our ability to visualize the protein samples. However, this method can only qualitatively determine the amount of cleavage obtained and that was where problems arose. Many of the samples resulted in skewed or distorted bands and vertical streaking of the protein, similar to that seen in Figure 19a, which can be attributed to salt containing samples. While changes in cleavage were observed in samples containing oxidants, numerous experiments were performed to produce a publishable image similar to Figure 19b.

While our results determined that methionine residues in tubulin and GAPDH were oxidized to methionine sulfoxide, there was still room to improve the procedure by eliminating the sodium formate by-product. Instead of using NaOH as the neutralizing base which resulted in the formation of sodium formate, ammonium hydroxide was used. Neutralization reactions

Figure 19. Detection of methionine sulfoxide
\(\beta\)-tubulin immunoblots comparing 0.83 mM concentrations of hydrogen peroxide, peroxynitrite, glycine-chloramine, and hypochlorous acid. Oxidation and cleavage procedures performed as explained in the methods section, using NaOH as the base. Samples separated by SDS-PAGE on a 7.5% polyacrylamide gel prior to transferring to a nitrocellulose membrane. Monoclonal anti-\(\beta\)-tubulin antibodies, horseradish peroxidase-conjugated secondary antibodies, and SuperSignal substrate were used for imaging.
using NH₄OH produce the volatile by-product, ammonia, and therefore should eliminate the problems previously seen. As seen in Figure 20, this slight alteration proved to be very successful. There is no longer any distortion of the protein bands, and the difference in migration distances previously seen when comparing samples containing the acid/base to a protein control, have also disappeared. It should also be noted that cleavage experiments using NH₄OH and stained using Coomassie Blue saw a slight improvement, however the overall consistency of these results remained inferior to immunoblotting.

While using CNBr is a fairly common method for proteolysis, it was necessary to create a procedure specific for our experimental purposes and institution. The significance of changing the base used in neutralization does not only lie in the improved visualization of experimental results, but in the simplicity of it.

![Figure 20. β-tubulin immunoblot comparing 0.250 mM and 0.500 mM glycine-chloramine, taurine-chloramine, and hypochlorous acid.](image)

Oxidation and cleavage procedures performed as explained in the methods section, using NH₄OH as the base. Samples separated by SDS-PAGE on a 7.5% polyacrylamide gel prior to transferring to a nitrocellulose membrane. Monoclonal anti-β-tubulin antibodies, horseradish peroxidase-conjugated secondary antibodies, and SuperSignal substrate were used for imaging.
Many publications using this method did not disclose specific details in the methods section, or included techniques that were not viable options for our laboratory. It was necessary to consider the required reaction conditions of this method, and apply them to our procedure. The problems that arose were not foreseen, and the attempts to correct them were focused on the analytical technique being applied, not the reaction. The improvements seen by this small change to the reaction just go to show that all aspects of an experiment need to be assessed when problem solving.
Appendix

Figure 21. Varying GAPDH concentration
Detection of interchain tubulin disulfides varying the concentration of GAPDH. Oxidized tubulin was separated by SDS-PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. Monomeric β-tubulin (50 kDa) is labeled as well as dimers and tetramers. Tubulin samples (1.5 μM protein, 30 μM cysteines) were treated with 500 μM H₂O₂ for 20 minutes at 25°C in the presence of 1.4, 2.8, 4.2, 5.6, 6.9, 8.3, and 9.7 μM GAPDH.

Figure 22. Oxidation of tubulin in the presence of ADH
Oxidized tubulin was separated by SDS-PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. Monomeric β-tubulin (50 kDa) is labeled as well as dimers and tetramers. Tubulin control samples (1.5 μM protein, 30 μM cysteines) and sample containing 3.7 μM ADH were treated with 500 μM H₂O₂ for 10, 20, or 30 minutes at 25°C.
Figure 23. Oxidation of tubulin in the presence of trypsin inhibitor
Oxidized tubulin was separated by SDS-PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. Monomeric β-tubulin (50 kDa) is labeled as well as dimers and tetramers. Tubulin control samples (1.5 μM protein, 30 μM cysteines) and sample containing 7.5 μM trypsin inhibitor were treated with 500 μM H₂O₂ for 10, 20, or 30 minutes at 25°C.

Figure 24. Oxidation of tubulin in the presence of lysozyme
Oxidized tubulin was separated by SDS-PAGE under nonreducing conditions on a 7.5% polyacrylamide gel, transferred to PVDF membrane and probed with anti-β-tubulin. Monomeric β-tubulin (50 kDa) is labeled as well as dimers and tetramers. Tubulin control samples (1.5 μM protein, 30 μM cysteines) and sample containing 10.7 μM lysozyme were treated with 500 μM H₂O₂ for 10, 20, or 30 minutes at 25°C.
References


