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Covalent Modification Of Recombinant Protein With Reactive Thiols

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COVALENT MODIFICATION OF RECOMBINANT PROTEIN WITH REACTIVE THIOLS

By
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ABSTRACT

SAWYER DULANEY AND BAILEY TAYLOR: Covalent Modification of Recombinant Protein with Reactive Thiols (Under the direction of Dr. Susan Pedigo)

Many diseases cause chronic and painful inflammation in different body systems. One of the front-line drug classes to treat such inflammation is Nonsteroidal Anti-Inflammatory Drugs (NSAIDs). Despite the benefits of oral administration of NSAIDs, there are drawbacks to their long-term usage because they can cause detrimental effects on off-target systems in the body such as the liver, kidney, or the lining of the intestinal tract. An alternative to NSAIDs is the usage of hydrogels for targeted drug delivery. Hydrogels can provide drug delivery in a specific portion of the site of inflammation, thus allowing higher doses of medication to be able to be given without the risks of damage to other body systems. Our lab has developed a Calmodulin: M13 peptide-based hydrogel system for this purpose which is the focus of this study.

The purpose of these experiments was to develop a method to modify CCLP3, the calmodulin component of our hydrogel, to create more sulfhydryl groups such that there are more attachment sites for future modification by a drug. There are three methods of modification explored in this study: the reaction of CCLP3 with Traut's Reagent (2-iminothiolane) in an aerobic environment; the reaction of CCLP3 with Traut's Reagent in an anaerobic environment; and, the reaction of CCLP3 with SATA (N-succinimidyl S-acetylthioacetate) in an anaerobic environment. Traut's Reagent and SATA have unique chemistries, each with its potential upsides and downsides. This work reports the results of the modification of CCLP3 with these reagents and the subsequent analysis of the products using Ellman's reagent.

The quantification of sulfhydryl group addition with Ellman's Reagent concluded that each method resulted in differing levels of modification to CCLP3. There are twenty-six potential sites

of modification on CCLP3, and the reagents provided total potential modification percentages of 11.7% for Traut's Reagent in an aerobic environment, 37.4% for Traut's Reagent in an anaerobic environment, and 19.1% for SATA in an anaerobic environment. After consulting the literature, we have determined two potential areas of improvement by increasing the molar ratio of reagent to CCLP3. In future experiments, we recommend adding a non-sulfur-containing reducing agent such as TCEP to prevent disulfide linkages while still allowing quantification of sulfhydryl groups using Ellman's Reagent.

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LIST OF ABBREVIATIONS

CaM	Calmodulin
MLCK	Muscle Light Chain Kinase
MMP	Matrix Metalloproteinases
HCl	Hydrochloric Acid
NaCl	Sodium Chloride
EDTA	Ethylenediaminetetraacetic Acid
SATA	N-succinimidyl S-acetylthioacetate
DMSO	Dimethyl Sulfoxide
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
Cys	Cysteine
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
Traut's Reagent	2-iminothiolane
Abs	Absorbance
MW	Molecular Weight
kD	Kilodaltons
D	Daltons
g	Grams
mg	Milligrams
cm	Centimeters
nm	Nanometers
mol	Moles
M	Molar
mM	Millimolar
μM	Micromolar
mL	Milliliter
μL	Microliter

Chapter 1

Introduction

I. Introduction

a. Background Information

Chronic and acute inflammation plague patients of all ages with devitalizing diseases. Oral Nonsteroidal Anti-inflammatory Drugs (NSAIDs) are the first-line treatment to reduce the symptoms of inflammation, despite the adverse effects that these oral medications can cause[1]. One of the largest potential issues with the oral route as a source of anti-inflammatory drug delivery is the vulnerability of the gastrointestinal tract and the organs that clear the drugs from the body, namely the liver and kidney[2]. NSAIDs also endanger the patient's cardiovascular functions leading to more prescribed medications[3]. Thus, paradoxically, oral NSAIDs cause systemic consequences that produce negative results by disrupting body systems that originally presented no issues. Therefore, it is important to find an alternative way to dispense NSAIDs that do not produce the pleiotropic effects that oral administration of medication presents.

Much effort in recent years has been devoted to targeted drug delivery systems or in other words, *in situ* drug delivery at specified sites in the body. Hydrogels have emerged as a major candidate to provide an alternative route to deliver NSAIDs specifically without the negative impacts presented with oral delivery. Hydrogels are inert materials that can be injected into inflamed tissues and then serve as an *in-situ* drug delivery depot. To create such a material, considerable molecular engineering is required including the design of the hydrogel matrix, which is a means to carry the drug in the matrix and a signal to release the drug in response to

inflammation. Work in this area is important because there are a considerable number of people that would benefit from the finding of an alternative route of medication delivery through hydrogels due to their unique properties and degradation activity.

Our lab has developed a promising copolymeric, protein-based hydrogel system intended to dispense NSAIDs in specific extracellular sites where inflammation is a problem. This copolymer comprises two polymers, one containing Calmodulin (CaM) and the other a peptide called M13. Since these proteins are from humans, they are unlikely to cause immune responses *in vivo*. M13 is a key peptide with 26 amino acids from striated skeletal muscle myosin light chain kinase (MLCK) that comprises a CaM binding site that is essential for calcium signal transduction pathways[4, 5]. M13 and CaM bind each other with high affinity in the presence of calcium, which is the basis of the noncovalent interactions between our copolymeric hydrogel. This hydrogel is then to serve as a scaffold for *in situ* drug delivery.

b. Our Experiment

The long-term goal of this experiment is to establish the chemistry for the use of the CaM: M13 hydrogel for in situ delivery of NSAIDs at the site of inflammation. The hydrogel would be a drug depot injected at the specific site of the inflammation such as at the sciatic nerve bundle of the knee joint. To attach NSAIDs to the hydrogel matrix, we will introduce thiol sites to the CaM-based copolymer, and then subsequently link NSAIDs to those thiols with hydrolyzable linkages. Once formed into a hydrogel, this would fulfill our goal of creating a copolymeric hydrogel for *in situ* drug delivery at specific sites of inflammation in the body.

Details of this proposed process are below (Figure 1.1). The main purpose of the experiment outlined therein is to create a covalently modified gel matrix for *in situ* drug delivery.

The first necessary step in the process (Figure 1.1 (a)) was to develop a process that was suitable for adding sulfhydryl groups to our CCLP3 protein as NSAID attachment sites. As CCLP3 only contains one cysteine, we were tasked with adding more sulfhydryl groups onto the protein for potential reaction sites. Sulfhydryl groups are important to our research as they ultimately provide the reactive group for the chemical linkage to naproxen, our NSAID of choice. Naproxen is activated in a separate set of experiments to make material 6 (Figure 1.1) and added to the thiolated mixture of already formed, and thiol-rich hydrogel in reaction (e). There is a lot of flexibility with sulfhydryl groups in that they can be easily oxidized or reduced, providing stability to the protein when needed yet still allowing the ability to be reduced for further reactions if needed.

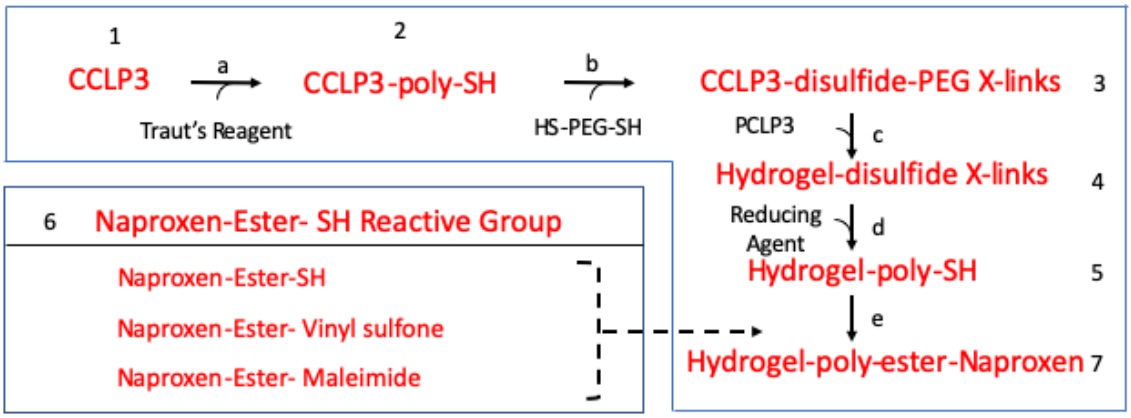

Figure 1.1: A potential reaction map of CCLP3 to a hydrogel containing naproxen

This scheme shows the activation of the CaM component of the hydrogel (CCLP3) with Traut's reagent[6] first (a), then it is treated with a bifunctional PEG spacer to create a 3D gel network (product 3). Mixing it with the M13 peptide component, PCLP3, in the presence of Ca^{2+} will promote hydrogel formation. Then the mixing of the prepared Naproxen (product 6) with activated hydrogel (product 5) will create a naproxen-modified hydrogel.

In the following sections, we will first address the breadth of uses of injectable hydrogels for a variety of purposes. Second, we will undertake a comprehensive review of the design elements of our copolymeric system.

II. Hydrogels

The purpose of this section is to provide a brief introduction to hydrogels. Hydrogels, a gel biomaterial with a hydrophilic nature, are 3D networks of hydrophilic polymers that are wetted by water and hold water in their stable structures. These hydrated networks of noncovalent interactions and covalent bonds prevent the collapse of the structure of hydrogels. They can be made of synthetic or natural polymeric components and mixtures of the two[7, 8]. Hydrogels may or may not be deformable, recover from injury, and degrade upon chemical or physiological signals. They can be used as scaffolds for tissue engineering, or depots for drug delivery as we are interested in developing. These ideas are discussed below.

A. Hydrogel structure

At the highest level of structure, hydrogels are classified by their interactions and linkages. Macromolecular hydrogels are defined by their ability to create covalent cross-linkages between polymeric components. Supramolecular hydrogels are formed through non-covalent interactions between polymeric components. They tend to have different characteristics that suit them to their applications. For instance, macromolecular hydrogels tend to not be as good at self-healing after injury, but they are stronger and purposed for bearing weight. Crosslinked hydrogels are insoluble; supramolecular hydrogels will erode. Thus, if the hydrogel should degrade as part of its function i*n vivo*, then a supramolecular hydrogel would be a better choice. Both forms of hydrogels have characteristics that contribute significantly to their functional attributes[9, 10].

B. Hydrogel Applications

Hydrogels have been applied in tissue engineering, wound dressing, and contact lenses[8]. These applications are very specific site-specific needs that each require tailored properties such as beyond biocompatibility, such as mechanical strength, pain relief, antiseptic delivery, and corrective properties for the contact lenses. Hydrogels can also be used for *in situ* drug delivery through transdermal patches, surgical implantation, and systemic delivery via intravenous infusion[11]. The latter of these two methods is problematic due to patient discomfort involved in surgery and potential hazard in intravenous injection. For that reason, there is great interest in injectable hydrogels that can stabilize and serve as a stable drug delivery depot in the extracellular matrix at the site of inflammation *in vivo*[12]. In terms of chronic inflammatory diseases that require a long duration of treatment and slow NSAID release, tunable, injectable hydrogels are the perfect solution. Previous applications of many drug delivery hydrogels have been limited for the usage of drug delivery because of their delicate properties and weak mechanical strength[10]. Two examples of how crosslinking agents can control injectable hydrogels are noted here. Brindha, et al (2019) explored the use of cross-linking agents as a means of transitioning supramolecular to macromolecular hydrogels to allow control over the viscoelasticity of the resultant gel[13]. The physical characteristics of protein-based hydrogels made from elastin are temperature dependent, including degradation by proteases, dependable composition, and viscoelasticity, and can be altered to adjust accordingly in a specific chemical component[14].

To create a responsive hydrogel, it is commonly desirable to design the hydrogel-specific elements according to the purpose. The characteristics of their interactions and bonds can permit a drug's release at sites of inflammation only when required by the body through signaling and activation of chemical processes (c.f. Devapally, et al[15]). The filamentous structure's chemical

cross-linking must be maintained for efficient usage in their purpose of drug delivery along with their biodegradable mechanism until they have slowly expended all their internal and useful components[16]. An influx in physiological calcium levels in extracellular space and the crosslinking of hydrogels help regulate the rate of expending all their materials which is attributable to calcium signaling that allows self-assembly of drug delivery.

Within the last century, research has been conducted by Senapati, et al (2018) to explore hydrogel's applications to chemotherapeutic solutions to solid, cancerous tumors. Often, chemotherapy, like NSAIDs, negatively harms other bodily regions free of cancer, but due to hydrogel's biocompatibility, they provide an alternative means to encapsulate and target-apply a hydrogel composed of biomacromolecules. The physiological pH and temperature, at which hydrogels perform a slow release of drug, can be altered to release faster at a more acidic pH attributable to its cross-linkages; however, hydrogels also characterize themselves due to minute cytotoxicity making hydrogels a 'promising candidate for the controlled delivery of chemotherapeutics' and a plausible solution to the negative side effects that currently conventional anti-cancer drugs have on interconnected body systems[17].

Hydrogels are highly applicable to a wide variety of physiological inflammatory situations. Atherosclerosis, which is a disease characterized by aberrant arterial fat deposition, is an inflammatory disease in arteries that are localized to a very specific compartment and inflammatory pathway. Yi, et al (2020) have designed a synthetic polymer that can be injected into the inflamed area to deliver a form of vitamin D to disrupt the aberrant immune response in the arteries associated with dendritic cells. The polymer works as a crosslinked network that entraps the vitamin D. In the extracellular space around the heart, the hydrogel network is oxidized into a micellar structure to allow for the dispersion of medication to be physiologically regulated by

changes in the oxidation levels. These hydrogels can then respond to changing oxidation levels through chemical transformation to provide a transport mechanism for the medication *in vivo* for weeks or months[16].

Through another application of hydrogels, we can recognize their importance in tissue reformation. Damaged tissue and skin wounds can vary by their degree of severity and ability to regenerate themselves because it involves regulating the inflammatory response to restore the properties of the extracellular matrix composed of proteins like collagen, polysaccharides, and glycosaminoglycans[18]. The extracellular matrix in this situation is responsible for cell proliferation, signaling, and repairs to the physiological system that are assisted by the presence of collagen, a component in the dermis and extracellular matrix connective tissue, but for a wound lacking collagen, many collagen-based skin restoration products are strenuous to apply and maintain care leading to irregular formations of skin. When collagen-based hydrogels are applied, their water-bearing properties along with restorative collagen create a fluidity filling the conformal layers of wounds that create a more normal-looking skin topography[18]. This allows for not only an appeasing physical appearance but also restoration of the nature of damaged epidermal tissue. They implemented a hydrogel drug vehicle that allowed it to adhere and promote endothelial cell proliferation that will produce and maintain the extracellular matrix.

III. Calmodulin Hydrogel

A. Overview

As we mentioned before, CaM and M13 provided ideal properties to engineer our hydrogel since they are naturally found in the body, high binding affinity in calcium[4, 5], thus reducing their immunogenicity and promoting gel formation *in vivo*. CaM provides extremely stable thermal

capabilities with a melting point of 115 °C in the presence of calcium, so it can boil to purify and bind without losing properties. CaM has two EF-hand helix calcium-binding sites in each of two domains for a total of 4 sites (Figure 1.1). When calcium binds, hydrophobic pockets in the N- and C-terminal domains become solvent accessible and then interact with target peptides. Hence, the CaM -M13 peptide interacts in this double mitten wrap action of the two domains of CaM around the peptide. The peptide is unstructured until CaM binds to it. M13 structure then becomes helical while CaM's structure is more compact as it wraps around the peptide as shown in Figure 1.2 below.

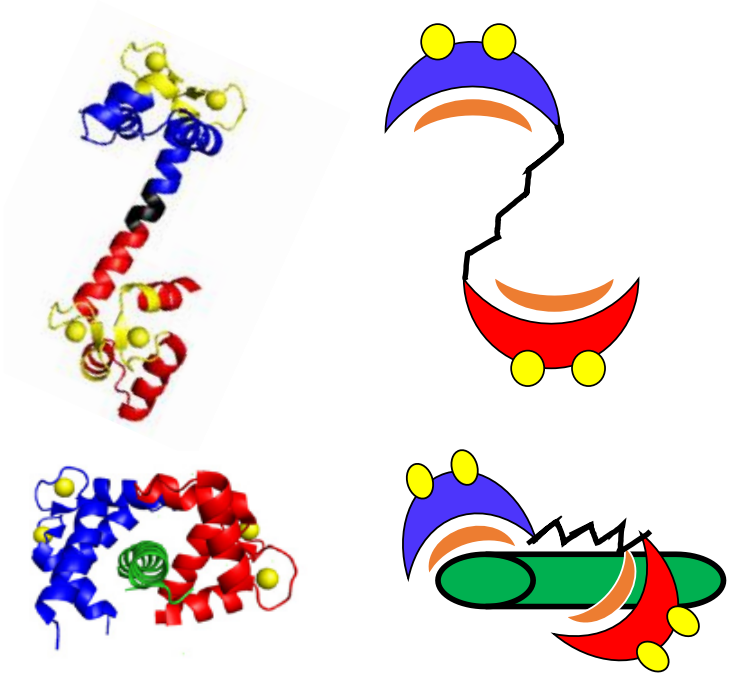

Figure 1.2: Ribbon and cartoon drawings calmodulin

Ribbon drawing of 3cln.pdb[19] with calcium-binding sites shown in yellow[20]. Helices lead into and out of each site. The "central helix" separates two independently folded domains. When calcium binds, the hydrophobic surface area is exposed at the base of each pair of sites (orange crescents in the cartoon). These hydrophobic surfaces interact with various calmodulin-binding agents such as trifluoperazine[21] and peptides from physiological partners (e.g. myosin light chain kinase[22] and ryanodine receptor[23]). In the ribbon[24] and cartoon drawings, the helical peptide from a physiological partner is represented as a green cylinder.

CaM-M13's self-assembling properties are spontaneous in the extracellular space due to free calcium ion levels varying between physiological concentrations of 1.1 mM to 1.5 mM[25-27]. These are approximately 100 to 1000 times higher than the binding affinities for the N- and C-terminal domains of CaM for calcium[4]. Thus, the linkage between CaM and M13 will develop in the presence of calcium ions that is predominantly in the extracellular (EC) space within a body system. The copolymer's ability to solubilize after lyophilization and to self-assemble allows for the optimization of genetically engineered components of the hydrogel. This section will cover the mechanisms of the synthetically modified proteins used in our experiments.

B. Engineered CaM and M13 Polymers

Two polymers, Calmodulin Collagen-Like Protein (CCLP) and Peptide Collagen-Like Protein (PCLP) were modified for the intent of this study and are represented by the schematic in Figure 1.3 below. CCLP3 has three repeating units of CaM while PCLP3 is named by three repeating segments of M13. They both have a general base of His6 sequences at the N-terminals that allow for His Tag purification, regions of collagen-seeming sequences between their different repeated segments that can be cleaved by physiologically occurring matrix metalloproteinases (MMPs), and C-terminal cysteine residues between each of the polymers used in covalent modifications.

CCLP3	
PCLP3	

Figure 1.3: Schematic of Polymers in Copolymeric Hydrogel

His Tag at the N-terminus (blue line) and calmodulin composed of 148 amino acids (orange) are followed by a collagen-like sequence with a consensus cleavage site represented by the green circle, which all compose the CCLP3. At the C-terminus of the protomer is a Cysteine that provides a thiol group that is modified in the specific chemistries explored in these studies. The PCLP3 protomer has the M13 peptide sequence (yellow) instead of Calmodulin and is composed of 26 amino acids at each M13 peptide.

IV. CCLP3, PCLP3, and Polymers

Physiologically, intracellular calcium levels range from 0.1 to 10 micromolar in the cytosol of muscle cells where CaM and M13 would normally associate. PCLP3 and CCLP3 in our hydrogel, which contains the sequences of M13 and CaM, vary extracellular calcium concentration between ~1 mM, as discussed above. This excess level of calcium will stabilize protein-protein interactions within the hydrogel. Intracellular CaM binds calcium ions at a high affinity because calcium concentrations are naturally low in the intracellular matrix, so calcium binds to both N-terminal and C-terminal sites of CaM to open the structure for binding of the target sequence. When it binds to CaM, CaM undergoes a conformational change that exposes hydrophobic areas at the base of binding sites, as seen in Figure 1.4 below. M13 from the skeletal muscle myosin light chain kinase is an appropriate choice of binding partner to CaM because of their high binding affinity and specific association. It was mentioned before that the bound complex makes M13 more

helical and CaM condensed, while the helix connecting the N- and C- terminal domains is unstructured and can adapt its shape to allow the hydrophobic pockets in the underside of the binding sites in each domain to interact specifically with the M13 peptide[22].

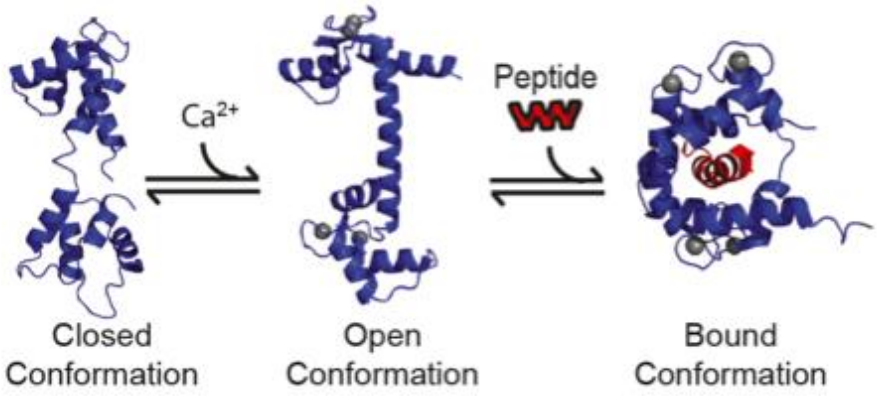

Figure 1.4: CaM binds with Peptide

The N and C terminal sites of the CaM-calcium complex are organized, and the hydrophobic pocket is exposed allowing for the binding of calmodulin to the intracellular protein's target sequences *in vivo*. Binding created the helical structure of the bound component.

With similar crosslinking as their basic protein components, CCLP3 and PCLP3 create noncovalent gel matrices, shown in **Figure 1.5** below. Based on previous research in this lab, our hydrogel is characterized by a few integral aspects. One aspect is that calcium ions are necessary to promote interactions for forming the supramolecular copolymeric hydrogels to form. Secondly, when used in vivo to deliver NSAIDs at the site of inflammation, we expect that the more acidic pH will increase the rate of hydrogel degradation due to the acid-activation of endogenous proteases[28]. Third, hydrogel formed from PLCP3 and CCLP3 showed only minor evidence of reshaping itself once it was deformed, reinforcing the importance of having the structure in the desired shape be applied. Lastly, the hydrogel did not swell "beyond the total volume of the mixed components," which emphasizes the boundaries to how much drug can be utilized in a single

hydrogel[29]. The formation of this study's copolymeric hydrogel, shown in Figure 1.5 below, occurs due to all of the characteristics aforementioned.

Figure 1.5: Formation of Hydrogel

Left: CaM-based polymer (CCLP3; n=3; blue) and M13-based polymer (PCLP3; n=3; green) are mixed in the presence of calcium. A complex physical co-polymeric "supramolecular" hydrogel form (right).

The next section of this review will cover details of the major materials and reactions used in this work. These include the protein that we studied and the chemistry behind our reactions of CCLP3 with Traut's Reagent and SATA for modifying the protein with additional -SH groups. Finally, we will descript the reaction of the -SH groups with Ellman's Reagent to determine the success of the modification of the protein.

Chapter 2

Chemistry

To acquire a better understanding of the reactions that take place throughout our experiment, this chapter will explain the chemistry behind those reactions. This chapter will have a specific focus on CCLP3 and the reactions involving Ellman's Reagent, Traut's Reagent, and SATA.

I. Characterization of CCLP3 and PCLP3

It is important to introduce the two components that comprise the hydrogel: CCLP3 and PCLP3. These are recombinant proteins designed in our laboratory by Drs. Fox and Pedigo after several iterations. The exact sequence of the Collagen-Like Sequence (GPQG/IWGQ) was chosen based on data regarding the most frequently cleaved sequence by the MMPs that are in most inflammatory processes[28, 30, 31]. The Arg-Cys-Arg sequence was chosen to place the single cysteine residue in an environment that would favor its reactivity. The exact sequences of both CCLP3 and PCLP3 are shown in Table 2.1 below. Notice the difference in the net charge on the two proteins. All studies in this thesis are on the CCLP3 construct.

	MW (g/mol)	Theoretical PI	Net Charge at pH 7	Extinction Coefficient at 280 nm ($M^{-1}cm^{-1}$)
CCLP3	58675	4.26	-68	32430
Amino acid Sequence MGSS**HHHHHH**SSGLVPRGSHMARMADQLTEEQIAEFKEAFSLFDKDGDGTITTKELG TVMRSLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRV FDKDGNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTA KGSGYGGPQGIWGQSGYGGSMADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMR SLGQNPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKD GNGYISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAKGSG YGGPQGIWGQSGYGGSMADQLTEEQIAEFKEAFSLFDKDGDGTITTKELGTVMRSLGQ NPTEAELQDMINEVDADGNGTIDFPEFLTMMARKMKDTDSEEEIREAFRVFDKDGNG YISAAELRHVMTNLGEKLTDEEVDEMIREADIDGDGQVNYEEFVQMMTAKGSGYGGP QGIWGQGSGYGS**RCR**				
PCLP3	19309	11.62	+22	35540
Amino acid Sequence MGSS**HHHHHH**SSGLVPRGSHMASMTGGQQMGRGSKRRWKKNFIAVSAANRFKKISS SGALELDSTQGPQGIWGQGGRRSGYGGSKRRWKKNFIAVSAANRFKKISSSGALELDS TQGPQGIWGQGSENSGKRRWKKNFIAVSAANRFKKISSSGALELDSTQGPQGIWGQGS ENSG**RCR**				

Table 2.1: Relevant Characteristics of CCLP3 and PCLP3

The Molecular Weight (MW) was calculated from the amino acid sequence, while the isoelectric point (PI), net charge at pH 7, and extinction coefficient were found from https://web.expasy.org/compute_pi/. Labels: Calmodulin sequence is in blue, **His6 sequence** is bolded, Collagenase Cleavage site is red underlined, The thrombin cleavage site is underlined black, the T7 tag is orange, M13 sequence is green, and the **cysteines** are red bolded.

II. Traut's Reagent

A. Traut's Reagent Background

Studies in this thesis were supported by work from earlier students in the Pedigo lab. Traut's Reagent is a cyclic thioimidate compound for thiolation or sulfhydryl addition[6]. The cyclic iminothiolane structure of Traut's opens to react with a free amine on proteins modify the protein with new sulfhydryl groups at the N-terminus and side chains of lysines of the protein. Traut's Reagent, which is known by its chemical name as 2-Iminothiolane HCl, was sought out to be a reliable reagent to allow the reactive primary amines on our protein to introduce new sulfhydryl groups and increase the number of reaction sites for NSAIDs while maintaining similar characteristics of the original protein. This reagent allows for spontaneous and efficient methods to react with primary amines at a pH range of 7-9 because it is very stable with acidic and neutral buffers that do not contain primary amines. These characteristics allow Traut's to be used with a variety of non-amino buffers. After the reaction is complete, it must be desalted using dialysis to remove small molecules and impurities, like salt, to purify the newly formed thiol addition version of CCLP3. Because of Traut's cyclic structure, the new sulfhydryl groups exposed on CCLP3 must be protected to prevent recyclization or oxidation. Its reversible nature contributes to the short life span before it reverts to a cyclic structure that is ineffective in attaching NSAIDs later. To prevent the reversible nature of the protein from cyclically reforming, or from reforming due to changes in pH not within range, we reacted modified CCLP3 with Ellman's Reagent, as a stable protector[32].

B. Traut's Chemistry

Traut's Reagent, also known as 2-iminothiolane, is a reagent used to modify primary amines such that a sulfhydryl is formed. The beginning reagents are shown in Figure 2.1A, and the final modified amine in Figure 2.1B. This is particularly useful for creating multiple sulfhydryl

sites on a protein for later reaction with thiol-reactive groups as attachment sites for drugs. In our experiment, we tested the success of this experiment with a sulfhydryl quantifying reagent like Ellman's Reagent.

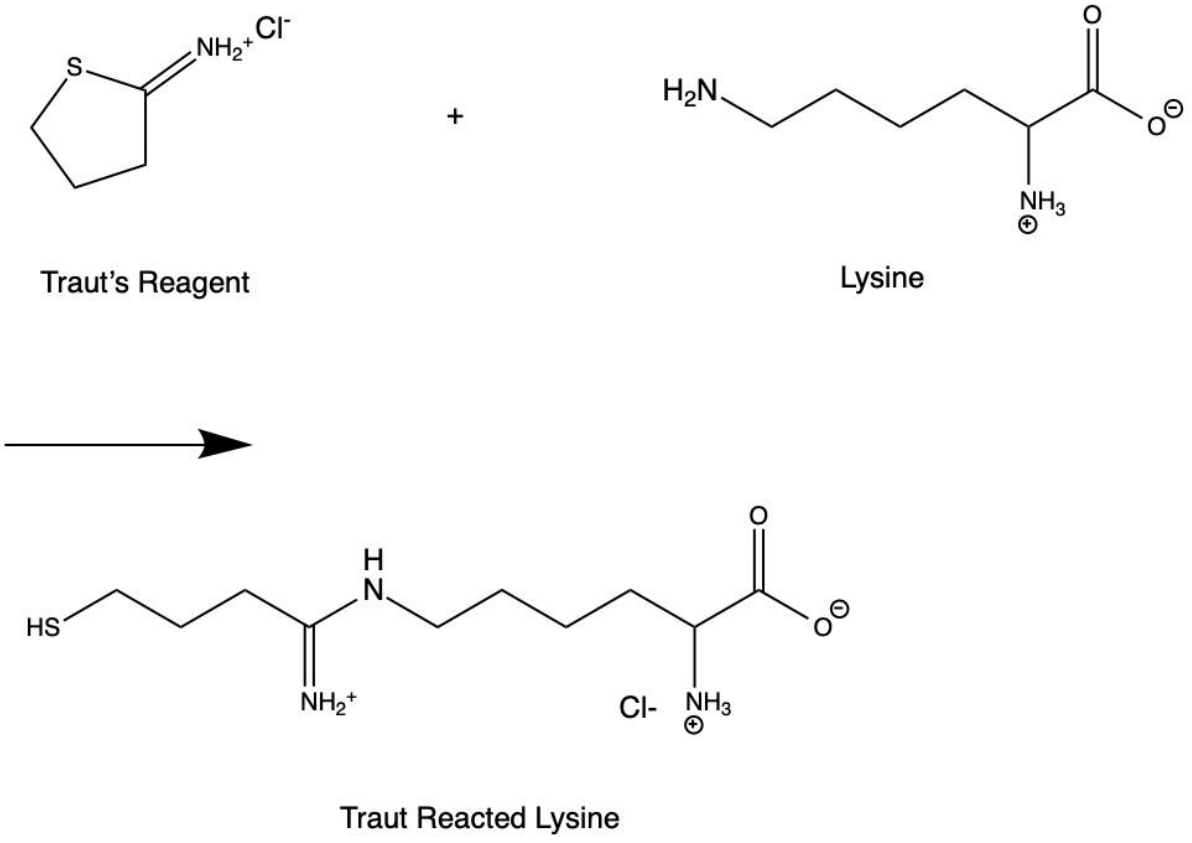

Figure 2.1: Reactivity of Traut's Reagent

(A) (Left) Traut's Reagent and (Right) the amino acid Lysine. As our protein has 24 lysines, Traut's Reagent will be immensely useful in creating sulfhydryl groups for further modification. (**B**) Lysine after reaction with Traut's Reagent.

As Figure 2.1 B shows, reacting Traut's Reagent with the lysine's ε amino group in CCLP3 allows a terminal sulfhydryl group to be formed, much like cysteine. The primary amine breaks open the 5-membered (four carbon one sulfur) ring, and then it forms an amidine functional group with the Traut's Reagent. Traut's Reagent also performs a similar reaction with the terminal amine

of the protein. This leads to an expected yield of 26 sulfhydryl groups: 1 terminal amine, 24 lysines, and 1 cysteine. The actual amount of sulfhydryl groups created will be quantified later once further reacted with Ellman's Reagent.

This reaction suffers from the problem that it is reversible. The sulfur can act as a nucleophile and attack the carbon of the amidine functional group and reverse the reaction. Thus, we fear the Traut-modified protein is unstable. (see Discussion) Thus, we sought a second means to modify the protein that afforded control over the reactivity of the thiol group.

C. SATA

SATA, or N-succinimidyl-S-acetylthioacetate, is another chemical that can modify primary amine groups into terminal sulfhydryl groups. Figure 2.2 shows the reaction between SATA and the amino acid lysine. SATA combines with the lysine by removing the N-hydroxysuccinimide group from SATA and forming an amide bond. SATA has the benefit of protecting the modified lysine from sulfur oxidation since the sulfur is protected until needed.

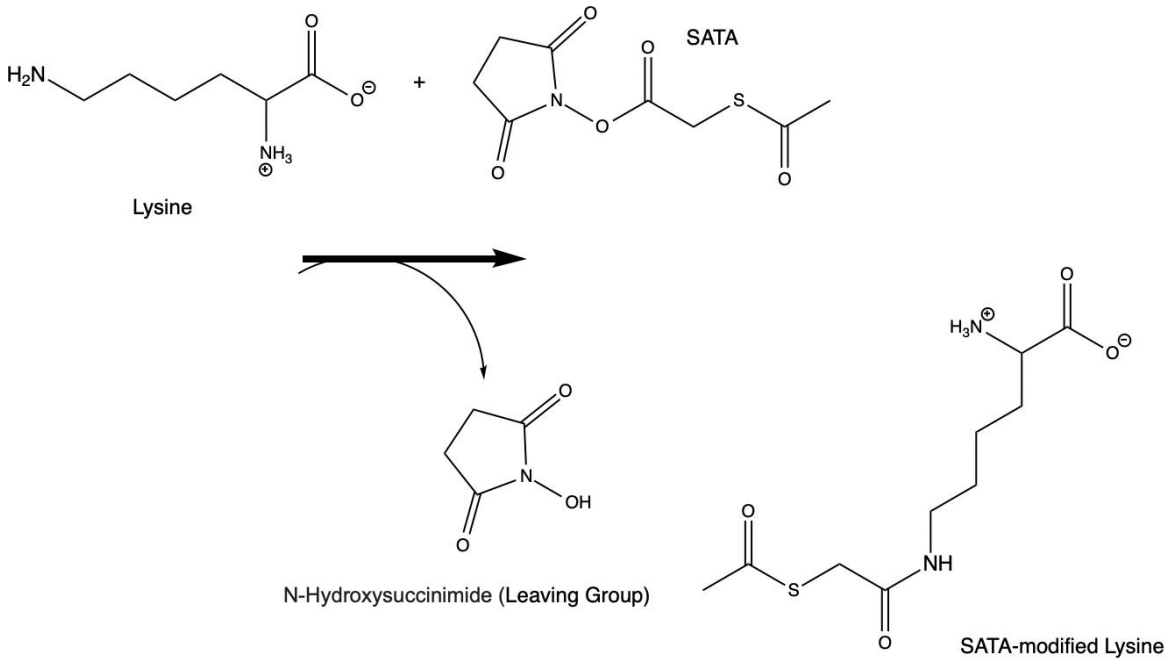

Figure 2.2: Lysine and SATA reaction

The reaction shown in Figure 2.3 shows the SATA-modified lysine becoming unprotected through its reaction with hydroxylamine. The hydroxylamine attacks the carbonyl carbon of the acetyl group protecting the sulfur, thus forming an amide bond with the SATA-modified lysine and N-hydroxy-acetamide becomes a leaving group. This leaves a sulfhydryl-modified lysine ready for further modification or quantification, like quantification with Ellman's Reagent.

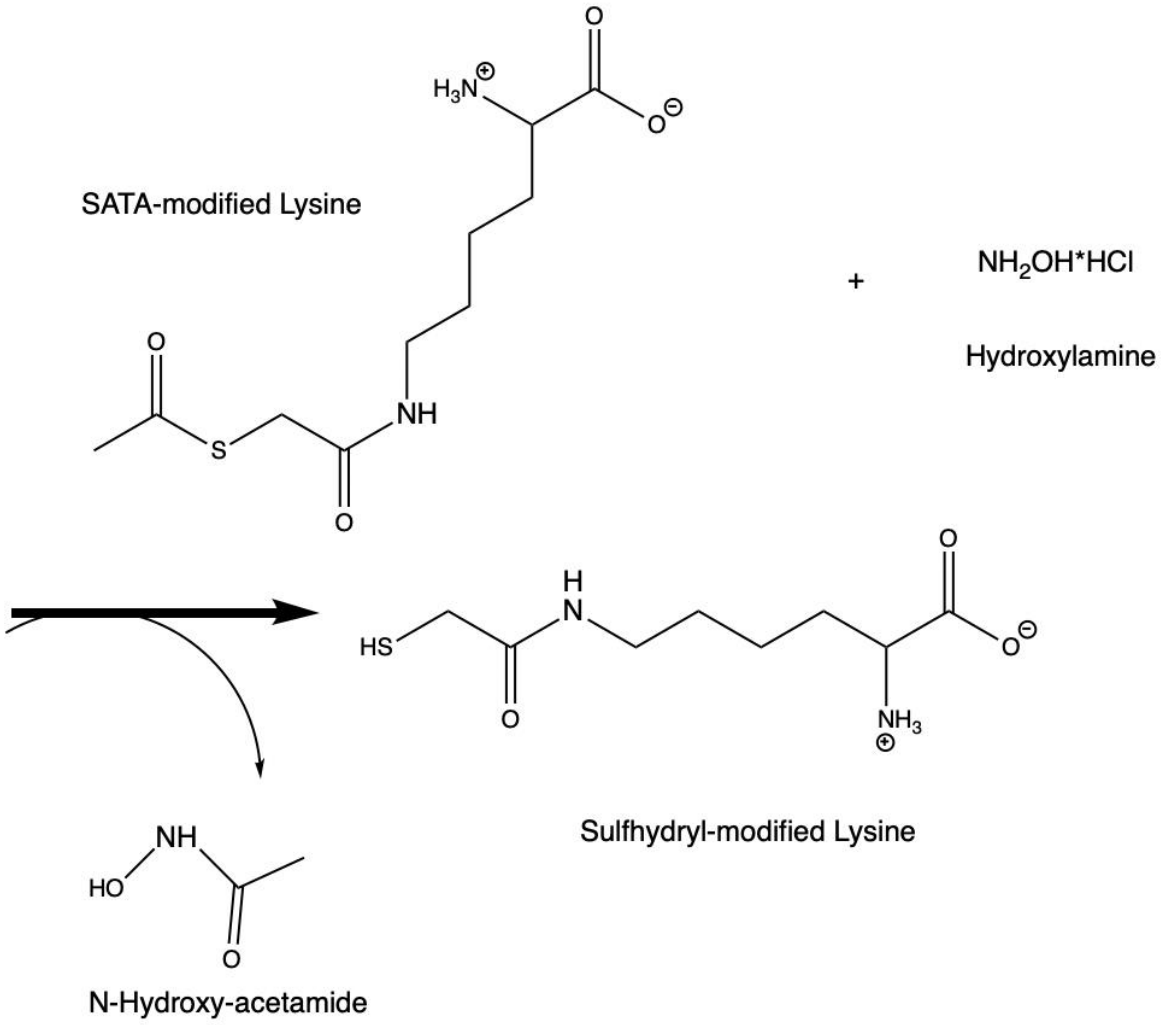

Figure 2.3: SATA-modified Lysine reaction with Hydroxylamine

This second reaction shows the deprotection of the sulfhydryl so that it can be subsequently reacted with a reactive drug linked to a maleimide group.

D. Ellman's Reagent

Throughout our experiments, we used Ellman's Reagent to quantify the efficiency of our sulfhydryl creating Traut's Reagent and SATA reactions. Ellman's Reagent is a good quantifier of sulfhydryl groups due to its reaction and clear color change in the visible region of the spectrum in the presence of sulfhydryl groups. The absorbance is measured at 412 nm. Figure 2.4 shows the

reaction of Ellman's Reagent and a sulfhydryl-containing compound (in our example cysteine). The sulfhydryl group attacks the disulfide bond of Ellman's Reagent, forming a mixed disulfide. The remaining product is 2-nitro-5-thiobenzoic acid (TNB^{2-}), which is fluorescent at 410 nm. This allows us to quantify the number of sulfhydryl groups in CCLP3.

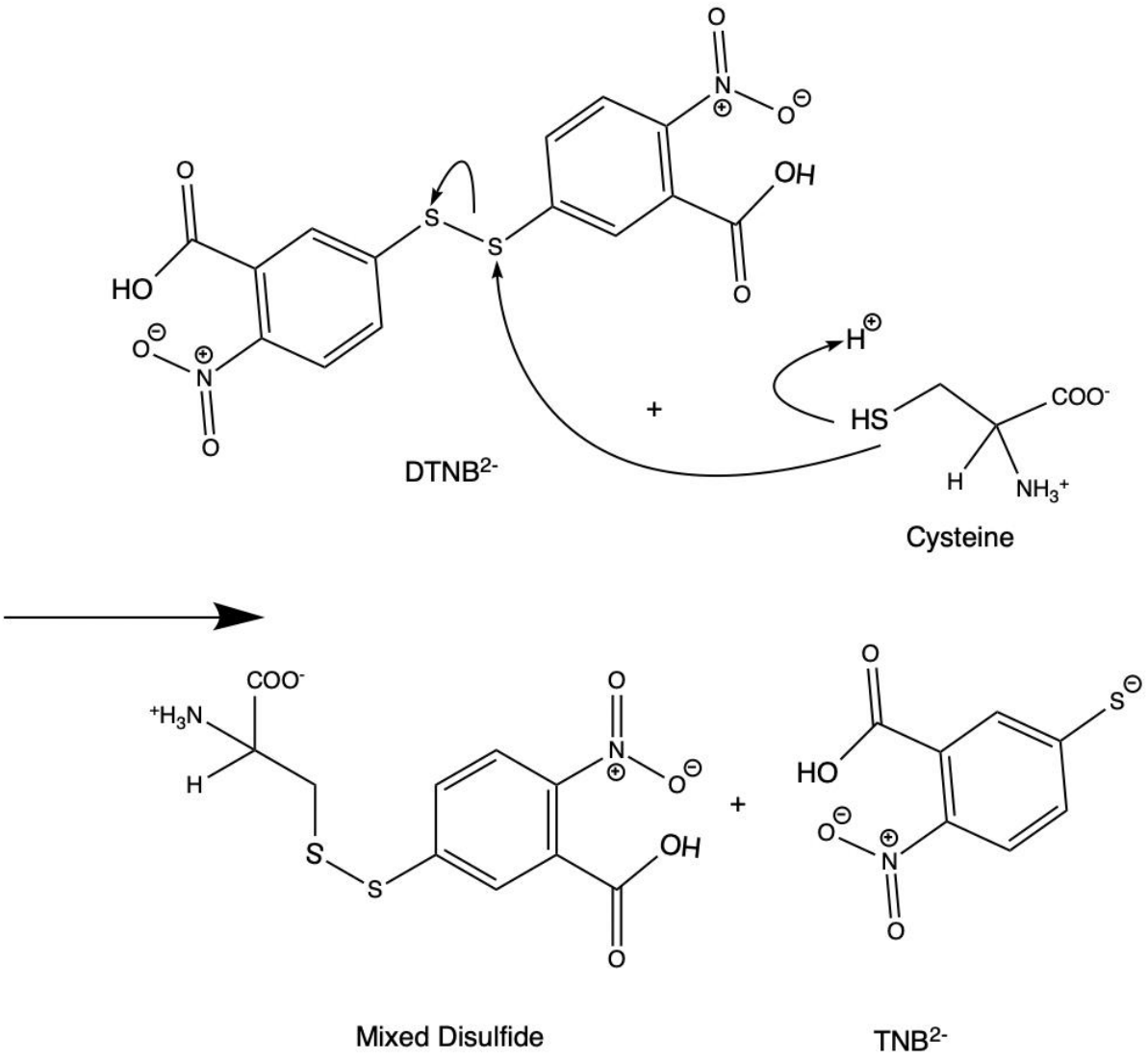

Figure 2.4: Ellman's reaction.

The reaction of Ellman's Reagent ($DTNB^{2-}$ / 5,5-dithio-bis-(2-nitrobenzoic acid)) and cysteine to a mixed disulfide and TNB^{2-} (2-nitro-5-thiobenzoate).

Chapter 3

Materials and Methods

I. Determining the Purity of Our Lyophilized CCLP3

Because CCLP3 was available as a dry powder, we were concerned about salt contamination in the lyophilized CCLP3 preparation. To determine the fraction of protein in our lyophilized CCLP3, and thus to determine the percent amount of CCLP3 that should be used in calculations, the following experiment was performed. We began by using Beer's Law to determine an appropriate concentration of CCLP3 needed to have an absorbance of 1 when read at 280 nm. By using the extinction coefficient of 32550 cm^{-1} M^{-1} [33], we determined that the required protein concentration would be 30.7 μM. Using this, along with a projected total volume of 1 mL of buffer and the molecular weight of CCLP3 being 58676 g/mol, it was determined that 0.018 g of CCLP3 was needed in 1 mL of buffer to achieve a 30.7 μM concentration. We then combined 0.018 g of CCLP3 with 1 mL of 10 mM HEPES and 140 mM NaCl buffer, pH 7.4, and read the absorbance spectrum from 200-350 nm on a Cary 50 Bio UV-Visible Spectrophotometer in a 1 cm quartz cuvette using the SimpleReads program. The specific absorbance at 280 nm and reported extinction coefficient above were then used to calculate the actual concentration of the prepared stock. The difference between the projected and prepared stock concentrations provided the percent purity of the CCLP3 protein preparation in terms of contaminating salts. The results of this experiment can be seen in Chapter 4.

II. SDS-PAGE to Assess Purity and Molecular Weight of CCLP3

To determine the purity and molecular weight of CCLP3 in the protein preparation, we performed a Tris-Glycine SDS-PAGE analysis of our protein stock of ten lanes with varying concentrations of CCLP3.

The gel was poured and polymerized in-house and comprised a 17% acrylamide: bisacrylamide (30:1) gel matrix, run under reducing and denaturing conditions. We created a running buffer made with 35 mL of 10x Tris-Glycine running buffer solution, 215 mL of water, and 3.5 mL of 10% SDS mixed in a large, graduated cylinder. We filled the chamber between the gel and the buffer dam with the running buffer to make sure the seal was watertight. For the sample dilutions, we used the 10 mM HEPES, 140 mM NaCl, and pH 7.4 buffer to create three dilutions of the protein stock (1:1; 1:10; and then 1:20). Each of these sample concentrations was diluted 1:1 in reducing, denaturing loading buffer containing bromophenol blue. Following this, we loaded the lanes in the gel. Lane 1 was created by using 5 μL of EZ-Run MW standard solution (FisherSci) to establish a calibration for the molecular weight of samples. Lane 2 was empty. Lanes 3-5 contained 2.5, 5, or 10 μL respectively of the 1:1 CCLP3 to buffer dilution. Lanes 6-8 contained 2.5, 5, or 10 μL respectively of 1:10 CCLP3 to buffer dilution. Finally, Lanes 9-10 contained 5 or 10 μL respectively of 1:20 CCLP3 to buffer dilution.

The gel was then run at 200 volts for sixty minutes at room temperature. At the thirty-minute mark, we noticed that the samples were moving down the gel and that the dye front was a thin blue line, indicating that the stacking process worked well, and the electrophoretic process was working normally. After the gel was completed, it was placed in the Coomassie blue staining solution overnight. The following day, the gel was destained in methanol, dilute acetic acid. The results of this experiment can be seen in Chapter 4.

III. Reacting CCLP3 with Traut's Reagent in an Aerobic Environment

Traut's reagent is the common name for 2-iminothiolane, a common reagent for modifying amines to create reactive sulfhydryl groups. This experiment required multiple iterations to optimize the protocol. These results can be found in Chapter 4.

A. Conversion of Primary Amines to Sulfhydryl Groups with Traut's Reagent

To be able to increase the number of reactive sulfhydryl groups in CCLP3, we used Traut's reagent to be able to convert the primary amine groups of the 24 lysines and the N-terminus of the protein to sulfhydryl groups. Along with the one cysteine already present in the protein, this would give us a theoretical yield of 26 sulfhydryl groups.

We began by using 5 mg of lyophilized CCLP3. A solution of CCLP3 was prepared at the concentration of 10 mg/mL in 0.1 M sodium phosphate, 1 mM EDTA, pH 8.0. Following this, Traut's reagent (2-iminothiolane) was prepared in 2 mg/mL concentration by adding 10 mg of Traut's to 1 mL buffer solution. The solution was proportioned in a 1:50 mole ratio (CCLP3: 2-iminothiolane) by adding 54 μL of Traut's solution to the 0.5 mL protein solution. Next, for 1 hour, the process was carried out in a shaker. Following this, unreacted Traut's reagent was removed by dialysis for 6 hours, with solvent replacement every two hours for a total of three rotations. At the end of these buffer exchanges, we projected a starting and ending concentration of 139 μM CCLP3; however, we had a starting concentration of 7 mM Traut's reagent and an ending concentration of 878 pM. The protein was then much more concentrated in the diluted sample with a negligible amount of Traut's reagent (approximately 0.00000629% of the solution was Traut's reagent). We then ran an SDS-PAGE experiment to assess the state of the protein. The

results of the gel can be seen in Chapter 4. We then used Ellman's reagent to determine the sulfhydryl groups in our newly modified protein.

B. Quantification of Sulfhydryl Groups using Ellman's Reagent

To quantify the number of sulfhydryl groups that were created from the reaction of CCLP3 with Traut's reagent, Ellman's reagent was used. Since Ellman's reagent is very reactive, highly specific for sulfhydryl groups at neutral pH, and has a high extinction coefficient, it was selected to quantify the number of sulfhydryl groups. Furthermore, the presence of sulfhydryl groups can be immediately determined since Ellman's reagent turns yellow in the presence of sulfhydryl groups.

We began by making a Reaction Buffer with the concentration of 0.1 M potassium phosphate, 1 mM EDTA, pH 8.0. Finally, we added water until the total is 500 mL of buffer. We then made Ellman's standards with dilute cysteine hydrochloride monohydrate as described in Table 3.1.

Table 3.1: The Ellman's Reagent Standards

Standard	Volume Reaction Buffer	Amount of Cysteine	Final Concentration
A	100 mL	26.34 mg	1.5 mM
B	5 mL	25 mL of A	1.25 mM
C	10 mL	20 mL of A	1.0 mM
D	15 mL	15 mL of A	0.75 mM
E	20 mL	10 mL of A	0.50 mM

F	25 mL	5 mL of A	0.25 mM
G	5 mL	20 mL F	0.20 mM
H	5 mL	15 mL G	0.15 mM
I	15 mL	10 mL H	0.06 mM
J	15 mL	15 mL I	0.03 mM
K	30 mL	0 mL of A	0.0 mM

Following this, we prepared a set of test tubes, each containing 50 µL of Ellman's Reagent Solution (4 mg Ellman's Reagent in 1 mL of Reaction Buffer) and 2.5 mL of Reaction Buffer. We then added 250 µL of each standard or unknown to the separate test tubes prepared previously. A volume of 250 µL of our protein went in the tube labeled L, and 250 µL of standards A-K were put in their respective tube as well. The test tubes were then mixed and incubated at room temperature for 15 minutes.

Following this, the absorbance was measured at 412 nm using the 1 cm quartz cuvette on the Cary 50 Bio UV-Visible Spectrophotometer and SimpleReads program. The values obtained were then plotted as the standards to generate a standard curve. Using the standard curve and the absorbance of the unknown, we were able to determine the concentration of sulfhydryl groups in the post-Traut's reagent reacted CCLP3. The results of both experiments can be found in Chapter 4.

IV. Reacting CCLP3 with Traut's Reagent in an Anaerobic Environment

After reacting the CCLP3 with Traut's in an aerobic environment, it was determined that a possible cause of the low cysteine per protein concentration was potential disulfide bonds forming after reaction with Traut's reagent. Thus, it was determined that it would be possible to prevent

the oxidation of sulfhydryl groups into disulfides if the experiment was carried out in an anaerobic environment.

A. Reacting CCLP3 with Traut's Reagent under Argon

The experiment was then undertaken under a controlled anaerobic environment that contained argon instead. We began by using 5 mg of lyophilized CCLP3. This was placed into a tube that was purged with argon before the CCLP3 was added. Following this, the buffer of 0.1 M potassium phosphate, 1 mM EDTA, pH 8.0, was placed into the tube via a syringe through the parafilm. Traut's reagent (2-iminothiolane) was prepared in 2 mg/mL concentration by adding 10 mg of Traut's to 1 mL of the buffer solution. The solution was proportioned in a 1:50 mole ratio (CCLP3: 2-iminothiolane) by adding 54 μL of Traut's solution to the 0.5 mL protein solution. Next, for 1 hour, the process was carried out in a shaker while still in the argon environment and sealed with parafilm. Following this, unreacted Traut's reagent was removed by dialysis for 6 hours, with a rotation every two hours for a total of three rotations. The entire dialysis procedure was performed with a continuous stream of argon being pumped into the beaker that was covered with parafilm, as seen in Figure 3.2.

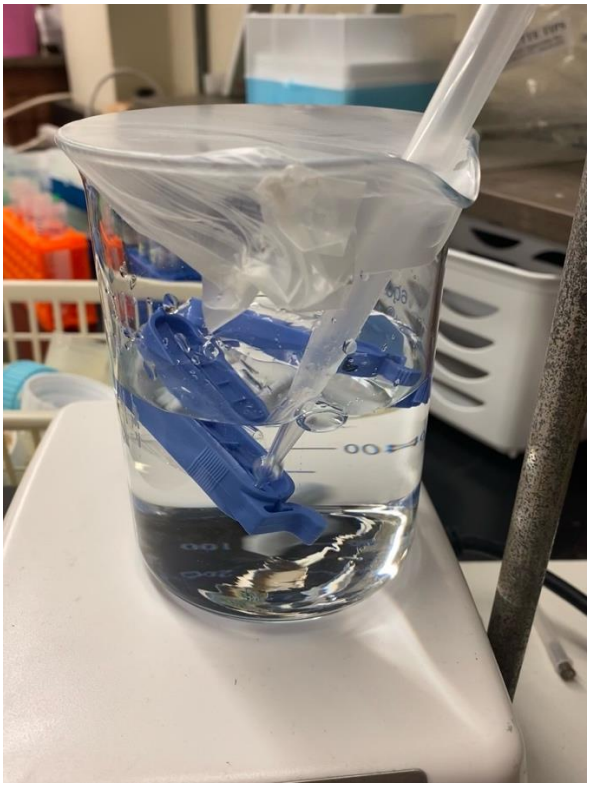

Figure 3.2: Photo Dialysis under Argon

As with the previous experiment, following the dialysis, we immediately proceeded with reacting with Ellman's Reagent. Each of the tubes was purged with argon before being filled with the Traut-reacted CCLP3. The reaction was carried out using the same standards in Table 3.1, the sole difference being that the reaction was carried out under constant sparging with Argon. The results were then analyzed using the Cary 50 Bio UV-Visible Spectrophotometer. These results can be seen in Chapter 4.

B. Reacting CCLP3 with SATA under Argon

After reacting CCLP3 with Ellman's Reagent in an aerobic environment, we realized that it was likely the oxidation of sulfhydryl groups that was causing Ellman's quantification to reveal

fewer sulfhydryl groups than expected. While reacting under argon was a potential solution to this problem, we thought it would be more simplistic if we used a reagent other than Traut's that could potentially inhibit such oxidation. Thus, we sought out SATA or N-succinimidyl S-acetylthioacetate. SATA is special because it protects the sulfhydryl group until it is deprotected with hydroxylamine for further reaction. This could produce a long-term solution for the storage of our reacted protein without having to worry about potential oxidation like you would with Traut's Reagent.

To prevent any oxidation potential during the reaction, this experiment was also carried out within an argon environment. We began by using 10 mg of lyophilized CCLP3. A buffer solution was created containing 0.1 M sodium phosphate buffer, 1mM EDTA, pH 8.0. A solution of CCLP3 and buffer was created by combining 10 mg of CCLP3 with 1 mL of the prepared buffer. We then dissolved 16 mg of SATA in 1 mL of DMSO resulting in an approximately 69.2 mM solution. This achieves a 250:1 molar ratio of SATA to protein (19.25 micromoles SATA: 77 nanomoles protein). Following this, we combined 1 mL of CCLP3 Solution with 556 µL of the SATA solution. We mixed the contents and incubated the reaction at room temperature for 30 minutes. The mixture was purified by dialysis for 6 hours, with a rotation every two hours for a total of three rotations and 500 mL of fresh buffer added each rotation (CCLP3 MW: 58.676 kDa, SATA MW: 231.23)

When it was time for quantification of the created sulfhydryl groups with Ellman's Reagent, we prepared the deacetylation solution. We created a deacetylation solution of 0.5 M Hydroxylamine, 25 mM EDTA, pH 7.4. We combined 1.0 mL of SATA-modified (acetylated) protein with 100 µL of the Deacetylation Solution. We then mixed the contents and incubate the

reaction for 2 hours at room temperature. Following this, we purified the mixture by dialysis under Argon for 2 hours.

As with the previous experiment, following the dialysis, we immediately proceeded with reacting with Ellman's Reagent. Each of the tubes was purged with argon before being filled with the SATA-reacted CCLP3. The reaction was carried out using the same standards in Table 3.1, the sole difference being that the reaction was carried out under constant sparging with Argon. The results were then analyzed using the Cary 50 Bio UV-Visible Spectrophotometer. These results can be seen in Chapter 4.

Chapter 4

Results

I. Characterizing the Lyophilized CCLP3 Stock

Figure 4.1 shows a spectrum of CCLP3 stock. The graph depicts the absorbance of our lyophilized at the wavelengths between 230-350 nm. As our protein contains 12 tyrosine, 3 tryptophan, and 1 cysteine, the anticipated extinction coefficient is 32550 M^{-1} cm^{-1} based on the equation that stays ε= aW + bY + cC where a, b, and c are 5690, 1280, and 120 M^{-1} cm^{-1}, respectively, and W, Y, and C are the number of tryptophan, tyrosine, and cysteine in CCLP3[33].

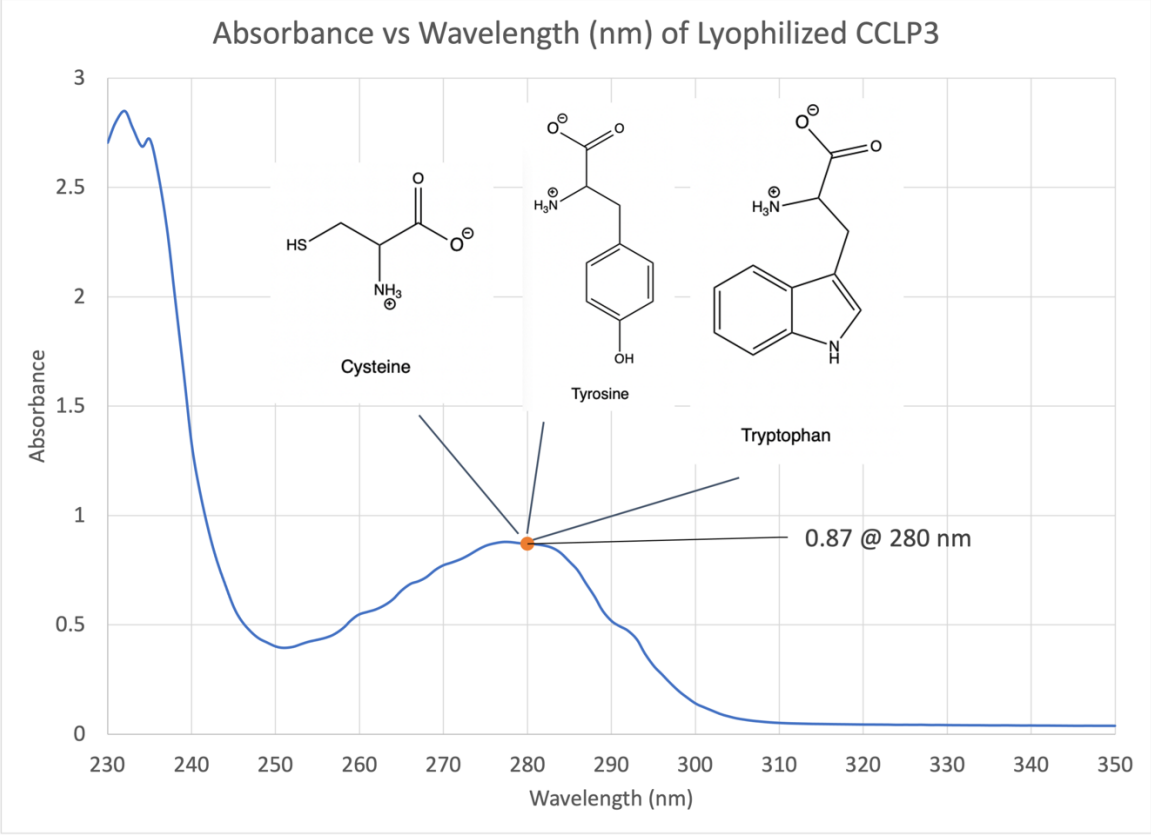

Figure 4.1: The Absorbance vs Wavelength of Lyophilized CCLP3

Using the extinction coefficient and Beer's Law of A=εbc, we calculated the concentration of CCLP3 to need an absorbance of 1, or 30.7 μM. Using this concentration, a spectrum was run on a Cary 50 Bio UV-Visible Spectrophotometer. The measured absorbance was found to be 0.87 at 280 nm, therefore the actual concentration of CCLP3 in solution was approximately 25.5 μM, or 87% protein by weight. For all future experiments, we considered these contaminating salts when calculating the total mass necessary to prepare a particular protein concentration.

II. Running an SDS-PAGE to Verify the Presence of CCLP3

To assess the approximate fraction of protein that is full-length CCLP3, we performed gel electrophoresis. Figure 4.2 shows the results of the SDS-PAGE gel of varying dilutions of CCLP3.

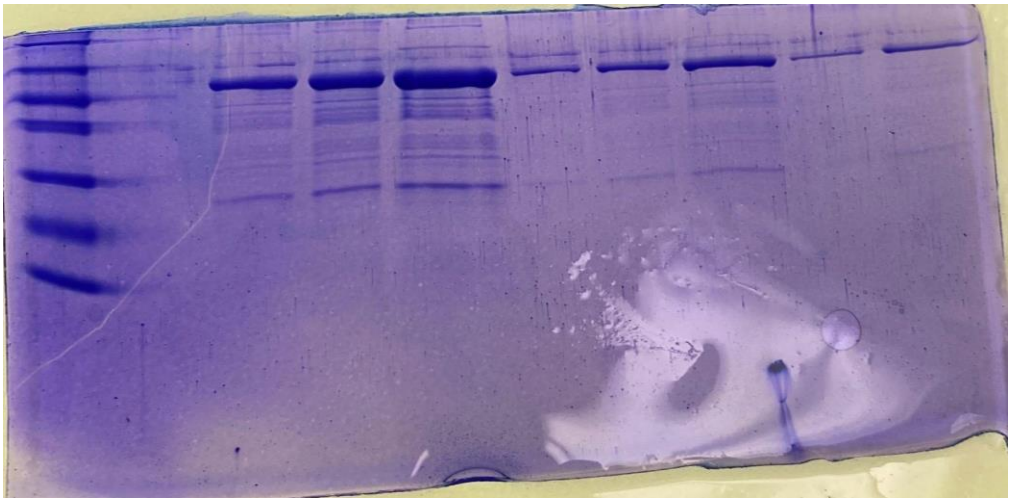

Figure 4.2: Reducing SDS-PAGE Gel Used to Verify Presence of CCLP3

Lane 1 contains the MW ladder (116, 66, 45, 35, 24, 18.4. 14.4 kD top to bottom), Lane 2 was skipped, Lanes 3-5 contained increasing amounts of 1:1 CCLP3 to buffer, Lanes 6-8 contained increasing amounts of 1:10 CCLP3 to buffer, and Lanes 9-10 contained increasing amounts of 1:20 CCLP3 to buffer.

The SDS-PAGE gel showed that CCLP3, with its molecular weight of 58676 g/mol was present in every sample, and it is approximately halfway between the 66 kD and the 45 kD

molecular weight ladder markers. Therefore, one can conclude that CCLP3 was present in our lyophilized sample with an expected molecular weight.

III. Reacting CCLP3 with Traut's Reagent in an Aerobic Environment

A. Basic Assessment of Procedure

We used SDS-PAGE to assess the integrity of the protein and the dialysis procedure. Figure 4.3 shows the results of the SDS-PAGE used to check for both CCLP3 after dialysis and the leakage of CCLP3 through the dialysis tubing during dialysis. The emptiness of lanes 2-7 shows that at no time did CCLP3 leak into the dialysis buffer. Furthermore, lanes 8-9 show that CCLP3 was present in the post-dialysis solution. Therefore, we expect the dialysis was successful at removing Traut's reagent (MW: 137.63 g/mol) since it is small, hydrophilic, and positively charged, yet the dialysis allowed CCLP3 (MW: 58676 g/mol) to remain in the dialysis tubing.

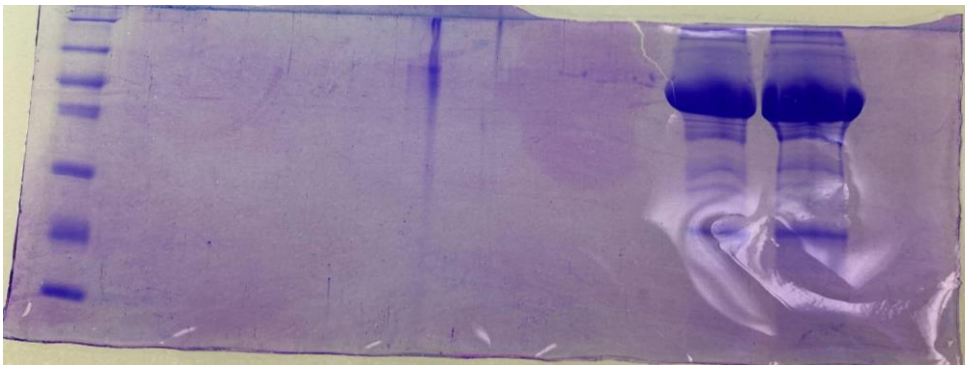

Figure 4.3: Reducing SDS-PAGE Used to Check for Presence of CCLP3 after Dialysis

Lane 1: MW Ladder (116, 66, 45, 35, 24, 18.4. 14.4 kD top to bottom).
Lanes 2 and 3: 5 and 10 µL respectively of the dialysis buffer were removed after 2 hours.
Lanes 4 and 5: 5 and 10 µL respectively of the dialysis buffer were removed after 4 hours.
Lanes 6 and 7: 5 and 10 µL respectively of the dialysis buffer were removed after 6 hours.
Lanes 8 and 9: 5 and 10 µL respectively of CCLP3 following dialysis after the reaction of CCLP3 and Traut's reagent.
Lane 10: Empty

B. Quantification of Sulfhydryl Groups using Ellman's Reagent

1. Experiment 1

Ellman's reagent is used to quantitate the success of the modification of the protein with Traut's reagent. Figure 4.4 shows the results of our cysteine calibration curve compared to the measured absorbance of Traut's Reagent reacted CCLP3.

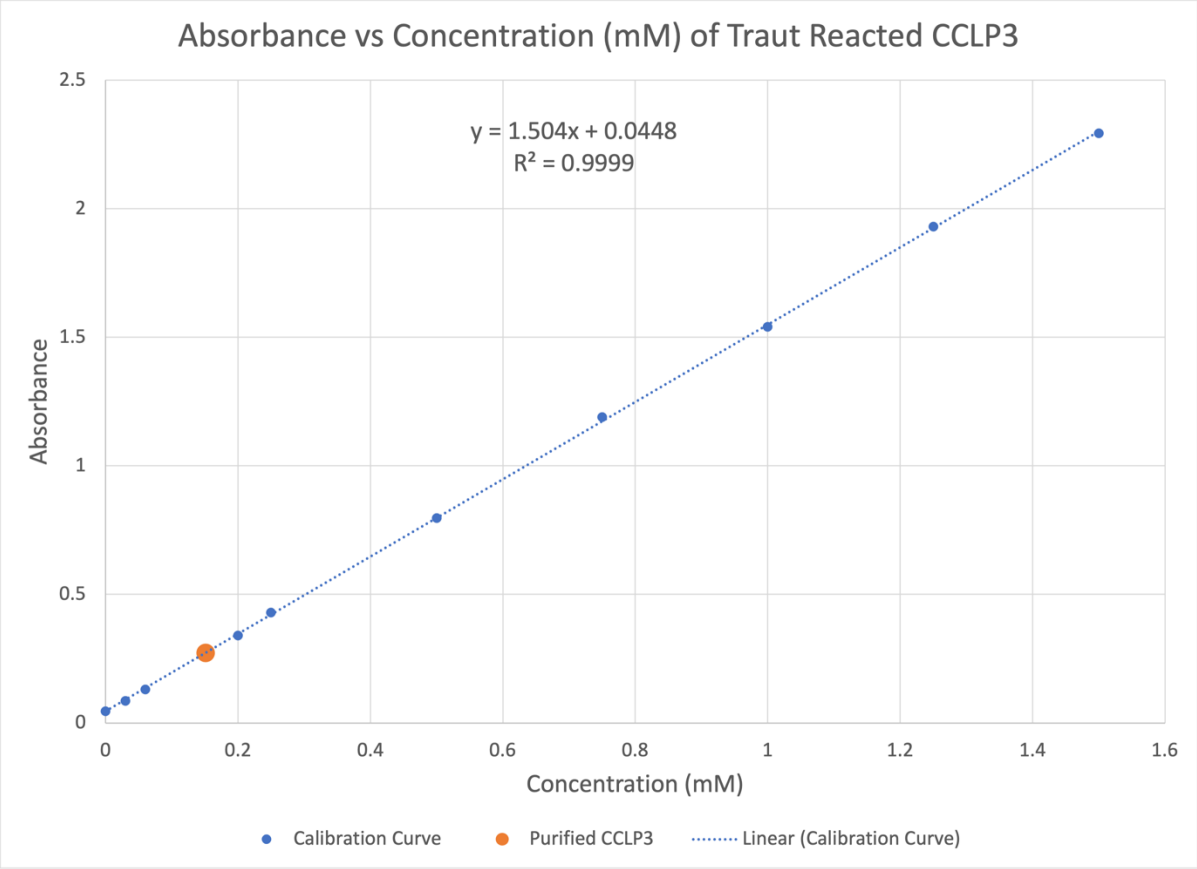

Figure 4.4: Ellman's Calibration curve and Sample- in air.

The Concentration (mM) vs Absorbance of a Cysteine Calibration Curve and our Purified CCLP3 Sample after reaction with Traut's Reagent

$$[Cys] = \frac{Abs}{\varepsilon * b}$$

$$\frac{\#Cys}{Protein} = \frac{[Cys]_{exp}}{[protein]_{dil}}$$

$$[protein]_{dil} = Stock * \frac{0.25\ ml}{2.8\ ml}$$

To find the number of cysteines per protein, one must do the previous calculations. The measured absorbance of the CCLP3 was 0.27227. Using Beer's Law and Ellman's Reagent's extension coefficient of 14140 M^{-1} cm^{-1} gives you a concentration of 19.26 μM cysteine in the CCLP3 sample.

Since we used 70.7 nmol of protein in the 2.8 mL solution it was determined that the concentration of protein was 6.3 μM in the whole solution. Therefore, when you divide the concentration of cysteine by the concentration of protein, one can determine that there are approximately 3.1 cysteine/protein molecule. This is about 11.7% of the expected 26 cysteines per protein molecule that should have formed after reaction with Traut's Reagent. Therefore, it was hypothesized that Traut's Reaction was volatile as Ellman's protocol was done the following day. To test this, the experiment was repeated the following day, and Ellman's protocol was done immediately after the conclusion of Traut's Reaction.

2. Experiment 2

Figure 4.5 shows the results of our cysteine calibration curve compared to the measured absorbance of our t's Reagent reacted CCLP3. The measured absorbance of the CCLP3 was 0.131. Using Beer's Law and the Ellman's Reagent's extension coefficient of 14140 M^{-1} cm^{-1} gives you a concentration of 9.26 μM cysteine in the CCLP3 sample.

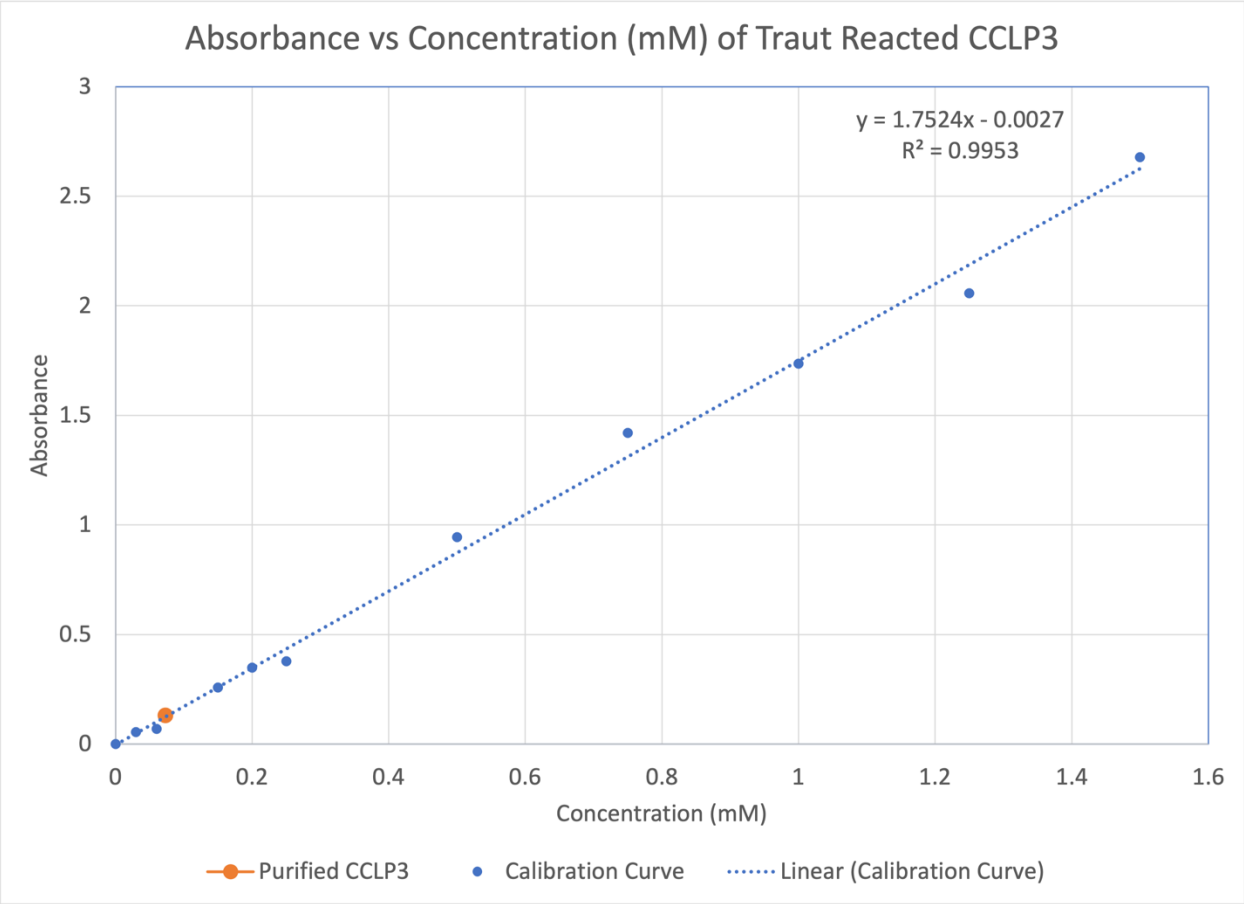

Figure 4.5: Ellman's Calibration curve and Sample- in air second trial.

The Concentration (mM) vs Absorbance of a Cysteine Calibration Curve and our Purified CCLP3 Sample after reaction with Traut's Reagent

As above, our protein concentration was 6.3 μM. Therefore, it was determined that there are approximately 1.5 cysteine/protein molecule. This is about 5.7% of the expected 26 cysteines per protein molecule that should have formed after reaction with Traut's Reagent. Therefore, our hypothesis about Traut's reaction being volatile was seemingly confirmed.

IV. Reacting CCLP3 in an Anaerobic Environment

A. Reacting CCLP3 with Ellman's Reagent under Argon

Figure 4.6 shows the results of our cysteine calibration curve compared to the measured absorbance of Traut's Reagent reacted CCLP3. The measured absorbance of the CCLP3 was 0.868. Using Beer's Law and Ellman's Reagent's extension coefficient of 14140 M^{-1} cm^{-1} gives you a concentration of 61.39 μM cysteine in the CCLP3 sample.

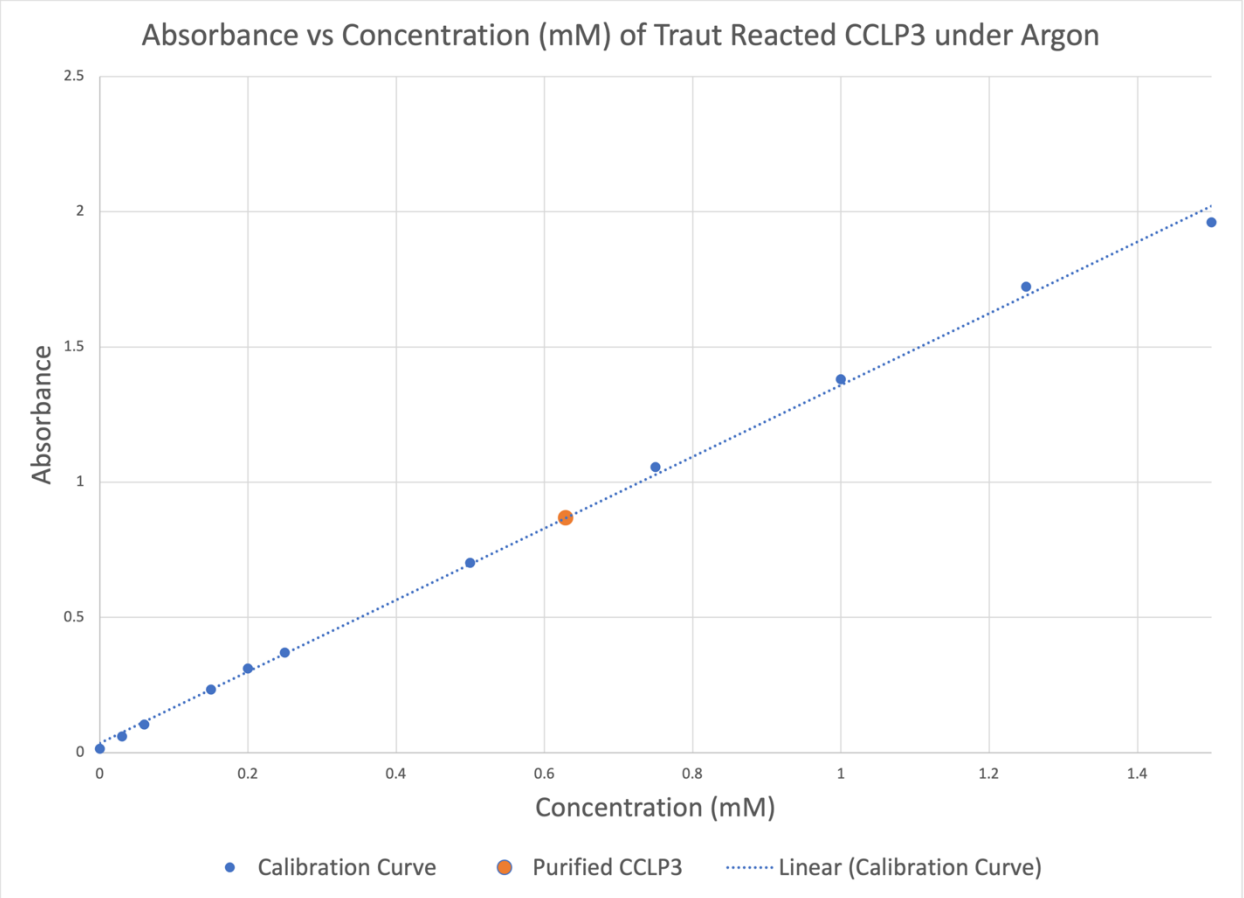

Figure 4.6: Ellman's Calibration curve and Sample- under Argon.

The Concentration (mM) vs Absorbance of a Cysteine Calibration Curve and our Purified CCLP3 Sample after reaction with Traut's Reagent under Argon

Like the aerobic reactions, our protein concentration was 6.3 µM. Therefore, it was determined that there are approximately 9.74 cysteine/protein molecule, about 37.4% of the expected 26 cysteines per protein molecule that should have formed after reaction with Traut's Reagent. Since the only variable we changed was whether the experiment was done in an aerobic or anaerobic environment, we determined that it was likely that the aerobic environment was not suitable for Traut's reaction and Ellman's quantification due to the oxidation of the created sulfhydryl groups.

B. Reacting CCLP3 with SATA under Argon

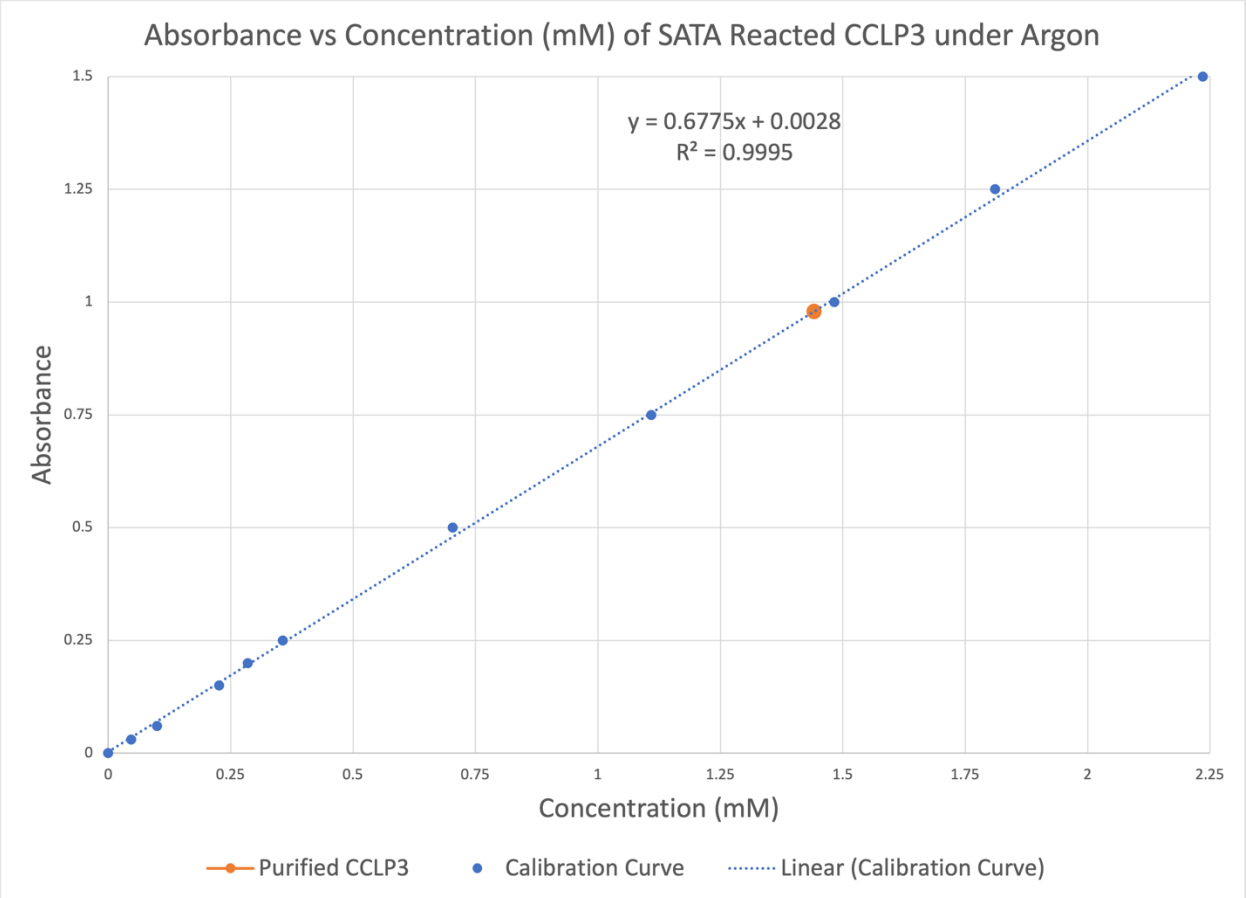

Figure 4.7: The Concentration (mM) vs Absorbance of a Cysteine Calibration Curve and our Purified CCLP3 Sample after reaction with SATA under Argon

Figure 4.7 shows the results of our cysteine calibration curve compared to the measured absorbance of our SATA reacted CCLP3. The measured absorbance of the CCLP3 was 0.979. Using Beer's Law and Ellman's Reagent's extension coefficient of 14140 M^{-1} cm^{-1} gives you a concentration of 69.23 μM cysteine in the CCLP3 sample.

Contrary to the previous reactions, because we used a differing amount of CCLP3 (10 mg), we determined there were 153 nmol CCLP3. Thus, it was determined the concentration of CCLP3 in our solution was 13.66 μM. Therefore, we calculated that there are approximately 5.1 cysteine/protein molecule, about 19.1% of the expected 26 cysteines per protein molecule that should have formed after reaction with SATA. Since SATA reactivity is partially determined by the molar concentration ratio with SATA to the protein to be reacted, this was likely the cause of our less reactive results. The efficiency of SATA will be discussed more in Chapter 5.

Chapter 5

Discussion

The main goal of this experiment was to create a process that was suitable for adding sulfhydryl groups to our CCLP3 protein such that potential drugs could be attached to our protein for use in a hydrogel. Sulfhydryl groups are rare in proteins as they are only found in one amino acid, cysteine. Most of the cysteines found in proteins are easily oxidized from the combination of two cysteines to form the amino acid cystine. Sulfhydryl groups are also reactive to multiple types of chemistries including reactions with themselves (as mentioned above) and reactions that involve them being the reducing agent of a reaction due to their easily donatable hydrogen. They furthermore are important in the creation of protein networks by the creation of sulfhydryl bonds between multiple cysteine groups. These bonds help establish a protein network between multiple proteins such as in a protein consisting of multiple units of itself.

We explored the use of two reagents that reacted with free amines such as those on the side chains of Lysines and the N-terminus of CCLP3, our protein of interest. The different methods used produced differing results that ranged from feasible for the addition of sulfhydryl groups to others that are less successful. Here we will discuss the different aspects of the results, while also expanding on what this could mean for future experiments.

I. Reacting CCLP3 with Traut's Reagent in an Aerobic Environment

The reaction of CCLP3 with Traut's Reagent carried out in an aerobic environment produced fewer modification sites than expected. The number of expected sulfhydryl groups after modification is twenty-six, accounting for the twenty-four lysines, one terminal amine, and one cysteine already present. However, using this method, we only achieved the creation of approximately 3.1 cysteines per protein molecule. We repeated the experiment and received similar results with 1.5 cysteines per protein molecule. Since there is already one cysteine in CCLP3, the expected results are much lower than anticipated as there were twenty-five amino groups that were to be converted to sulfhydryl groups with Traut's Reagent. Thus, we expected that there was some other complication causing the error.

Based on the chemistry of both Traut's Reagent and sulfhydryl groups in general, we hypothesized that there was potential oxidation of the sulfhydryl groups into a disulfide linkage. The formation of a disulfide would deactivate the reagent, and the protein wouldn't be modified. This would cause a lower-than-expected result following the quantification of sulfhydryl groups with Ellman's Reagent. Therefore, we determined that the experiment should be repeated in an anaerobic environment.

II. Reacting CCLP3 with Traut's Reagent in an Anaerobic Environment

The reaction of CCLP3 with Traut's Reagent in an anaerobic environment produced better results than the aerobic reaction, as expected. With approximately 9.74 sulfhydryl/protein, there was more than a 3x improvement compared to the aerobic reactions. Thus, we determined that there was some validity to our hypothesis that there was oxidation of sulfhydryl groups in the

aerobic reactions. Therefore, we recommend that for future experiments all reactions be carried out in an anaerobic environment, particularly under an inert gas atmosphere like argon.

Even though the results were greater than reactions in aerobic conditions, the results provided only about 37.4% of the expected 26 reaction sites/protein. We believe this may have something to do with the fact the reaction took place with only a 1:50 mole ratio of CCLP: 2-iminiothiolane (Traut's Reagent). This is only a 1:2 mole ratio of Traut's Reagent to reaction sites. By potentially increasing the ratio of Traut's Reagent, we predict there could be greater reactivity. Another issue is the potential oxidation of sulfhydryl groups, as no matter, the environment used there is always the potential for the possible introduction of oxygen to the environment. One of the possible ways to mitigate this issue is the addition of a reducing agent, like dithiothreitol (DTT). This could be added at the beginning of the process and removed during the dialysis step. Furthermore, a reducing agent like tris(2-carboxyethyl)phosphine (TCEP) could be used the following dialysis as it would not interfere with the quantification of sulfhydryl groups by Ellman's Reagent as it contains no sulfhydryl groups itself.

III. Reacting CCLP3 with SATA under Argon

Our third and final attempt at adding sulfhydryl groups used SATA rather than Traut's Reagent. This attempt was also undertaken in an anaerobic environment, even though SATA sulfur is protected in the form of a thioester, and therefore oxidation was not expected to occur until it was deprotected with hydroxylamine later in the experiment. Our results produced an approximately 5.1 sulfhydryl/protein molecule. Although this was lower than Traut's Reagent modification in an anaerobic environment, it was higher than Traut's Reagent modification in an

aerobic environment, making it our second-best option for sulfhydryl creation in terms of raw results.

Furthermore, the efficiency of SATA is based upon the molar ratio to the protein to be modified[30]. We used a 250:1 molar ratio of SATA: protein, however, a higher molar ratio would be more efficient. When reacted with bovine serum albumin (BSA), a 250:1 mole ratio of SATA: BSA had a 54.41% efficiency of converting primary amines to sulfhydryl groups[6]. Sulfhydryl incorporation increased with greater amounts of SATA, but with decreasing efficiency[6]. Compared to our 19.1% efficiency of turning CCLP3 primary amines into sulfhydryl groups, we may have also potentially created oxidized sulfhydryl groups during the Ellman's Reagent quantification portion of the experiment. Thus, by both increasing the molar ratio and adding a reducing agent after the deprotection of the sulfhydryl group with hydroxylamine, a future experiment could potentially see greater results in sulfhydryl conversion.

IV. Future Studies: Applying NSAIDs to the Hydrogel

In continuation of our work to apply to future experiments and to advance our knowledge on the usage of hydrogels as an *in-situ* source of drug delivery, our results can be applied to allow linkages of our sulfhydryl groups to NSAIDs. With the assistance of Dr. Watkins's lab and specifically Mr. Blaine Derbigny, experiments are advancing by applying a standard N-Hydroxysuccinimide (NHS) ester protocol. NHS is an organic compound, composed of ester terminal groups, and used for amine coupling reactions in bioconjugation, or in other words, to create covalent bonds, via amide linkages, between amine-containing molecules to exteriors of other molecules[34]. Finding a protocol that allows for a high percentage yield of linkages to further

explore its ability to function and maintain properties *in vivo* is a top priority for advancing this work.

Throughout these experiments, our team employed many strategies to target sulfhydryl groups specifically disulfide formation, which is reversible, but relevant to the oxidizing conditions in the extracellular space. Our polymers are engineered to have CCLP3. CCLP3 thiols are involved in the chemical bond to form the higher-order structure of polymers discussed and can be unavailable for modification with a drug. For this reason, we applied Traut's reagent in an aerobic environment and Traut's Reagent and SATA in an anaerobic atmosphere to initiate the presence of thiols for the development of biomaterials[35, 36], which essentially converts the polypeptide into a poly thiol template through reaction with amines. Under basic conditions, a nonreactive cyclic compound forms, [37, 38,] and thus, the timing and order of completion were an utmost priority in this lab.

In Figure 5.1, we outlined the continuing process that future labs will apply to their protocols. From the scheme below, they will prepare a stable and modified drug through a reaction *N*-(2-hydroxyethyl) maleimide, a linking group that creates an ester to the carboxylate of the drug, that is tethered to maleimide through a 2-carbon chain. This reaction will be carried out in an aprotic solvent to ensure that the maleimide is not deactivated by water. In addition, they can easily lengthen the carbon chain to reduce restrictive or steric effects of the protein backbone on drug release. Separately, using our findings, CCLP3 will be treated with Traut's reagent which will react with amines to create up to 25 -SH sites. The Naproxen-ester-S-Maleimide will then be added to create f-CCLP3. *Formation of the Hydrogel*: f-CCLP3 would then be added to PCLP3.

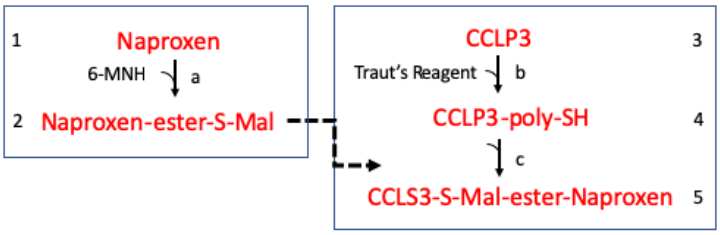

Figure 5.1: Strategy for the modification of thiols

Modification of Polymer Scheme: Right: React CCLP3 with Traut's reagent to create CCLP3-poly-SH. Left: Activate naproxen by forming an ester linkage to the carboxylate using 6-MNH (6-Maleimidohexanoic acid N-hydroxy succinimide ester), which links the naproxen to maleimide. Mixing Products 2 and 4 will yield functionalized CCLP3

V. Conclusion

After exploring a variety of ways to construct CCLP3 to add thiols, we found that Traut's Reagent in an anaerobic environment proved the most beneficial in our goal of creating a reactive CCLP3 that could be tested once Naproxen is modified. The importance of our work is clearly shown with the prevalence of overprescribing NSAIDs in the world. Through research, we have acknowledged the benefits that hydrogels can provide to a system that continuously approaches complications with the standard way of ingesting the medication. Further studies can be conducted to increase the overall modification capabilities of each polymer as well as hydrogels. Drug compounds using the maleimide linkage can be constructed, creating a drug-modified polymer. Our study has prepared the next step in our overall goal, which is to create hydrogel that can serve as a drug depot for specified regions in the body.

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