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Fluorescently-Labeled Glutathiones to Quantify Cysteine Reactivity

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Using Fluorescently-Labeled Glutathiones to Quantify Cysteine Reactivity

A thesis submitted in partial fulfillment of the requirement
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by

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I. Abstract

The purpose of this study was to quantify cysteine reactivity with fluorescently-labeled glutathiones. Previous work on fluorescein-labeled glutathiones had established that they undergo S-glutathionylation with GAPDH and tubulin cysteines. In the current study, the proteins GAPDH and LDH were reacted with Fluorescein-labeled and dansyl-labeled glutathiones under different reaction conditions identical to steric, salt, and pH environments in a cell. These reactions were monitored via UV/Vis spectroscopy to obtain quantitative data on reactivity of GAPDH and LDH cysteines. Reactions were determined qualitatively through nitrocellulose membrane dot blots and SDS-PAGE gels. Cysteine reactivity with fluorescently-labeled glutathiones was shown to be influenced by sterics, salt concentration, and pH. Reactivity was higher in conditions of low steric hindrance and in high salt concentration. Interestingly, reactivity was decreased at a pH higher than physiological pH. By establishing that the change in reactivity of protein cysteines in various cellular conditions can be investigated via reaction with fluorescent glutathiones and subsequent monitoring by UV/Vis spectroscopy, I have demonstrated that cysteine reactivity of the proteins GAPDH and LDH can be quantified by fluorescently-labeled glutathiones.

II. Introduction

A. Cysteine Structure and Function

Cysteine is one of the 20 common amino acids in proteins and is notable for its functional importance. It serves a role in protein structure regulation, as a catalyst in active sites, and as a cofactor binder (Marino & Gladyshev, 2012).[1] Cysteine is also unique because it can form disulfide bonds between intracellular thiol species including proteins and peptides to modulate protein conformation. One only has to look at mutation studies where a cysteine residue has been deleted from a protein to understand the importance of this amino acid to protein structure and function. For instance, a cysteine mutation in the *ret* proto-oncogene causes medullary thyroid cancer and Hirschsprung's Disease, a condition where the ganglion cells of the digestive tract are absent (Ito et al., 1997)[2]. The unique structure and functions of cysteine translates into its important role in various cellular processes. This makes cysteine a critical residue to study in relation to protein function

B. Cysteine as a Nucleophile

Cysteine's reactivity is largely dependent on the pKa of its side chain (Bulaj et. al, 2009).[3] Cysteine is distinctive in that it has a pKa of 8.3, close to that of physiological pH, making it easy for minor perturbations in the local microenvironment to change the protonation state of the thiol group. At a pH lower than the side chain pKa, the amino acid is overwhelmingly surrounded by protons, which cause the side chain to become protonated. At a pH above the thiol pKa, the side chain becomes deprotonated to $–S^-$. Thiolate anion is a stronger nucleophile, so the residue's reactivity is increased. Highly conserved cysteines are typically buried in the protein's hydrophobic core (Rose, Geselowitz, Lesser, Lee, & Zehfus, 1985)[4]

The pKa of the thiol side chain of cysteine can vary depending on the residue's location on a protein (Pace et al., 2009)[5] For example, if the cysteine residue is on the exterior of the protein it will have a lower pKa. The deprotonated thiol will thus favorably interact with the water surrounding the protein. Conversely, a buried cysteine will have a higher pKa and therefore be in a neutral state. As a neutral species, cysteine is better able to interact with the hydrophobic core of the protein.

The local electrostatic environment of the cysteine also determines its pKa. For example if the cysteine is near an arginine residue, its pKa decreases to favor the anionic form. The deprotonated thiol can then interact favorably with the positively charged side chain of the arginine. In one study it was found that tubulin cysteine reactivity increased around positively charged amino acids due to their stabilizing effect on the high electron density of the deprotonated thiol (Britto et al., 2002).[6] This suggested that the cysteine reactivity of tubulin was influenced by its local electrostatic environment.

Cysteine's reactivity makes it susceptible to oxidation by reactive oxygen species, which can lead to loss of enzymatic activity in many different proteins. Thus, it is important to study the reactivity of cysteines as it relates to various enzymatic processes within the cell, including glycolysis.

C. Glycolysis

Glycolysis is the metabolic process in which one molecule of glucose is converted to two molecules of pyruvate. The energy captured in glycolysis is temporarily stored in two molecules of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide (NADH). There are 10 reactions in the glycolytic pathway, occurring in two stages. During the first stage glucose is phosphorylated and cleaved to form two molecules of glyceraldehyde-3-phosphate. In the second

stage glyceraldehyde-3-phosphate is converted to pyruvate. In the anaerobic mechanism of glycolysis, pyruvate is converted to lactic acid. Lactate dehydrogenase and glyceraldehyde 3-phosphate dehydrogenase are two important glycolytic enzymes that will be discussed further below (McKee and McKee, 2012). [7]

D. LDH Structure and Function

Lactate dehydrogenase (LDH) catalyzes the conversion of pyruvate to lactic acid via the cofactor NADH (Figure 1). It is a tetramer comprised of 331 amino acids with two isozymes: heart type and muscle type. It is activated during glycolysis under conditions of low oxygen. Under aerobic conditions, the hydrogen of NADH is transferred to oxygen to create water. However, under anaerobic conditions, there is a buildup of NADH. LDH removes excess NADH by catalyzing the reduction of pyruvate to lactic acid, which oxidizes NADH to NAD+. NAD+ is thus regenerated so that glycolysis can continue to function (Mckee and Mckee, 2012).[8]

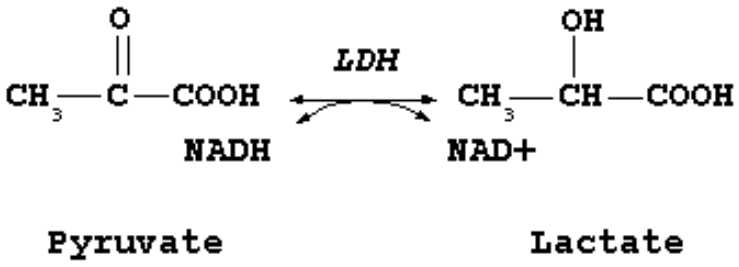


Figure 1 Conversion of pyruvate to lactate is catalyzed by lactate dehydrogenase

During the catalytic mechanism of LDH, NADH binds to Arg106 and Asn138 while pyruvate binds Arg106, Arg169, and Thr 248 (Holmes & Goldberg, 2009). [9] Upon binding of pyruvate in the active site of LDH, a hydride ion is transferred from the dihydronicotinamide ring of NADH to the carbonyl carbon of pyruvate. A proton is transferred from His193 to the carbonyl oxygen of pyruvate, creating lactate (Ferrer et al., 2008)[10] The negative carboxyl group of pyruvate participates in a stabilizing electrostatic interaction with the arginine residues of the

LDH active site (Figure 2) (Hou et. al, 2000).[11] Cys162 in LDH is known as the "essential" thiol because of its location in the active site. While it does not interact with the binding of NADH, its modification has been implicated in LDH inactivation (Gold & Segal, 1965).[12]

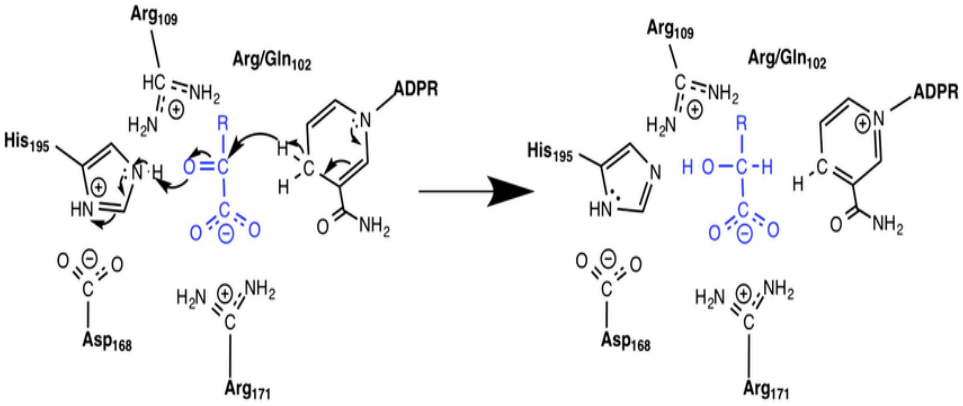


Figure 2 Active site mechanism of LDH. A hydride ion of NADH is transferred to the carbonyl carbon of pyruvate and a proton from His 195 is transferred to pyruvate to create lactate. Arg108=Arg 106, His195=His193, Arg171=Arg169 in rabbit muscle LDH.

E. LDH Cysteine Function

Rabbit muscle LDH contains five cysteine residues: Cys34, Cys130, Cys162, Cys184, and Cys292 (Figure 3). Cumming et al. identified LDH as a target for thiol oxidation (2004).[13] Cys 152 has been established as the "critical cysteine" of LDH. Cys152 (Cys162 in rabbit muscle LDH) is the primary target for oxidizing agents because of its close proximity to the active site of LDH. In 2005 Pamp et al. conducted a study to investigate Cu (II)-mediated inactivation of LDH. They found that NADH increased Cu (II) access to the critical cysteine of LDH, facilitating cysteine oxidation by Cu (II). (Pamp et al., 2005).[14] This study exhibits the importance of further investigating reactive cysteines in LDH that may be susceptible to oxidation.

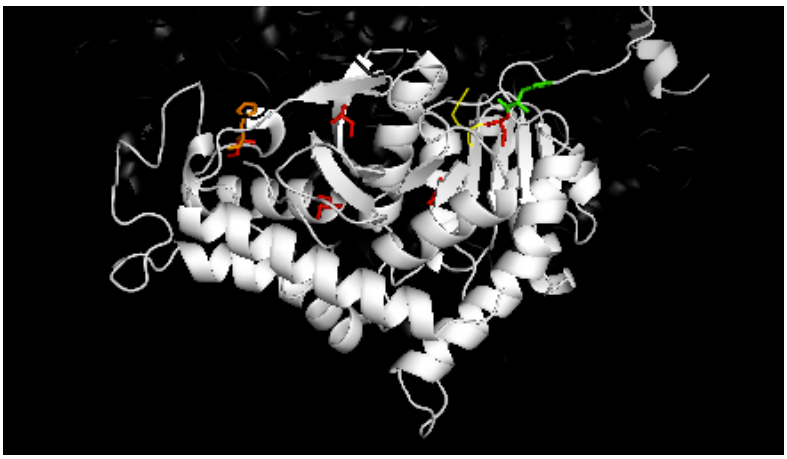


Figure 3 Subunit of LDH with important residues tagged. Cys34, Cys130, Cys162, Cys184, Cys292 (red), His129 (orange), His185 (green). Visual pulled from PyMol PDB 4I9U

F. GAPDH Structure and Function

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is a tetramer with each identical domain containing a total of 333 amino acids (Applequist et al., 1995).[15] The first domain, composed of the first 148 amino acids, is involved in the binding of NAD+ and is arranged in a βαβ-sheet pattern. Because it contains the active site of the enzyme, it is highly conserved and catalytic. The second domain is arranged in a β-sheet pattern. (Dugaiczyk et al., 1983).[16]

GAPDH participates in the sixth step of glycolysis (Mckee and Mckee, 2012).[17] It catalyzes the synthesis of 1,3-Bisphosphoglycerate from glyceraldehyde-3-phosphate via a reversible redox reaction (Figure 4).

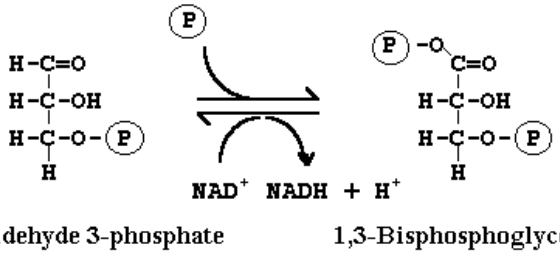


Figure 4 Conversion of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate is catalyzed by glyceraldehyde 3-phosphate dehydrogenase

An NAD+ is reduced to NADH. In the conversion of glyceraldehyde-3-phosphate (G3P) to 1,3-bisphosophateglycerate (BPG), Cys149 acts as a nucleophile to remove a hydride from G3P. G3P forms a tetrahedral hemithioacetal intermediate with the thiol group. The hydride ion is transferred to a NAD+ molecule bound to the active site. The newly formed BPG then dissociates from the active site (Figure 5) (Soukri et al., 1989).[18] This catalytic nature of Cys149 is just one of the many functions this reactive residue plays in the activity of GAPDH.

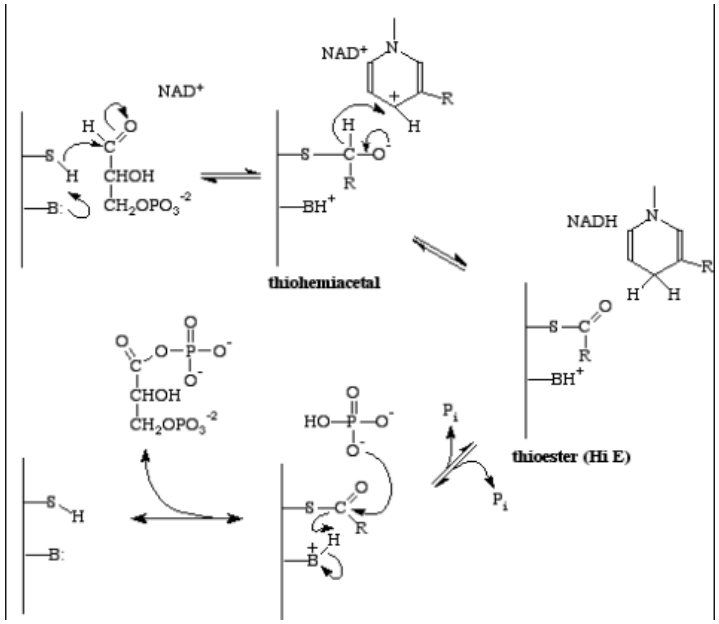


Figure 5 Active site mechanism of GAPDH. Cys149 attacks aldehyde carbon of glyceraldehyde-3-phosphate. NAD+ is reduced to NADH upon accepting proton from thiohemiacetal. Newly-formed thioester acts as acid to deprotonate nearby basic residue. Phosphate attacks thioester to create 1,3-bisphosphateglycerate.

G. GAPDH Cysteine Function

Rabbit muscle GAPDH has four cysteine residues: Cys149, Cys153, Cys244, and Cys281 (Figure 6). Though it is a glycolytic enzyme, GAPDH has a myriad of functions throughout the cell, and its cysteines are integral to its role.

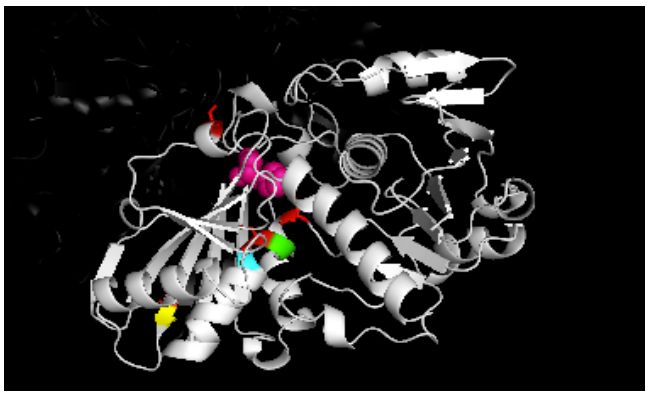


Figure 6 Subunit of GAPDH with important residues tagged. Cys 149, Cys153, Cys244, Cys281 (red), His176 (pink), Asn148 (green), Arg245 (yellow). Proximity of cysteine to basic residues increases reactivity. Visual pulled from PyMol, PDB 1JOX.

The active site of GAPDH contains a cysteine residue at position 149. Cys149 is flanked on either side by an arginine and lysine. Most importantly, it is in close proximity to His176. The distance between the sulfur atom of the thiol and the closest nitrogen atom of histidine in the active site of GAPDH is on average 4.03 angstroms. This close proximity favors deprotonation of the thiol group, making the cysteine more nucleophilic and prone to oxidation (Zaffagnini et al., 2013)[19] In addition to its catalytic role, Cys149 has been implicated in other cellular processes. There is evidence suggesting the role of Cys149 in protein aggregation as a result of oxidative stress (Nakajima et al., 2009).[20] This was discovered through a mutagenesis study that looked at the aggregation levels of GAPDH with C149S, C153S, C244A, and C281S mutants after addition of oxidant. Of these mutations, C149S mutants did not aggregate, suggesting that the active site cysteine of GAPDH plays an essential role in aggregation of GAPDH upon oxidation. GAPDH cysteines also play an important role in ROS signaling cascades. Cys152 of GAPDH in *Schizosaccharomyces pombe* (Cys153 in rabbit muscle GAPDH) is implicated in peroxide stress signaling of the sensor kinase Mak2/3 (Morigasaki et al., 2008).[21]

These studies demonstrate how integral cysteine reactivity is to the glycolytic and non-glycolytic functions of GAPDH.

H. Glutathione Background

Glutathiones (GSH) act as endogenous antioxidants that eliminate ROS, highly reactive molecules containing oxygen that can wreak havoc on a cell by oxidizing various proteins. Diseases caused by ROS include Alzheimer's and Parkinson's Disease (Berlett & Stadtman, 1997).[22] Oxidation of cysteine by ROS creates sulfenic acid (-SOH), which is an unstable and irreversible modification that can cause permanent damage to proteins. However, because of its high reactivity, sulfenic acid favorably reacts with glutathione to form disulfides (Forman et al., 2004).[23]

Glutathiones work in tandem with other enzymes to maintain redox homeostasis. Thioredoxins (Trxs) are redox proteins that catalyze the reversible reduction of protein disulfide bonds. Trxs collaborate with glutathioredoxins (Grx) to reduce protein mixed disulfides. Trx1 is normally found in the cytosol however, during oxidative stress, it localizes to the nucleus (Circu & Aw, 2010).[24]

I. Glutathione Structure

Glutathione is a peptide composed of cysteine, glycine, and glutamine. It is the most abundant intracellular low molecular weight thiol (López-Mirabal & Winther, 2008).[25] In the cytosol, reduced glutathione levels are maintained by glutathione reductase, *de novo* GSH synthesis, and exogenous GSH uptake (Meister & Tate, 1976).[26] It is present in mammalian cells in the range of 1-10 mM. In the cytosol, it interacts with protein cysteines via S-glutathionylation, where the reduced cysteine thiol is reversibly and covalently modified (Dalle-Donne et al., 2009).[27] During this covalent modification, the nucleophilic cysteine attacks GSH to create a disulfide bond. S-glutathionylation targets cysteines in close proximity to basic residues such as lysine, arginine, and histidine because these cysteines are more likely to be in

their anionic, nucleophilic state (Grek et al., 2013).[28] Because these cysteines are more nucleophilic, they are also more likely to be oxidized by reactive oxygen species (ROS). Typically, GSH exists in a ratio of 100:400 with its oxidized form, GSSG. This ratio determines the redox environment of the cell (Dalle-Donne et al., 2009).[29] Under conditions of oxidative stress, there is not enough GSH to protect critical cysteines via reversible S-glutathionylation because the GSSG form is prevalent (Gilbert, 1995).[30]

J. Fluorescein-Labeled Glutathiones To Study S-Glutathionylation

Landino et al. showed that fluorescein-labeled glutathiones could be used to study the S-glutathionylation reaction. In this study, the glutathiones were modified at the amino group by the fluorescent molecule fluorescein isothiocyanate (FITC) and then purified by chromatography, washed with methanol, and collected in a series of fractions. This created singly-bound fluorescent glutathione (FGSSG) and doubly-bound fluorescent glutathione (FGSSGF). The purified product was then analyzed for correct reaction between FITC and glutathione on a TLC plate. Concentration of FGSSG (F1) and FGSSGF (F2) was determined using absorbance measurements at 498 nm and comparison to a FITC standard. The purified fluorescein-labeled glutathione was reacted with GAPDH and tubulin to determine the extent of the reaction with protein cysteines. The extent of the reaction was detected by nitrocellulose dot blot and SDS-PAGE under non-reducing conditions. Addition of dithiothreitol (DTT), a reducing agent, removed fluorescent glutathione from protein cysteines, demonstrating that F1 and F2 bind reversibly at cysteine residues. On the other hand addition of H_2O_2 enhanced labeling of GAPDH and tubulin by fluorescent glutathione due to the reaction of the intermediate RSOH with the glutathione thiol (2010).[31]

This study exhibits the simplicity and elegance of fluorescent glutathione synthesis and their use in quickly and accurately studying S-glutathionylation reactions in proteins. This reaction can be monitored by both absorbance and gel electrophoresis assays. Absorbance measurements give quantitative data on protein cysteine reactivity, and gel electrophoresis gives qualitative data protein cysteine reactivity. This allows for the relative comparison between protein cysteine reactivities.

K. Fluorescently-Labeled Glutathiones to Quantify Cysteine Reactivity

Protein cysteines react with the oxidized form of GSH (GSSG) via thiol-disulfide exchange to produce oxidized protein and GSH (Figure 7). The ability to break this disulfide bond depends on the reactivity of the nucleophilic protein cysteine (Gilbert, 1995)[32]. This reactivity is therefore influenced by steric hindrance, the pH microenvironment of the nucleophile, and the local electrostatic environment surrounding the nucleophile.

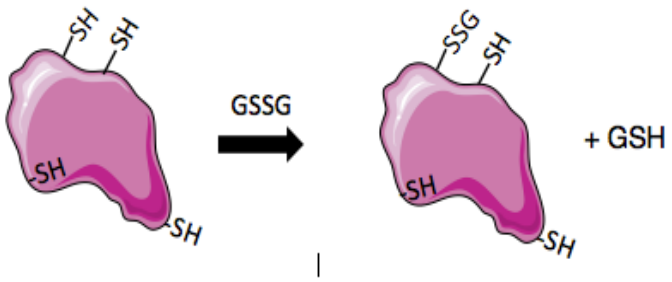


Figure 7 S-glutathionylation of protein cysteine via thiol/disulfide exchange

It is expected that F1 would react half as much with the protein cysteine compared to F2, however due to steric hindrance this is not the case (Figure 8). The extra bulky FITC molecule hinders the nucleophilic cysteine from attacking the disulfide bond of F2. Furthermore, Landino et al. found that F1/F2 were too hindered to accurately reflect GSSG reactivity with tubulin or GAPDH (Landino et al., 2010).[33] A promising alternative was dansyl, a fluorescent molecule that is attractive in its low molecular weight and high fluorescence.

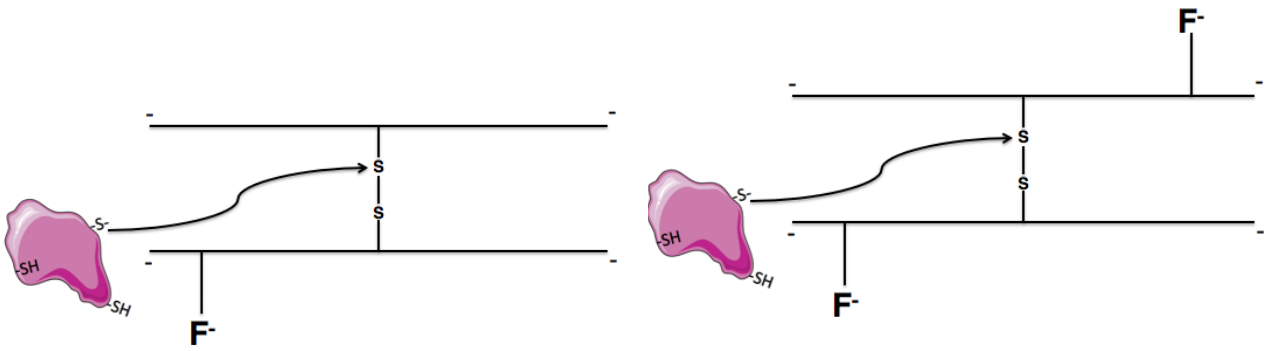


Figure 8 Visual created in Microsoft Word depicting attack of nucleophilic protein cysteine on disulfide linkage in FGSSG (F1, left) and FGSSGF (F2, right). Because of extra FITC group, reactivity of cysteine with F2 is decreased due to steric hindrance.

In 2012, undergraduates in the Landino group were able to synthesize and purify the dansyl-labeled glutathiones DGSSG (D1) and DGSSGD (D2). These were shown to be reduced, oxidized with H_2O_2, and reduced again, demonstrating the reversibility of the thiol-disulfide exchange between dansyl-labeled glutathione and protein cysteines.

In the current study, the fluorescently-labeled glutathiones F1, F2, D1, and D2 were reacted with GAPDH and LDH to quantify cysteine reactivity. Absorbances of reactions with F1/F2 were monitored via UV/Vis spectroscopy (Figure 9). This method was chosen over fluorescence spectroscopy because fluorescein-labeled glutathiones absorb at a wavelength (498

nm) that is in the visible range of the electromagnetic spectrum and because of the high molar absorptivity of FITC. Due to the low molar absorptivity of dansyl-labeled glutathione, reactions of D1 and D2 with GAPDH and LDH cysteines were monitored qualitatively via nitrocellulose dot blots. Nitrocellulose membranes were chosen because they preferentially bind molecules greater than 14kDa and can easily be visualized on a ChemiDoc imaging system. Various intracellular conditions were modeled using different pH and ionic conditions. To mock cellular conditions, 13.3 mM NaCl was used. Cellular concentrations of NaCl do not exceed 0.2 M (Lodish et al., 2000)[34]. The samples were monitored via UV/Vis spectroscopy to investigate reactivity of protein cysteines with F1 and F2 under various cellular conditions. As expected, it was found that protein cysteine reactivity with F1 was higher than that of F2, likely due to steric hindrance. Reactivity of GAPDH cysteines was increased with F1 compared to the F2. Interestingly, reactivity of GAPDH cysteines was decreased in phosphate buffer pH 8.0 for reactions with F1 and F2. Fluorescein-labeled glutathiones were also reacted with yeast whole extracts and visualized on nitrocellulose membrane dot blots. SDS-PAGE could not be used to qualitatively established reactivity of fluorescein-labeled glutathiones with yeast because of the similar molecular weight of GAPDH and LDH.

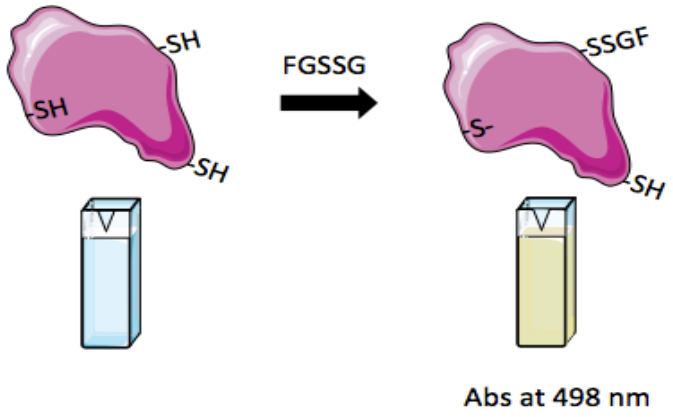


Figure 9 Visual depicting reaction of protein cysteine with FGSSG (F1) and subsequent absorbance measurement via UV/Vis spectroscopy. FGSSG absorbs at 498 nm.

L. Relevance of Current Study

Fluorescently-labeled glutathiones provide an elegant, simple method to quantify protein cysteine reactivity. Here, they were used to study reactivity of cysteines under various cellular conditions. The local steric, pH, and electrostatic environment influence cysteine's reactivity and ability to participate in thiol-disulfide exchange. Quantifying the reactivity of cysteines in GAPDH and LDH is important because of the implication of reactive cysteines in various cellular processes.

III. Methods and Materials

A. Purification of GAPDH and LDH

Commercially available rabbit muscle GAPDH and LDH were treated with the reducing agent dithiothreitol (DTT) to reduce any oxidized cysteines and then desalted on a gel filtration column to remove DTT. Their concentrations were calculated by absorbance measurements to be 1.16 mg/ml for GAPDH and 2.13 mg/ml for LDH.

B. Preparation of Fluorescently-Labeled Glutathiones

FGSSG (F1) and FGSSGF (F2) were previously prepared by J. Zuercher according to the method created by Landino et al. (2010). Fluorescently-labeled glutathiones were re-suspended in 100 ul 0.1 M phosphate buffer pH 7.4. 2 ul of F1 and F2 was diluted in 198 ul 6 M Guanadine HCl pH 6.8 and concentration of diluted sample was determined via UV/Vis spectroscopy. DGSSG (D1) and DGSSGD (D2) were synthesized by a undergraduate student in 2014 and concentration was determined in the same way as above.

C. Visualization of Fluorescein-Reacted Proteins on Nitrocellulose Membranes

Samples of 15 uM GAPDH were prepared under similar conditions to Table 1, but at half the total volume (7.5 ul instead of 15 ul). To ensure F1 or F2 had not bound to the nitrocellulose membrane instead of reacting with the protein, 6.6 mM DTT was added to samples and allowed to react for 3 minutes. The samples were incubated for 30 minutes at 37 C. Samples were blotted on a nitrocellulose membrane and washed for 8 minutes in phosphate buffered saline (PBS). Gels were visualized with the BIO-RAD ChemiDoc XRS+ imaging system.

D. Reaction of GAPDH and LDH with Fluorescein-Labeled Glutathiones

Samples of 15 uM GAPDH were prepared under similar conditions to Table 1. Concentrations of F1 and F2 were five times and ten times the concentration of cysteines for

GAPDH and LDH. The samples were vortexed immediately and allowed to incubate at 37C for 30 minutes. Next, 60 ul of 100% ethanol was added to the samples and they were frozen at 4C for 2 hours. The samples were centrifuged for 15 minutes after which the supernatant was removed. 75 ul of 80% ethanol was added to each sample and the samples were put on ice for 10 minutes. The samples were again centrifuged for 5 minutes and the supernatant was again removed. 75 ul of 80% ethanol was added to each sample and the samples were put on ice for 5 minutes. This was repeated twice, after which the supernatant was removed and the samples were allowed to dry for 10 minutes. 100 ul of 6M Guanadine-HCl pH 6.8 was added to each sample to re-suspend the fluorescently-reacted protein (Figure 1).

Table 1: Typical Reaction Conditions for GAPDH

Sample	GAPDH (ul)	F1 or F2 (ul)	0.1 M pH 7.4 Phosphate Buffer (ul)
1	6.9	2.0	6.1
2	6.9	2.0	6.1
3	6.9	4.0	4.1
4	6.9	4.0	4.1
5	6.9	3.3	4.8
6	6.9	3.3	4.8
7	6.9	6.6	1.5
8	6.9	6.6	1.5

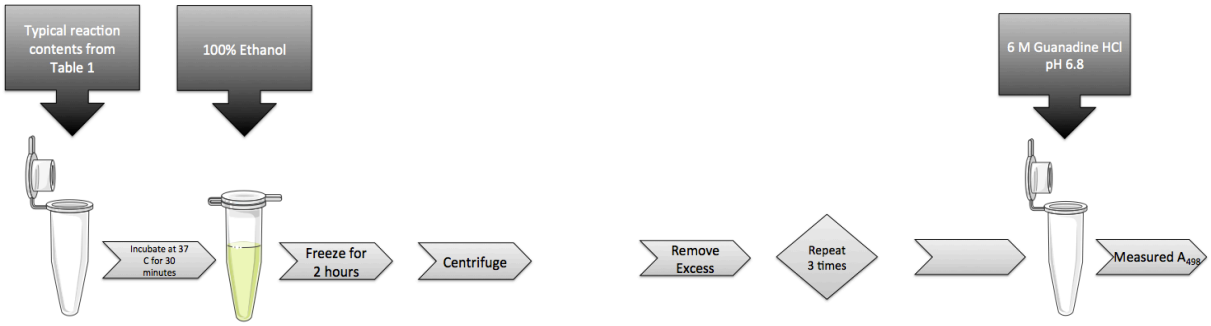


Figure 7 Visual representing preparation of GAPDH (or LDH) reacted with fluorescently-labeled glutathiones F1 and F2 and subsequent absorbance measurement

To examine effects of pH on cysteine reactivity, F1 and F2 were either prepared in 0.1 M phosphate buffer pH 7.4 or pH 8.0 and reacted with 15 uM GAPDH in the same method above. GAPDH was reacted with concentrations of F1 and F2 at ten times the amount of cysteines. The samples were brought to 15 ul with 0.1 M phosphate buffer pH 7.4.

To examine electrostatic effects on cysteine reactivity, 15 uM GAPDH was reacted with F1. 1 ul of 13.3 mM NaCl was added to this solution and 0.1 M phosphate buffer pH 7.4 was added to give a total volume of 15 ul. This sample was repeated but with iodoacetamide added 5 minutes after addition of 1 ul 13.3 mM NaCl to ensure the iodoacetamide was not added so early that it prevented the F1 from tagging the protein cysteines (Figure 2). Additional samples of GAPDH with 1 ul of 0.5 mM iodoacetamide added after addition of 100% ethanol were prepared to rule out denaturation of GAPDH after addition of ethanol.

Figure 8 Visual depicting reaction conditions for reaction of GAPDH cysteines with F1 under high salt concentrations.

An Oceans Optics UV Spectrometer was calibrated with 100 ul of 6M Guanadine HCl pH 6.8 and used to measure absorbance of the samples at 498 nm.

E. Visualization of Fluorescein-Labeled Glutathione Reaction by SDS-PAGE

Samples of 15 uM GAPDH were prepared under similar conditions to Table 1. Samples were incubated for 30 minutes at 37C. After incubation, 15 µL of SB (-) was added to each sample, and the total volume was loaded onto a 10% 1.5 mm SDS-polyacrylamide gel. The gel ran for 90 minutes at 90 V before visualization with the BIO-RAD ChemiDoc XRS+ imaging system.

F. Yeast Preparation and Purification

Yeast culture was grown in autoclaved YPD media (5g yeast extract, 10g peptone, 450 ml H2O, then 50 ml glucose after autoclaving) and incubated overnight at 30 C in a ThermoScientific MaxQ shaker. The overnight culture was centrifuged at 3000 rpm for 10 minutes at 4 C using a ThermoScientific ST 16R Centrifuge. The supernatant was decanted and 1 ml of deionized water was added to re-suspend yeast. The solution was centrifuged for 10 seconds at 13000 rpm in an Eppendorf 5415C microcentrifuge. The supernatant was removed

and 1 ml of extraction buffer (10 mM pH 7.5, 0.5 mM ATP, 0.2 mM CaCl2, 0.05% Triton X-100) was used to re-suspend the yeast samples. The solution was centrifuged for an addition 10 seconds at 13000 rpm and supernatant was removed. 850 ul of lysis buffer (1 ml of extraction buffer with 10 ul of Halt protease inhibitor cocktail) was added to yeast to re-suspend them. This solution was transferred to a bead ruptor tube with standard bead ruptor beads filled to the 0.25 ml mark. Yeast extractions were bead-rupted in a Bead Ruptor 24 Homogenizer for 15 seconds on medium power with 1 minute ice baths in between ruptions. This was repeated four times. Yeast extract was transferred to separate tube and centrifuged for 5 minutes at 4C at 13000 rpm in an Eppendorf Minispin Microcentrifuge. Extract was transferred to separate tube and centrifuged again for 10 minutes at 4C at 13000 rpm.

IV. Results

A. Reaction of Cysteine with Fluorescein-Labeled Glutathione

Previous studies have already established reaction of fluorescein-labeled glutathiones with GAPDH and tubulin cysteines (Landino et al, 2010). To observe reaction of fluorescent glutathione with protein, 2 ul solutions of GAPDH with fluorescein-labeled glutathione and 0.1 M phosphate buffer pH 7.4 were spotted on a nitrocellulose membrane, washed with PBS for 6 minutes, and visualized with the BIO-RAD ChemiDoc XRS+ imaging system. Samples of GAPDH with F2 exhibited darker spots than those with F1 (Figure 1, Table 10). 2 ul samples of GAPDH with fluorescein-labeled glutathione and 6.6 mM dithiothreitol (DTT) were spotted on nitrocellulose membrane to establish binding of the fluorescein-labeled glutathione to the protein and not the gel (Figure 2, Table 1). These exhibited diminished or no spots under UV light for FGSSG (F1), but not for FGSSGF (F2). These results indicated some of the F2 was binding to the nitrocellulose membrane. Similar results were obtained for LDH with F1 and F2 (Figures 3 & 4, Table 10). For both proteins, binding of fluorescent glutathione was concentration-dependent.

Because binding of F2 to the nitrocellulose membrane could not be ruled out, an SDS-PAGE was run to qualitatively establish binding of the fluorescent glutathiones with GAPDH and LDH. 15 ul solutions of GAPDH with fluorescein-labeled glutathione in phosphate buffer pH 7.4 were run on a 10% gel (Figure 1, Table 11). BioRad imaging showed higher intensity bands for GAPDH reacted with F1 compared to F2 (Figure 1). Similar results were observed for LDH **(**Figure 2, Table 11 & Table 1**).** This suggested increased reaction of GAPDH and LDH cysteines with F1 compared to F2.

Sample	[F1] or [F2] (uM)	F1 or F2
1	290	F1
2	290	F1
3	580	F1
4	580	F1
5	290	F2
6	290	F2
7	580	F2
8	580	F2

Table 9 Reaction conditions for SDS-PAGE of GAPDH reacted with F1 and F2 in phosphate buffer pH 7.4

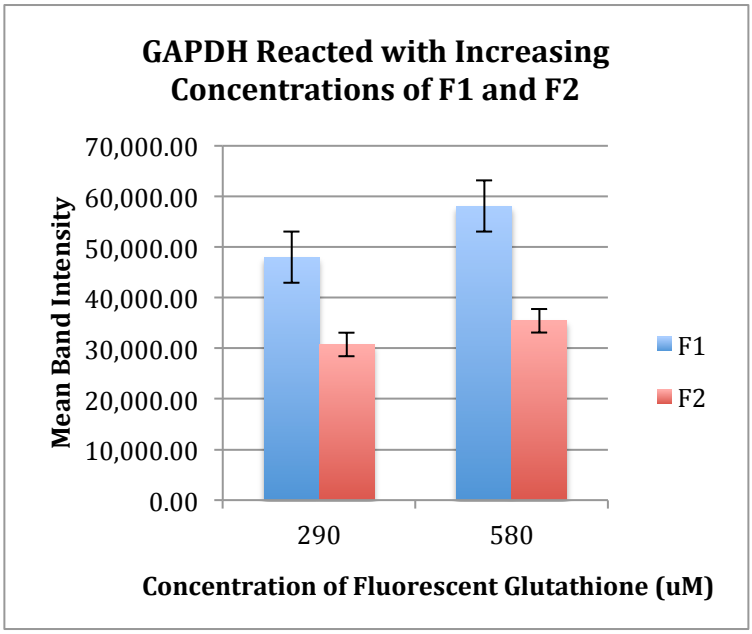


Figure 1 Mean band intensity of GAPDH reacted with F1 and F2 in phosphate buffer pH 7.4. GAPDH reacted with F1 produced higher intensity bands compared to GAPDH reacted with F2. Data represents average of four trials

Reaction of fluorescein-labeled glutathione with GAPDH was quantitatively determined using UV/Vis spectroscopy (Figure 2). Absorbance measurements of 100 ul solutions of Guanadine HCl, 15 uM GAPDH and fluorescein-labeled glutathione in phosphate buffer pH 7.4 were determined. Absorbance for GAPDH reacted with F1 was higher than those for GAPDH in F2, suggesting increased reactivity of GAPDH cysteines with F1 (Table 2).

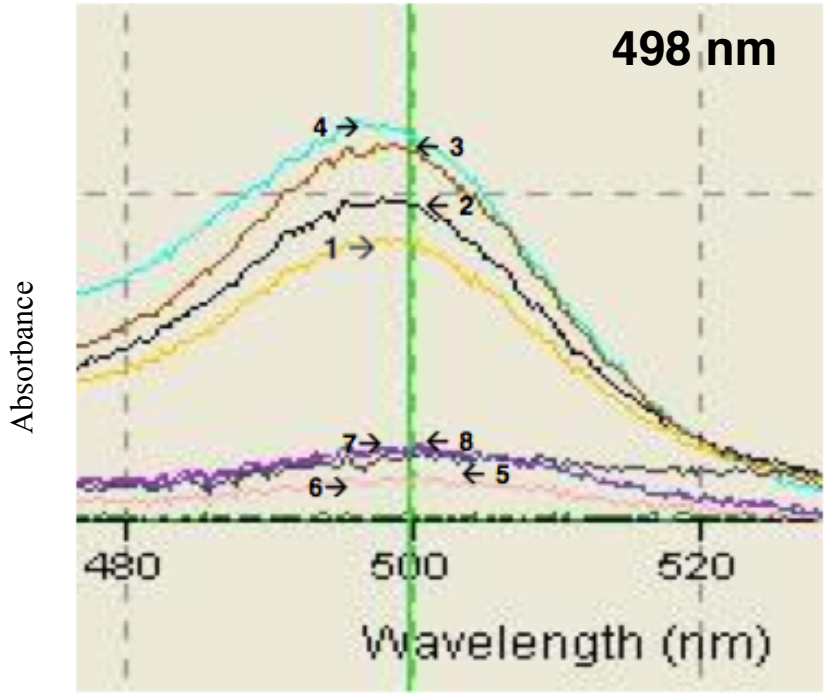


Figure 2 Absorption spectrum of GAPDH reacted under conditions identical to Table 2.

Sample	[F1] or [F2] (uM)	F1 or F2	Abs at 498 nm
1	290	F1	0.176
2	290	F1	0.197
3	580	F1	0.232
4	580	F1	0.240
5	290	F2	0.053
6	290	F2	0.038
7	580	F2	0.069
8	580	F2	0.059

Table 2 Absorbance values for trial of GAPDH reacted with F1 and F2 in phosphate buffer pH 7.4. Absorbance increased for GAPDH reacted in F1 relative to F2.

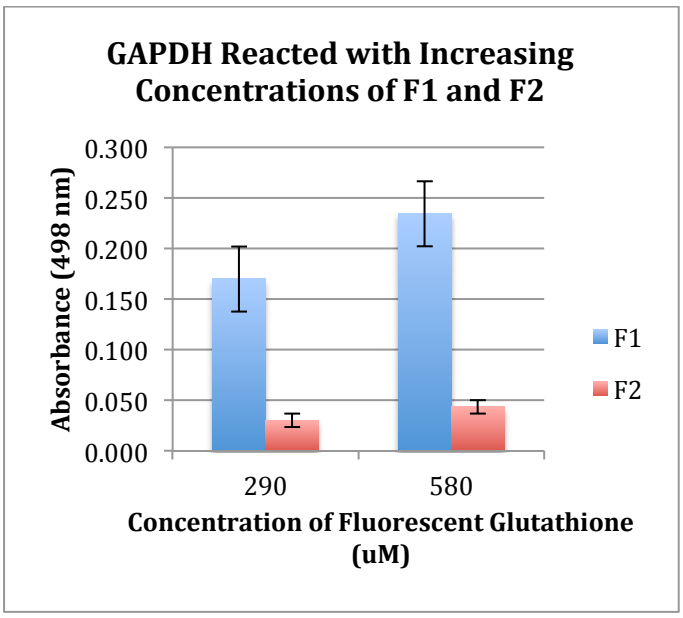


Figure 3 Graph depicting absorbance values of GAPDH reacted with F1 and F2 in phosphate buffer pH 7.4. Absorbance increased for GAPDH reacted with F1 relative to F2. Data represents average of four trials

LDH was reacted with fluorescein-labeled glutathiones under conditions identical to Table 3, spotted on a nitrocellulose membrane (Figures 3 & 4, Table 10), and run on an SDS-PAGE (Figure 2, Table 11). Differential absorbance values were also observed for LDH (Figure 4, 5, 6, and Table 4). For both proteins, fluorescent glutathione binding was concentration-dependent. Taken together, these results suggest that the protein cysteines of both GAPDH and LDH reacted preferentially with F1 compared to F2.

Sample	[F1] or [F2] (uM)	F1 or F2
1	375	F1
2	375	F1
3	750	F1
4	750	F1
5	375	F2
6	375	F2
7	750	F2
8	750	F2

Table 3 Reaction conditions for SDS-PAGE of LDH reacted with F1 and F2 in phosphate buffer pH 7.4

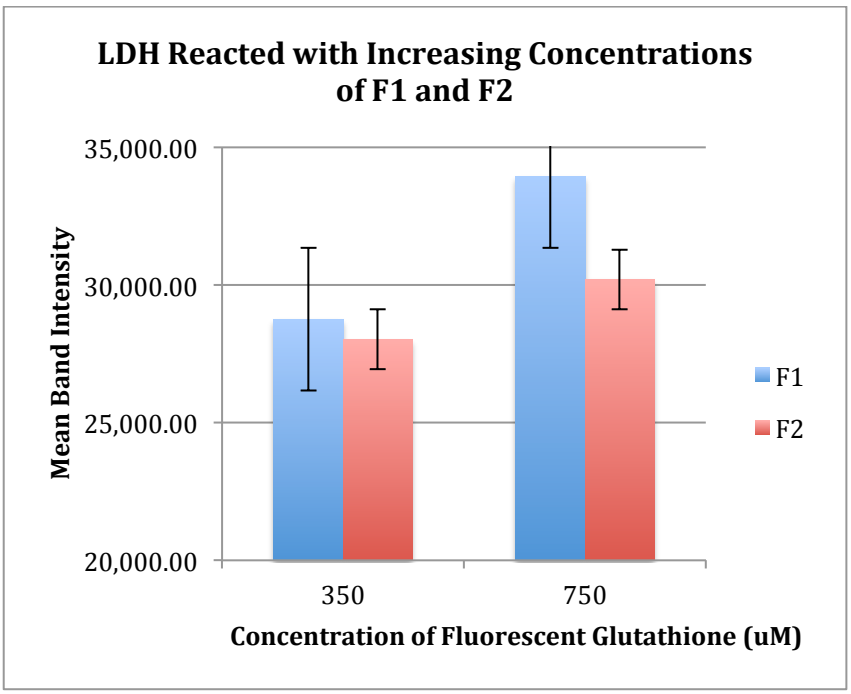


Figure 4 Graph depicting mean band intensity of LDH reacted with F1 and F2. LDH reacted with F1 produced higher intensity bands compared to LDH reacted with F2. Data represents average of four trials

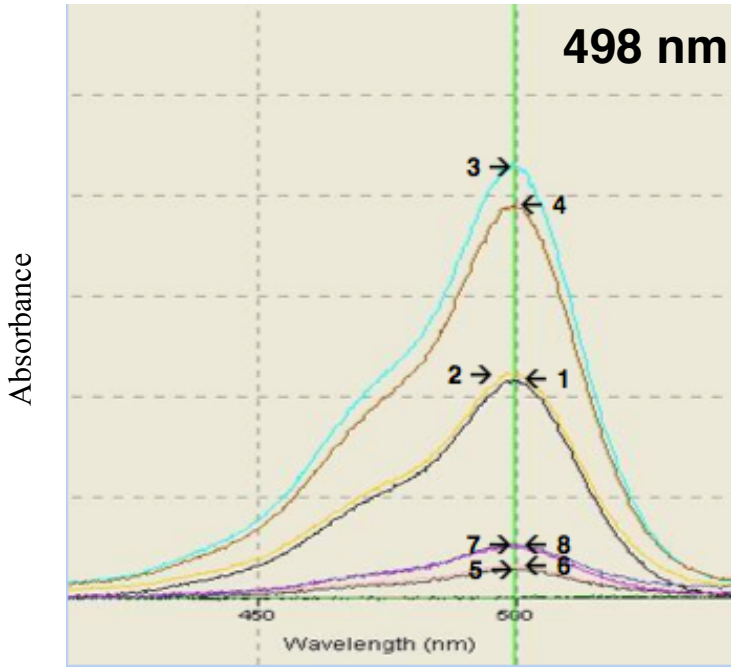


Figure 5 Absorption spectrum for LDH reacted under conditions identical to Table 4.

Sample	[F1] or [F2] (uM)	F1 or F2	Abs at 498 nm
1	375	F1	0.168
2	375	F1	0.178
3	750	F1	0.273
4	750	F1	0.263
5	375	F2	0.116
6	375	F2	0.111
7	750	F2	0.186
8	750	F2	0.199

Table 4 Absorbance values for LDH reacted with F1 and F2 in phosphate buffer pH 7.4. Absorbance increased for LDH reacted in F1 relative to F2.

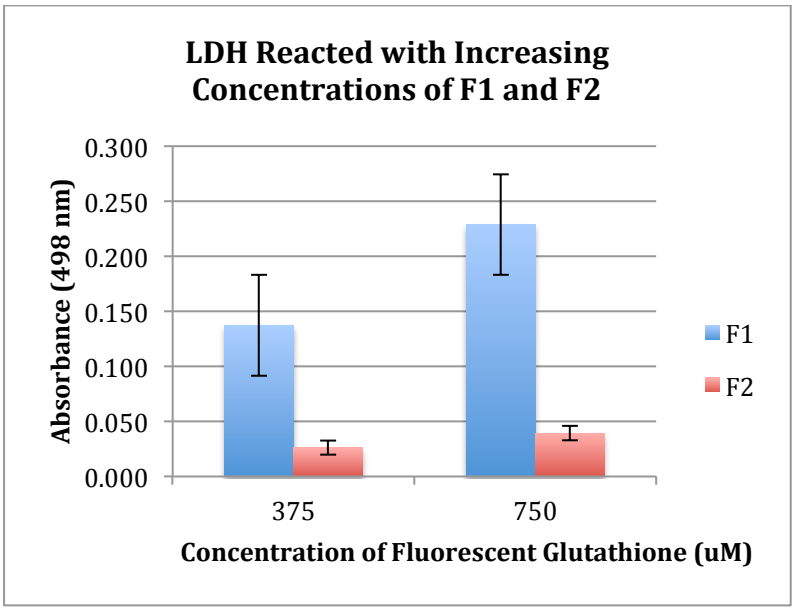


Figure 6 Graph depicting absorbance values of LDH reacted with F1 and F2 in phosphate buffer pH 7.4. Absorbance increased for LDH reacted with F1 relative to F2. Data represents average of four trials

These results suggest increased reactivity of GAPDH and LDH cysteines with F1 rather than F2. This is likely to due to steric effects, as F2 is a far bulkier molecule with two FITC moieties whereas F1 is less bulkier with only one FITC moiety.

B. Reaction of Cysteine with Fluorescein-Labeled Glutathione under High Salt Concentration

Absorbance measurements of GAPDH reacted with F1 and increasing concentration of NaCl showed increased absorbance when compared to solutions without NaCl (Figure 7 and Table 5)

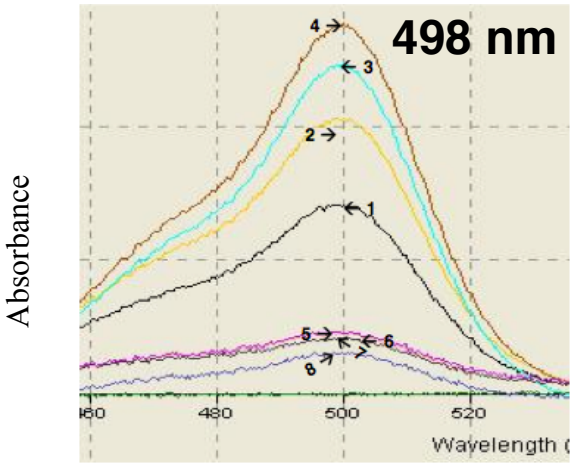


Figure 7 Absorption spectrum for GAPDH reacted under conditions identical to Table 5.

Sample	[F1] or [F2] (uM)	F1 or F2	Nacl (M)	Abs at 498 nm
1	290	F1	0	.279*
2	290	F1	0	.410
3	290	F1	.2	.492
4	290	F1	.2	.552
5	290	F2	0	.072
6	290	F2	0	.064
7	290	F2	.2	.067
8	290	F2	.2	.058

Table 5 Absorbance values for GAPDH reacted with F1 and F2 in increasing concentrations of NaCl. Absorbance increased for GAPDH reacted with F1 in 13.3 mM NaCl. *pipetting error during preparation of sample 1.

To further investigate if the addition of NaCl was denaturing the GAPDH, 1 ul of the cysteine-chelating agent iodoacetamide (33.3 mM IAM) was added to a separate 15 uM GAPDH sample after addition of F1 and 13.3 mM NaCl. In one sample set IAM was added 0 minutes after

addition of NaCl and in another it was added 5 minute after to allow NaCl to react with the protein. To rule out denaturation of GAPDH upon addition of 100 % ethanol during the precipitation process 33.3 mM IAM was added after addition of ethanol were carried out (Table 6). Samples showed a decrease absorbance with immediate addition of IAM after 13.3 mM NaCl whereas samples where NaCl was allowed to react with protein for five minutes produced a higher absorbance value. This suggests addition of NaCl exposes hidden GAPDH cysteines for reaction with F1. Absorbance measurements of samples in which IAM was added immediately after addition of ethanol were close to those of only GAPDH with F1 in 0.1 M phosphate buffer pH 7.4, suggesting the addition of ethanol does not denature the protein (Figure 8, Figure 9, and Table 6). Taken together, these results suggest addition of 13.3 mM NaCl to GAPDH changes tertiary interactions in such a way that F1 is able to react with an otherwise buried cysteine.

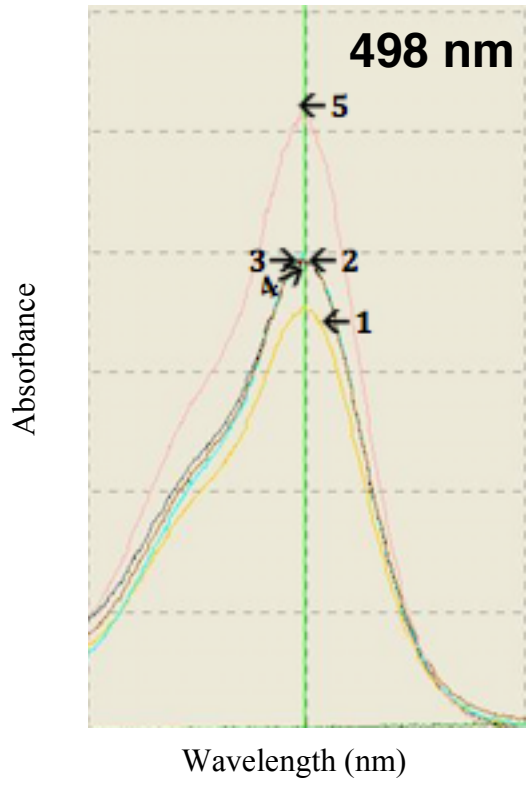


Figure 8 Absorption spectrum of GAPDH reacted with F1 and 13.3 mM NaCl identical to conditions in Table 6.

Sample	F1 (uM)	0.2 M NaCl (ul)	0.5 mM IAM (ul)	Time of Reaction (min)	Abs at 498 nm
1	580	1	1	0	0.705
2	580	1	1	5	0.777
3	580	1	1	10	0.789
4	580	1	1	15	0.788
5	580	1	-	5	1.016

Table 6 Absorbance values of GAPDH reacted with F1 under various conditions of 13.3 mM NaCl and 33.3 mM IAM. Incremental increase in duration of reaction between 13.3 mM NaCl and GAPDH reacted with F1 produced fluctuating absorbance values.

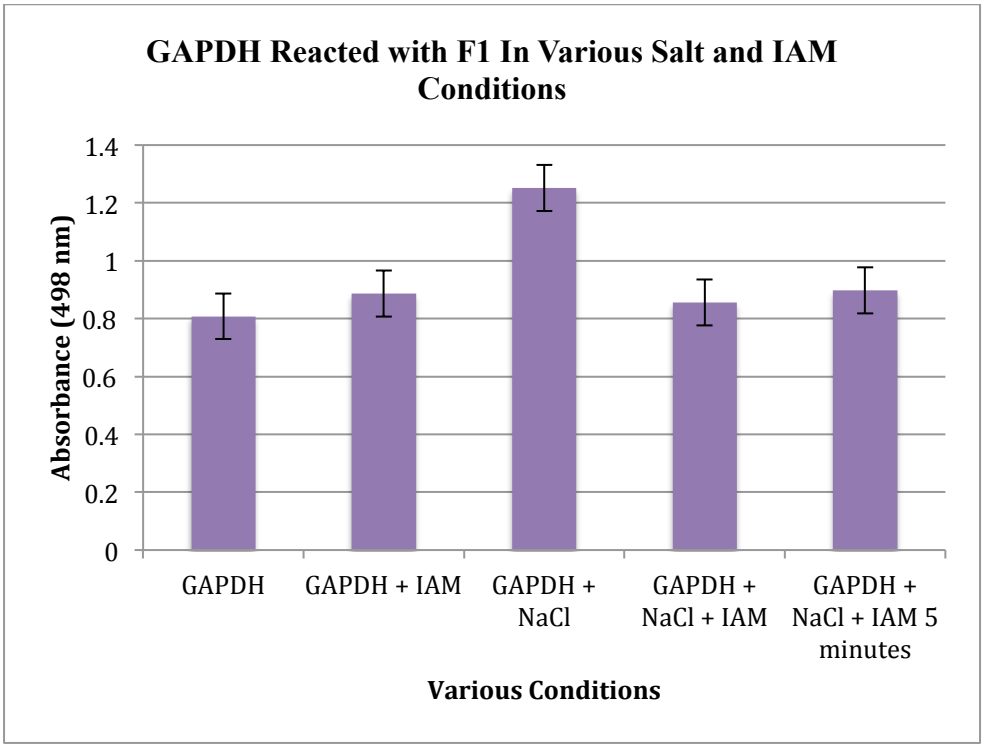


Figure 9 Graph depicting absorbance values of GAPDH reacted with F1 under various conditions of 13.3 mM NaCl and 33.3 mM IAM. Incremental increase in duration of reaction between 13.3 mM NaCl and GAPDH reacted with F1 produced fluctuating absorbance values. Data represents average of four trials.

These results were qualitatively backed by SDS-PAGE (Figure 3, Table 11). Solutions prepared according to Table 7 were run on a 10% gel. Mean band intensity was increased for GAPDH reacted with F1 and 13.3 mM NaCl (Figure 10).

Sample	F1 (uM)	0.2 M NaCl	0.5 mM IAM	Reaction Time Before Addition of IAM (min)
1	580	-	-	-
2	580	-	-	-
3	580	-	1 ul	-
4	580	-	1 ul	-
5	580	1 ul	1 ul	0
6	580	1 ul	1 ul	5
7	580	1 ul	-	-

Table 7 Reaction conditions for SDS-PAGE of GAPDH reacted with F1 under various conditions of 13.3 mM NaCl and 0.5 mM IAM.

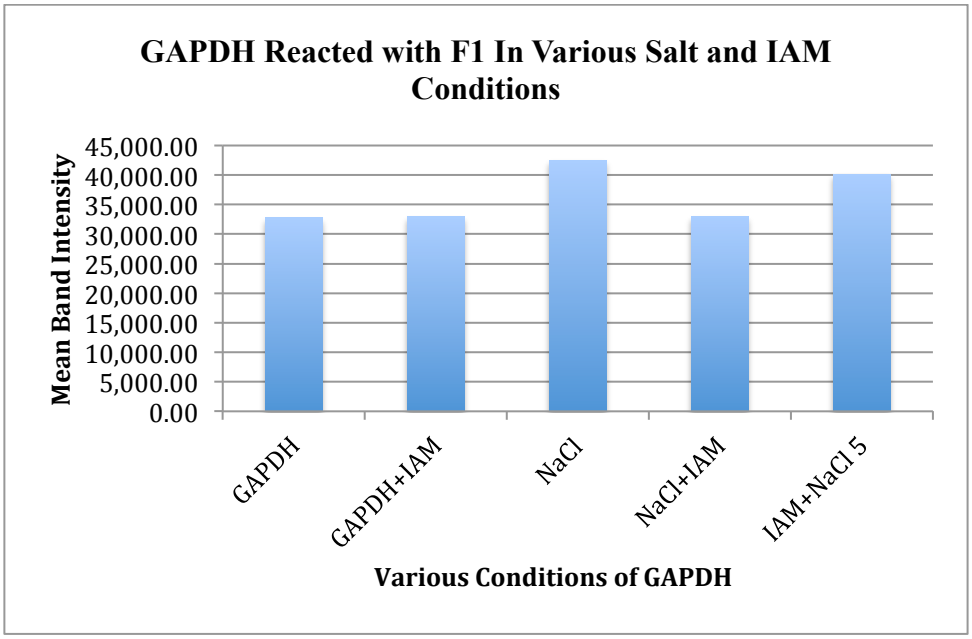


Figure 10 Graph depicting mean band intensity of GAPDH reacted with F1 under various conditions of 13.3 mM NaCl and 33.3 mM IAM. Incremental increase in duration of reaction between 13.3 mM NaCl and GAPDH reacted with F1 produced fluctuating intensity values.

Taken together, the results suggest addition of 13.3 mM NaCl likely uncovered a previously buried cysteine. Upon reaction with F1, GAPDH reacted with 13.3 mM NaCl produced higher absorbance because the now uncovered cysteine was able to react with F1.

C. Reaction Between Fluorescein-Labeled Glutathione and GAPDH Cysteines in Different pH

Dot blots of GAPDH reacted with F1 and F2 in 0.1 M phosphate buffer pH 7.4 and 8.0 were carried out with 2 ul samples spotted on nitrocellulose membrane. These were washed in

PBS for 6 minutes and visualized with the BIO-RAD ChemiDoc XRS+ imaging system. Samples in pH 8.0 for GAPDH in F1 and F2 yielded lighter spots than samples in pH 7.4 (Figure 5, Table 10).

Absorbance of GAPDH reacted with F1 and F2 in phosphate buffer pH 7.4 was increased relative to samples prepared in phosphate buffer pH 8.0 (Figure 11, Figure 12, and Table 9).

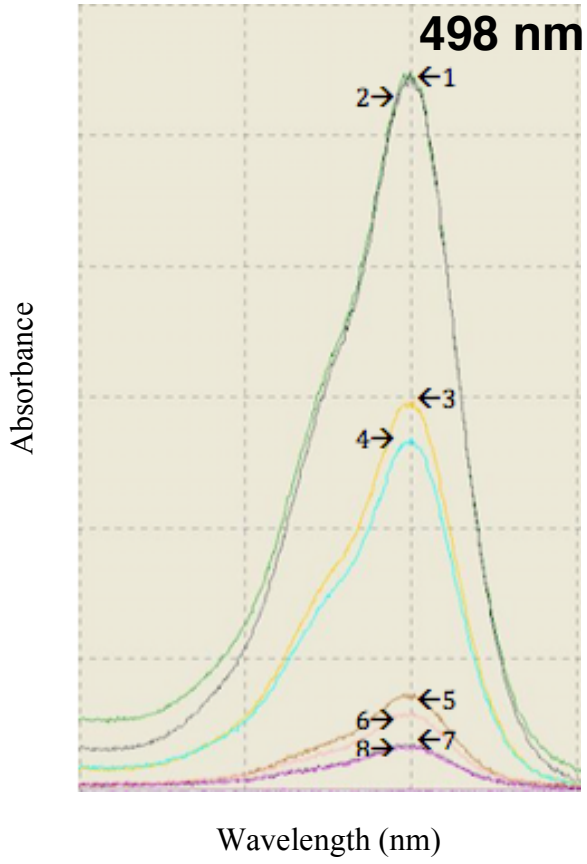


Figure 11 Absorption spectrum of GAPDH reacted under conditions identical to Table 9

Sample	F1 or F2	pH	Abs at 498 nm
1	F1	7.4	1.086
2	F1	7.4	1.082
3	F1	8.0	0.587
4	F1	8.0	0.536
5	F2	7.4	0.143
6	F2	7.4	0.113
7	F2	8.0	0.068
8	F2	8.0	0.065

Table 9 Absorbance values for trial of GAPDH reacted with F1 and F2 at phosphate buffer pH 7.4 and pH 8.0. Data suggest absorbance decreased for GAPDH reacted in pH 8.0 compared to pH 7.4 for both F1 and F2.

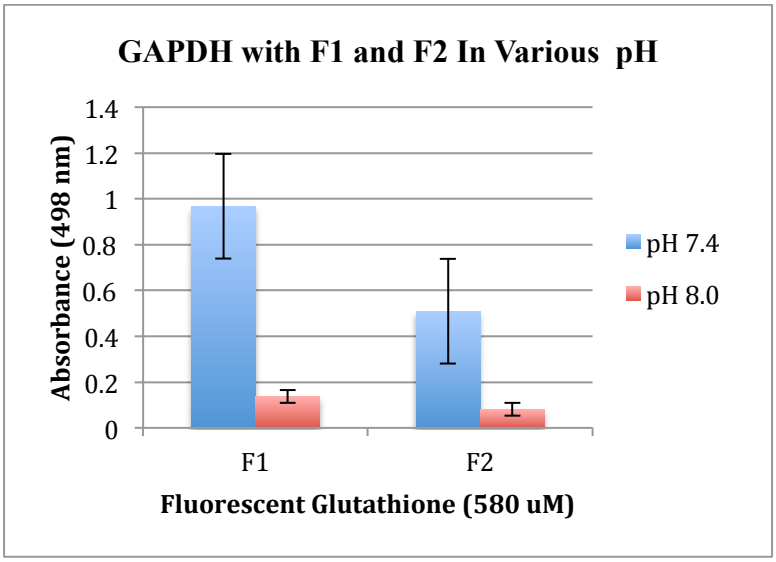


Figure 12 Graph depicting absorbance values of GAPDH reacted with F1 and F2 in phosphate buffer pH 7.4 and pH 8.0. Absorbance increased for GAPDH with F1 and F2 in phosphate buffer pH 8.0. Data represents average of four trials

To qualitatively determine pH effects on protein cysteine reactivity with fluorescein-labeled glutathiones, GAPDH was reacted under conditions identical to Table 9. F1 and F2 were prepared in phosphate buffer pH 7.4 and pH 8.0. These samples were run on a 10% gel (Figure 4,

Table 11). Imaging showed higher mean band intensities for F1 and F2 in pH 7.4 compared to F1 and F2 in phosphate buffer pH 8.0 (Figure 13).

Sample	pH	F1 or F2
1	7.4	F1
2	7.4	F1
3	8.0	F1
4	8.0	F1
5	7.4	F2
6	7.4	F2
7	8.0	F2
8	8.0	F2

Table 9 Reaction conditions for SDS-PAGE of GAPDH reacted with F1 and F2 in phosphate buffer pH 7.4 and pH 8.0.

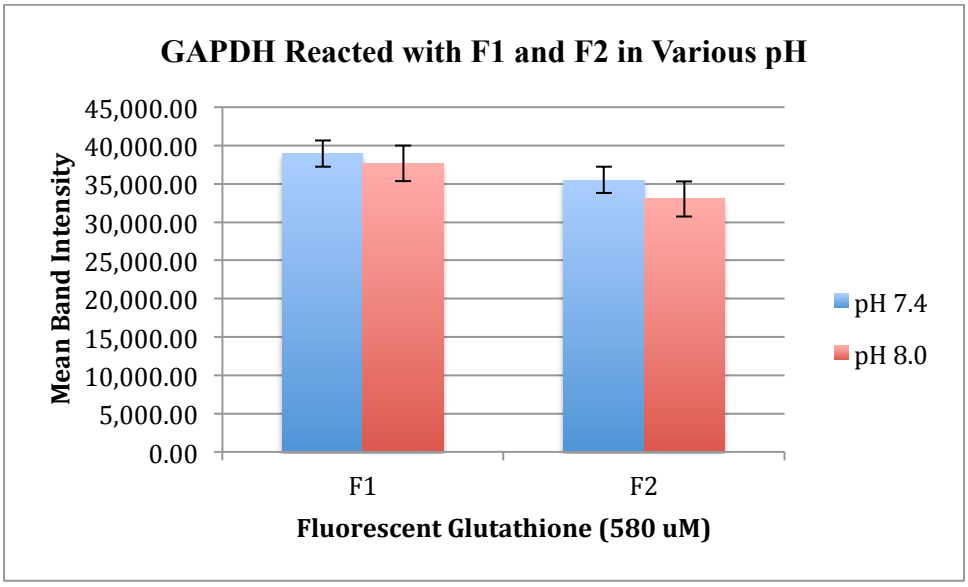


Figure 13 Graph depicting mean band intensity of GAPDH reacted with F1 and F2 in phosphate buffer pH 7.4 and 8.0. GAPDH reacted with F1 and F2 in phosphate buffer pH 8.0 produced lower intensity bands compared to phosphate buffer pH 7.4. Data represents average of four trials

The pH experiments suggest reactivity of GAPDH cysteine decreased in pH 8.0 relative to pH 7.4.

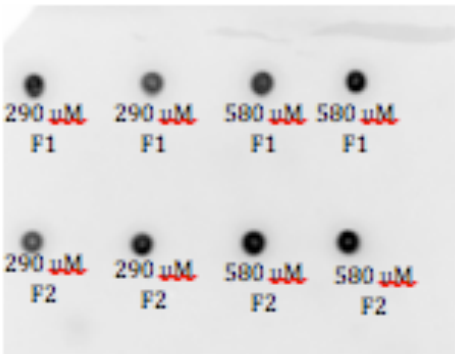
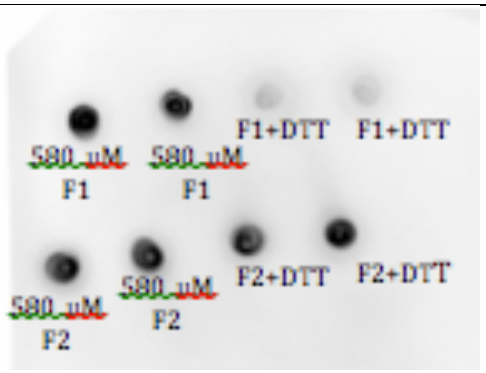
D. Reaction of GAPDH and LDH with Dansyl-labeled Fluorescent Glutathione

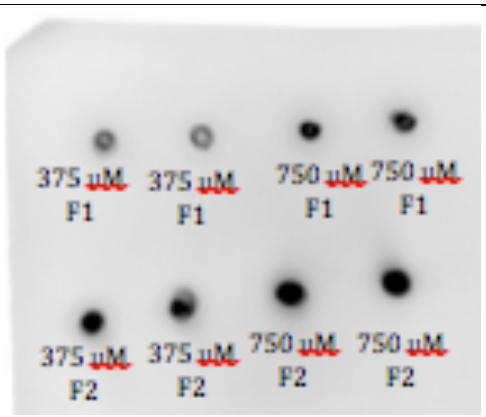
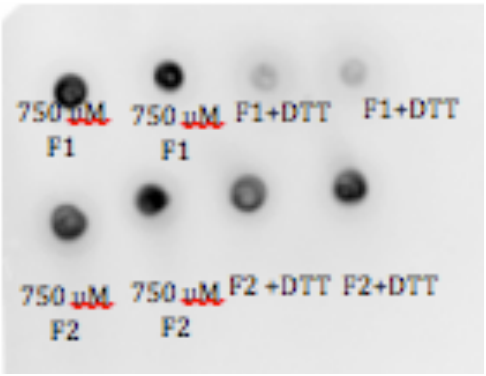
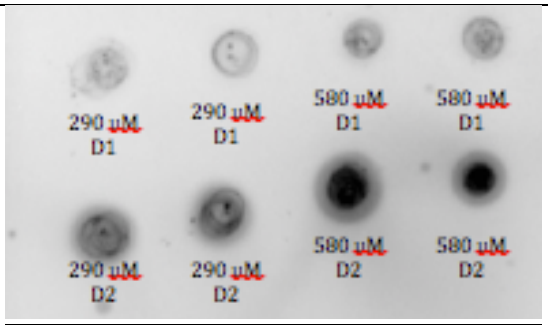
Due to the low molar absorptivity of dansyl ($\varepsilon = 4300\ M^{-1}cm^{-1}$), absorbance values could not be used to establish reactivity trends for protein cysteines. Therefore, nitrocellulose dot blots were performed to qualitatively establish reactivity of dansyl-labeled glutathione with GAPDH and LDH. 2 ul solutions of GAPDH with dansyl-labeled glutathione and 0.1 M phosphate buffer pH 7.4 were spotted on a nitrocellulose membrane, washed with PBS for 6 minutes, and visualized with the BIO-RAD ChemiDoc XRS+ imaging system. Samples of GAPDH with DGSSGD (D2) exhibited darker spots than those with DGSSG (D1) (Figure 6, Table 10). 2 ul samples of GAPDH with dansyl-labeled glutathione and 6.6 mM dithiothreitol (DTT) were spotted on nitrocellulose membrane to establish binding of the fluorescent glutathione to the protein and not the gel. These exhibited diminished or no spots under UV light for both D1 and D2 (Figure 7, Table 10). Similar results were obtained for LDH with D1 and D2 (Figures 8 & 9, Table 10). For both proteins, binding of dansyl-labeled glutathione was concentration-dependent.

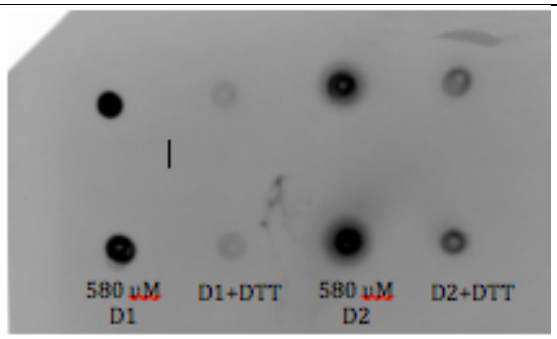
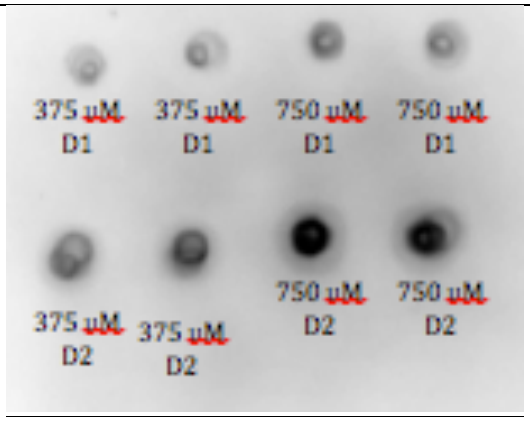
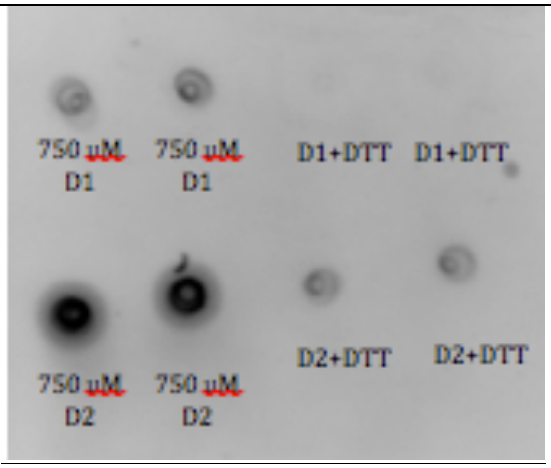
E. Reaction of Yeast Whole Extracts with F1 and F2

Increasing concentrations of yeast extracts were reacted with F1 and F2 in 0.1 M phosphate buffer pH 7.4 to qualitatively determine binding of fluorescein-labeled glutathione to potential cysteines contained in extracts. 2 ul solutions of extract with fluorescein-labeled glutathione were spotted on nitrocellulose membrane, washed with PBS buffer for 6 minutes, and visualized with the BIO-RAD ChemiDoc XRS+ imaging system. Additional samples of fluorescein-labeled glutathione with yeast extract and 6.6 mM DTT were also spotted on the gel to rule out fluorescently-labeled glutathione binding to gel. Reaction of F1 and F2 to yeast extract was confirmed with spots increasing in darkness with increase in yeast extract concentration. Spots were diminished with DTT (Figure 10, Table 10). These results suggest the

fluorescein-labeled glutathiones reacted with cysteines contained in yeast extracts. This result indicates extraction of proteins in yeast was successful and that these proteins contain reactive cysteines.

Table 10: Dot Blots of Various Reactions with GAPDH and LDH	
1	
2	

3	 375 uM F1 375 uM F1 750 uM F1 750 uM F1 375 uM F2 375 uM F2 750 uM F2 750 uM F2
4	 750 uM F1 750 uM F1 F1+DTT F1+DTT 750 uM F2 750 uM F2 F2 +DTT F2+DTT
5	 GAPDH +F1 pH 7.4 GAPDH +F1 pH 8.0 GAPDH+F2 pH 7.4 GAPDH+F2 pH 8.0
6	 290 uM D1 290 uM D1 580 uM D1 580 uM D1 290 uM D2 290 uM D2 580 uM D2 580 uM D2

7	 580 uM D1 D1+DTT 580 uM D2 D2+DTT
8	 375 uM D1 375 uM D1 750 uM D1 750 uM D1 375 uM D2 375 uM D2 750 uM D2 750 uM D2
9	 750 uM D1 750 uM D1 D1+DTT D1+DTT 750 uM D2 750 uM D2 D2+DTT D2+DTT

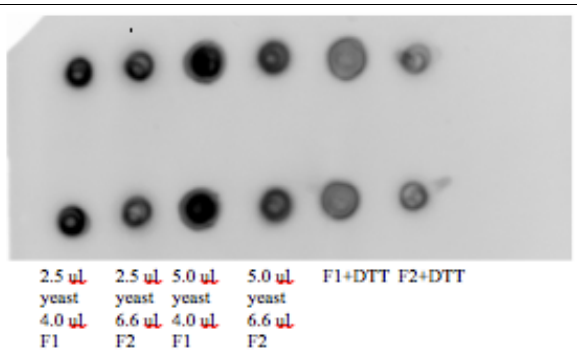
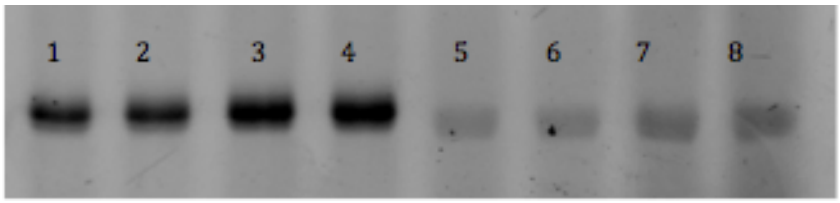
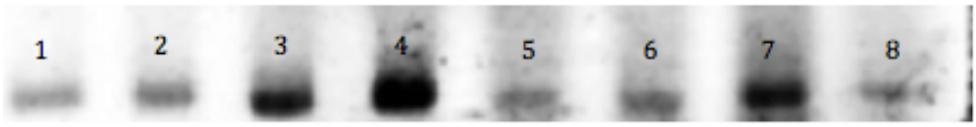
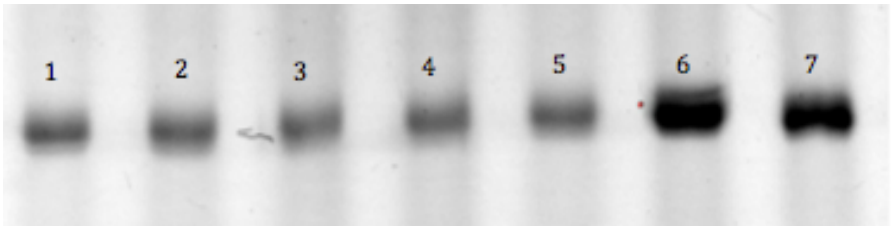
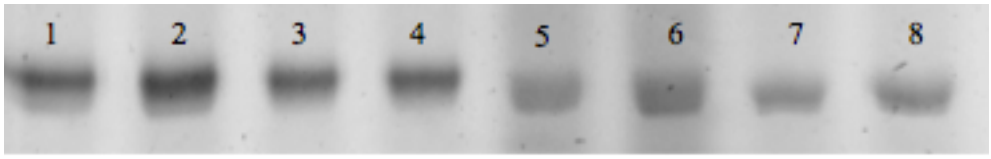
10	

Table 11: SDS-PAGE of Various Reactions with GAPDH and LDH

1	
2	
3	
4	

V. Data Analysis

GAPDH and LDH Reacted with F1 and F2

Data analysis showed that the highest concentration of F1 reacted with 35% of GAPDH cysteines while the highest concentration of F2 reacted with 6% of GAPDH cysteines (Table 1). 26% of LDH cysteines reacted with highest concentration of F1 while 5% of LDH cysteine reacted with highest concentration of F2 (Table 2).

Percent Cysteines Reacted for GAPDH with F1 and F2		
F1 or F2	**[F1] or [F2] (uM)**	**Percent cysteines reacted**
F1	290	25%
	580	35%
F2	290	5%
	580	6%

Table 1 Percentage of GAPDH cysteines that reacted with F1 and F2 for increasing concentrations determined from theoretical F1- and F2-reacted GAPDH.

Percent Cysteines Reacted for LDH with F1 and F2		
F1 or F2	**[F1] or [F2] (uM)**	**Percent cysteines reacted**
F1	375	16%
	750	26%
F2	375	3%
	750	5%

Table 2 Percentage of LDH cysteines that reacted with F1 and F2 for increasing concentrations determined from theoretical F1- and F2-reacted LDH.

GAPDH Reacted with F1 and F2 in pH 7.4 and pH 8.0

F1-reacted GAPDH in 0.1 M phosphate buffer pH 7.4 reacted with 109% of GAPDH cysteines, compared to pH 8.0 where 76% of GAPDH cysteines reacted with F1. 20% of GAPDH cysteines reacted with F2 in 0.1 M phosphate buffer pH 7.4 compared to 12% in 0.1 M phosphate buffer pH 8.0.

Percent Cysteines Reacted for GAPDH in Different pH		
F1 or F2	**pH**	**Percent cysteines reacted**
F1	pH 7.4	109%
	pH 8.0	76%
F2	pH 7.4	20%
	pH 8.0	12%

Table 3 Percentage of GAPDH cysteines that reacted with F1 and F2 in different pH determined form theoretical F1- and F2-reacted GAPDH.

GAPDH Reacted with F1 in 13.3 mM NaCl

Percentage of cysteines reacted could not be determine from theoretical data because data analysis indicated over 100% of cysteines had been tagged for F1-reacted GAPDH with 13.3 mM NaCl. A possible source of error could have occurred in the removal of excess fluorescent-glutathione with 80% ethanol. If excess fluorescent-glutathione was present, the absorbance measurements would be higher than theoretical values. Furthermore, if concentration of F1 was measured incorrectly via absorbance, a higher than normal concentration would produce higher than expected absorbance values.

VI. Discussion

A. Steric Hindrance Influences Cysteine Reactivity

Reaction of GAPDH and LDH with F1 produced higher absorbance values compared to reaction with F2. These results were backed qualitatively by SDS-PAGE gels where average band intensity for GAPDH and LDH reacted with F1 was increased relative to GAPDH and LDH reacted with F2. This suggests protein cysteine reactivity is limited due to steric hindrance, due to the larger size of F2 compared to F1. As Figure 1 suggests, F2 has two molecules of FITC, each with four rings. Compared to F1, this molecule is bulkier and therefore it is more difficult for the nucleophilic cysteine to attack the disulfide bond of the fluorescent glutathione (Figure 1). F1-reacted GAPDH exhibited an 81% increase in average absorbance values compared to F2-reacted GAPDH, for the highest concentration of fluorescent glutathione. F1-reacted LDH exhibited an 83% increase over F2-reacted LDH for the highest concentration of fluorescent glutathione. This suggested higher reactivity of GAPDH and LDH cysteines with F1 compared to F2. GAPDH cysteines exhibited a 9% increase in reacted cysteines over LDH for the highest concentration of F1-reacted protein, suggesting increased reactivity of GAPDH cysteines compared to LDH cysteines. This is surprising considering LDH has one more cysteine than GAPDH. A potential explanation could be a higher degree of burial of LDH cysteines. This would hinder F1 from reacting with the protein cysteines (Figure 11). GAPDH and LDH cysteine's differential reactivity with the fluorescein-labeled glutathione can be monitored by UV/Vis spectroscopy. This data can then be used to quantitatively determine cysteine reactivity.

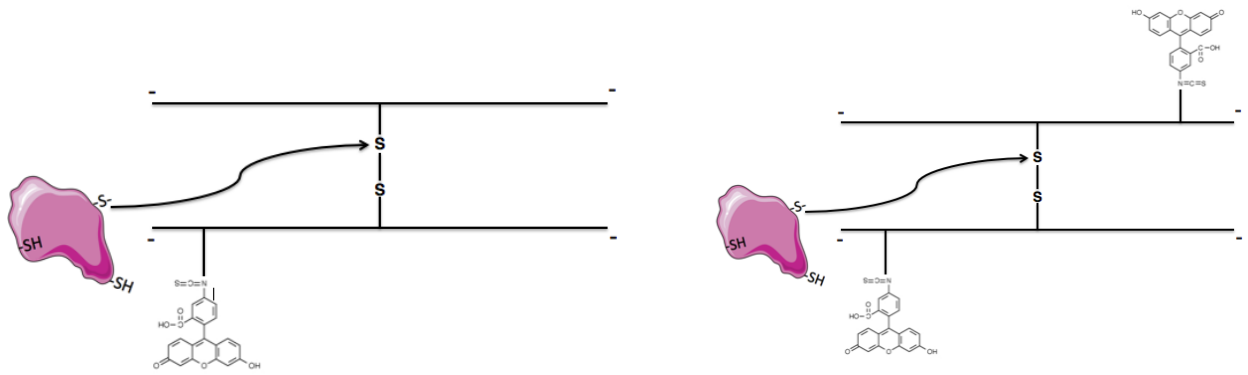


Figure 10 Visual depicting nucleophilic attack of protein cysteine on disulfide linkage in F1 (left) and F2 (right). Steric hindrance causes decrease in reactivity of cysteine with F2, which translates to a decrease in absorbance for F2-reacted cysteines.

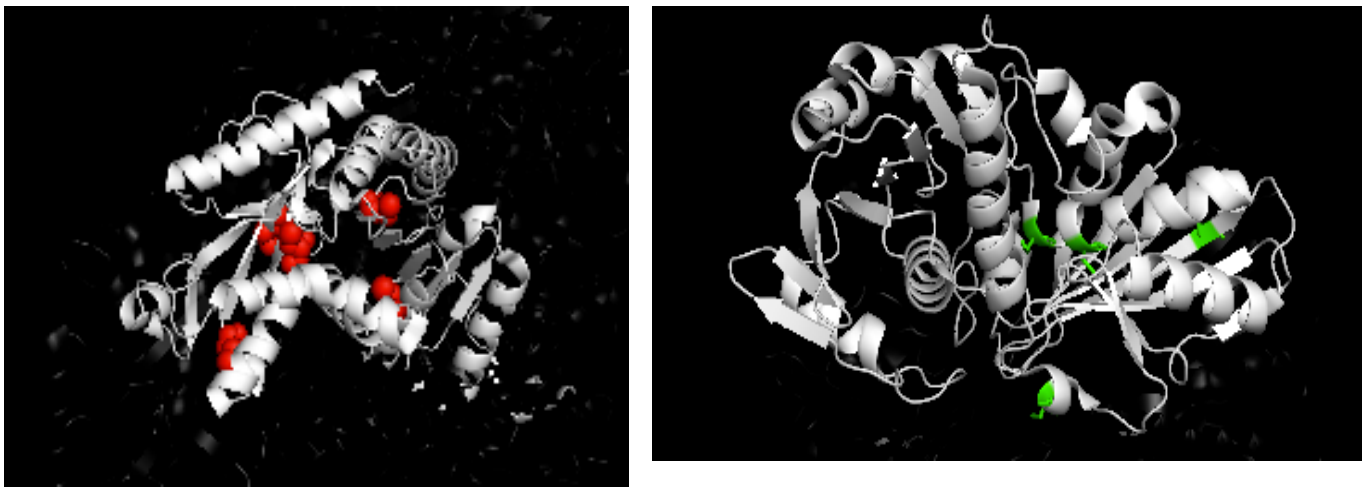


Figure 11 A potential explanation for increased reactivity of GAPDH cysteines (right, green) compared to LDH cysteines (left, red) could be the higher degree of burial of LDH cysteines compared to GAPDH cysteines.

B. Salt Concentration Influences Cysteine Reactivity

Absorbance values of GAPDH reacted with F1 were increased upon addition of 13.3 mM NaCl. Samples of F1-reacted GAPDH with IAM added immediately after addition of 13.3 mM NaCl produced average absorbance values that were 3% different from F1-reacted GAPDH with IAM and . These results were backed qualitatively with nitrocellulose dot blots and SDS-Page gels. Furthermore, addition of IAM five minutes after the 13.3 mM NaCl reacted with the F1-reacted GAPDH exhibited average absorbance values 5% higher than F1-reacted GAPDH with IAM added immediately after the 13.3 mM NaCl. Furthermore, F1-reacted GAPDH in 13.3 mM

NaCl produced a 35% increase in absorbance compared to F1-reacted GAPDH, indicating increased reactivity of GAPDH cysteines in high salt concentration. Taken together, these results suggest addition of NaCl exposed a cysteine that reacted with the F1, which explains the increase in absorbance. Given that highly conserved cysteines are typically buried in the hydrophobic core of the protein, it is likely that the disruption of tertiary interactions within GAPDH exposed a previously buried cysteine. There is strong evidence to suggest that this cysteine could be the highly conserved residue Cys281 (Figure 2). This thiol group is particularly buried and flanked by the charged residue Asp282, which has the potential to be involved in electrostatic interactions with surrounding residues. Disruption of these interactions by NaCl would expose the buried cysteine to react with fluorescein-labeled glutathione. It is likely that after five minutes of reaction with NaCl, the previously hidden cysteine was exposed more and reacted with F1 while the other cysteines were prevented from reacting with F1 due to the addition of IAM. (Figure 3). Additional time-dependency experiments would need to be performed to further distinguish the effects of NaCl on GAPDH cysteine reactivity. The interaction between NaCl and GAPDH cysteines is easily monitored via UV/Vis spectroscopy, which can be used to quantitatively determine the extent of GAPDH cysteine reactivity with fluorescently-labeled glutathione.

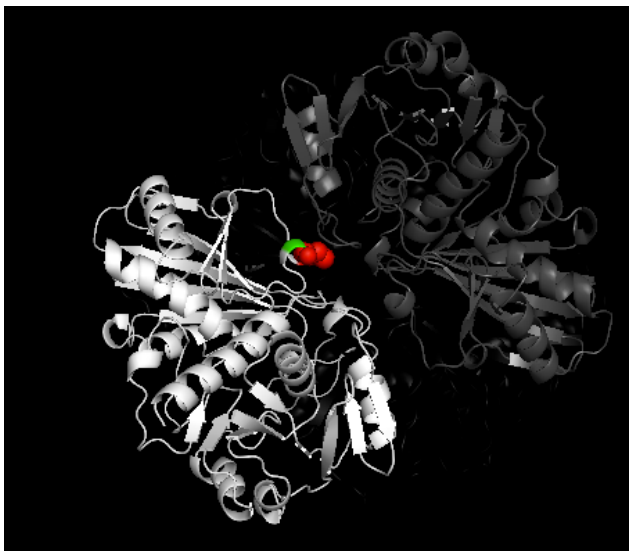


Figure 12 Visual pulled from PyMol (PDB 1JOX) of two subunits of rabbit muscle GAPDH for clarity. The buried residue Cys281 (red) is flanked by charged residues Asp282 (green). The charged residue presumably interacts with surrounding residues via electrostatic interactions. Addition of NaCl would disrupt these interactions and expose the previously buried cysteine to react with F1.

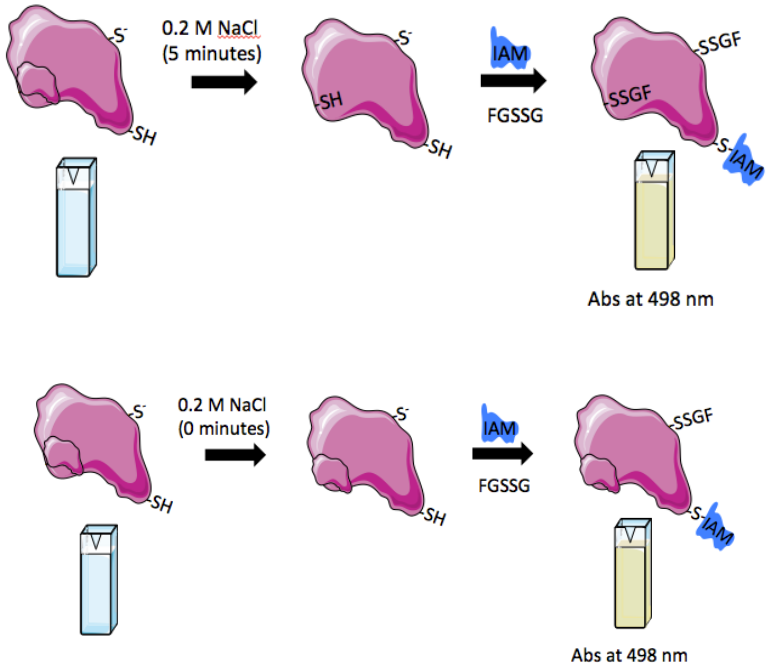


Figure 13 Visual depicting reaction conditions where GAPDH (pink) was reacted with 13.3 mM NaCl for 5 minutes after which cysteine-chelating agent IAM was added and sample was reacted with F1 (FGSSG). Average absorbance was increased relative to sample where IAM was added immediately after NaCl, suggesting buried cysteine was uncovered during five-minute reaction.

C. The Local pH Microenvironment Influences Cysteine Reactivity

Absorbance of GAPDH reacted with F1 in 0.1 M phosphate buffer pH 8.0 was decreased relative to GAPDH reacted with F1 in 0.1 M phosphate buffer pH 7.4. Similar results were obtained for GAPDH reacted with F2. These results were backed qualitatively with nitrocellulose dot blots and SDS-Page gels, which exhibited decreased intensity for samples of GAPDH reacted with F1 and F2 in 0.1 M phosphate buffer pH 8.0. This likely resulted because at pH 8.0, the a majority of the cysteine residues are likely in their anionic form (the pKa of cysteine is 8.3). While it is expected that cysteine will be more reactive since it is in its nucleophilic state, this is not the case. The added negativity from the nucleophilic cysteines creates charge-charge repulsion between the protein and FGSSG, with its five negative charges. The same is true for GAPDH reacted with FGSSGF, which has six negative charges (Figure 4). Furthermore, average absorbance values of F1-reacted GAPDH in 0.1 M phosphate buffer pH 7.4 produced a 30% increase compared to pH 8.0. Average absorbance values of F2-reacted GAPDH showed a 41% increase in pH 7.4 compared to pH 8.0. These results suggest increased reactivity of GAPDH cysteines in pH 0.1 M phosphate buffer pH 7.4 compared to pH 8.0. Differential reactivity of GAPDH cysteines in different buffers can be monitored via UV/Vis spectroscopy, which can then given quantitative data on reactivity of protein cysteines.

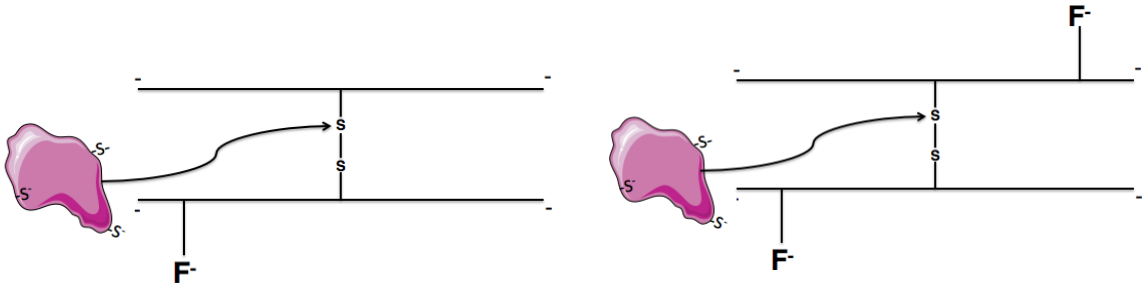


Figure 14 Visual depicting reaction of GAPHD with F1 (left) and F2 (right) in phosphate buffer pH 8.0. At pH 8.0, cysteines are in their anionic state, which means they repel the negatively charged F1 (4 negative charges) and F2 (5 negative charges), leading to decreased reactivity of GAPDH cysteine with F1 and F2.

D. Dansyl-Labeled Glutathiones Are a Less Sterically-Hindered Alternative to Fluorescein-Labeled Glutathione

In contrast with nitrocellulose dot blots of GAPDH reacted with F1 and F2, GAPDH reacted with D1 and D2 showed darker spots for D2-reacted GAPDH compared to D1-reacted GAPDH. Similar results were obtained for LDH. This is likely due to decreased steric hindrance of dansyl compared to FITC. These results suggest dansyl-labeled glutathione is a promising alternative to fluorescein-labeled glutathione. Because of its decreased steric hindrance, dansyl-glutathione thiol/disulfide exchange with protein cysteines can more accurately reflect protein cysteine reactivity.

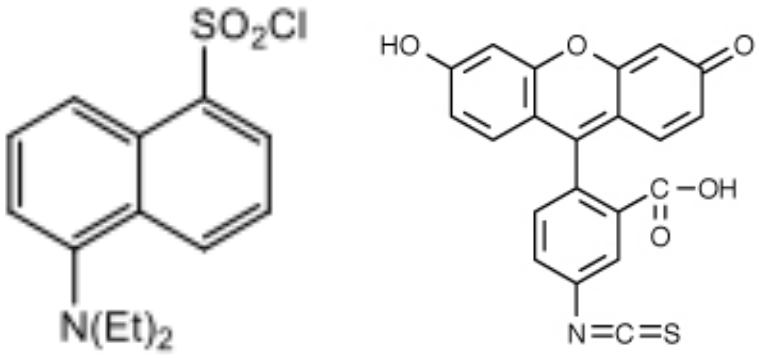


Figure 15 Structure of dansyl (left) to FITC (right). Dansyl is structurally more sterically hindered.

E. Fluorescein-Labeled Glutathiones Used to Identify Reactive Cysteines in Yeast Whole Extracts

Nitrocellulose dot blots of F1- and F2-reacted yeast whole extracts exhibited increasing darkness with increase concentration of yeast extract. Addition of DTT exhibited diminished or no spots for F1- and F2-reacted yeast extract. This data suggests that fluorescein-labeled glutathiones can be used to identify reactive cysteines in yeast whole extracts.

VII. Conclusion

Cysteine is a highly reactive residue that can easily be oxidized. While cysteine's oxidation is important to regular cellular functioning, over-oxidation by ROS can be damaging to cellular protein structure and functioning. Cysteines react with the oxidized form of glutathiones (GSSG) via thiol-disulfide exchange. This exchange is also carried out with fluorescently labeled glutathiones, whose amino termini are modified by addition of fluorescent molecules. Cysteine's reactivity with fluorescein-labeled glutathiones is influenced by the local steric, salt, and pH environment. This reaction can be monitored via UV/Vis-spectroscopy, nitrocellulose membrane dot blots, and SDS-PAGE.

In this study, fluorescently-labeled glutathiones were used to quantify cysteine reactivity in the proteins GAPDH and LDH. Nitrocellulose gel dot blots and SDS-PAGE qualitatively established GAPDH and LDH reactivity with F1 and F2. Binding of F2 to nitrocellulose membrane could not be ruled out, however SDS-PAGE gels showed higher intensity bands for F1 compared to F2 for both GAPDH and LDH. Absorption spectra produced higher absorbance peaks for GAPDH and LDH reacted with F1 compared to F2. These results suggested increased cysteine reactivity with F1 due to the large steric hindrance of F2. Additional trials are required to statistically establish higher reactivity of GAPDH and LDH cysteines with F1 over F2. Furthermore, reactivity of GAPDH cysteines with F1 and F2 was increased compared to LDH cysteines, potentially due to a higher degree of LDH cysteines compared to GAPDH. A potential future area of study is comparing the oxidation of GAPDH compared to LDH cysteines to establish increased reactivity of GAPDH cysteines.

SDS-PAGE qualitatively established increased reactivity of GAPDH and F1 under high NaCl concentration. These results were quantitatively established via UV/Vis spectroscopy

where absorption spectrum peaks and absorbance values were higher for F1-reacted GAPDH in a high concentration of NaCl compared to F1-reacted GAPDH without NaCl. A time-dependency study with the cysteine-chelating agent IAM resulted in decreased absorbance for F1-reacted GAPDH in a high concentration of NaCl, suggesting that the protein's tertiary interactions were disrupted by the high salt concentration. Additional trials are required to statistically establish higher reactivity of F1-reacted GAPDH in high salt concentration. Furthermore, a broader range time-dependency study that extended reaction of GAPDH with NaCl to 10 or 15 minutes would strengthen this finding.

SDS-PAGE and nitrocellulose membrane dot blots qualitatively established decreased reactivity of F1- and F2-reacted GAPDH in 0.1 M phosphate buffer pH 8.0. These results were quantitatively established through UV/Vis spectroscopy where absorption spectrum peaks and absorbance values were decreased for GAPDH reacted with F1 and F2 prepared in 0.1 M phosphate buffer pH 8.0. This pH is close to the pKa of cysteine residues, so it is expected GAPDH cysteines would be in their anionic, nucleophilic states. However, it is likely that due to the overall negative charge on GAPDH in physiological pH, the added negativity caused by the anionic cysteines could be repelling the negatively charged F1 and F2 molecules. This would decrease reactivity of the cysteines overall, thus decreasing absorbance of the F1- and F2-reacted GAPDH in pH 8.0. Additional trials are required to statistically establish decreased reactivity of F1- and F2-reacted GAPDH in 0.1 M phosphate buffer pH 8.0.

Differential reactivity of GAPDH and LDH cysteines with fluorescein-labeled glutathiones under various steric, salt, and pH environments translated to differential peak values on absorption spectra. This finding suggests fluorescein-labeled glutathiones are a simple and elegant way to quantify cysteine reactivity. Fluorescein-labeled glutathiones are quick and easy

to synthesize, have a high molar absorptivity, and can qualitatively and quantitatively be determined through nitrocellulose membrane dot blots, SDS-PAGE, and UV/Vis spectroscopy. SDS-PAGE qualitatively established increased reactivity of GAPDH and LDH with D2 compared to D1. This likely resulted because of the decreased steric hindrance of D2 compared to F2, as dansyl is a low molecular weight fluorescent molecule. This established dansyl-labeled glutathiones as a promising alternative to fluorescein-labeled glutathiones. However, the molar absorptivity of dansyl is too low for UV/Vis to accurately detect the absorbance of its reaction with GAPDH or LDH. Thus, another promising alternative with better spectral properties is 7-methoxy-4-methylcoumarin-3-carboxylic acid, succinimidyl ester (mCSE), a fluorescent molecule that absorbs at 358 nm with a molar absorptivity of 25600 $M^{-1}cm^{-1}$ (See Figure 1). mCSE is of intermediate size compared to dansyl and FITC isothiocyanate. mCSE has already been shown to label one or both termini of GSSG.

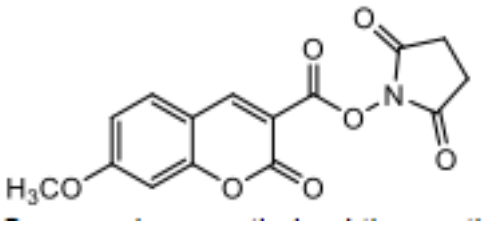


Figure 1 Structure of fluorescent molecule mCSE. A=358 nm, ε=25600 $M^{-1}cm^{-1}$

Nitrocellulose membrane dot blots qualitatively established reactivity of F1 and F2 with yeast extracts prepared during Summer 2015. This finding was important because it indicates presence of reactive cysteines in the yeast whole extracts. This suggests fluorescently-labeled glutathiones can be used to predict presence of proteins in whole extracts quickly and easily, rather than using a more invasive and time-consuming method such as SDS-PAGE. A future are of study could compare cysteine reactivity between various proteins contained in yeast whole

extracts. This would involve SDS-PAGE analysis of yeast whole extract reacted with fluorescently-labeled glutathione and stopped by IAM after 5 minutes, 10 minutes, 15 minutes, etc. reactions. Mean band intensities of these gels could then be compared to establish increased reactivity of fluorescently-labeled glutathione with a cysteine-containing protein in the extracts.

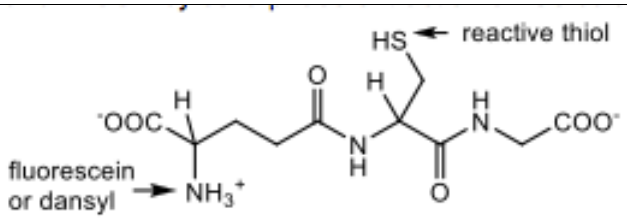
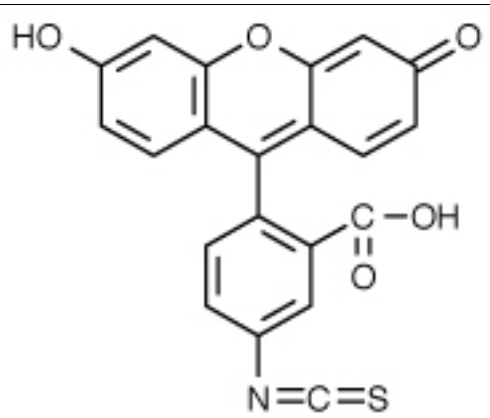
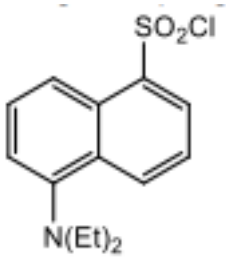
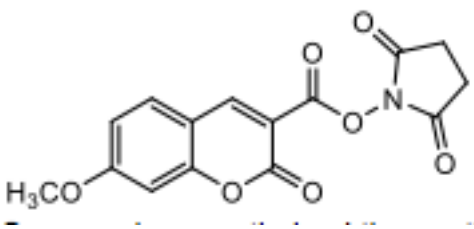
Nitrocellulose membranes proved useful in quickly and easily determining reaction of protein with fluorescent-glutathione, however binding of fluorescent-glutathione to membrane for F2, D2, and yeast extracts could not be ruled out by reaction with DTT. It is suspected that a longer reaction time between F2-reacted protein and DTT was needed to produce a lighter spot. An additional source of error is the visualization of SDS-PAGE. While pipetting error was diminished by preparing doubles of each sample during a trial, it can not be ruled out. Another potential source of error is the method of protein cysteine reaction with fluorescently-labeled glutathiones. For example, if the protein requires more than 30 minutes to react with fluorescently-labeled glutathione, lower absorbance values would be produced.

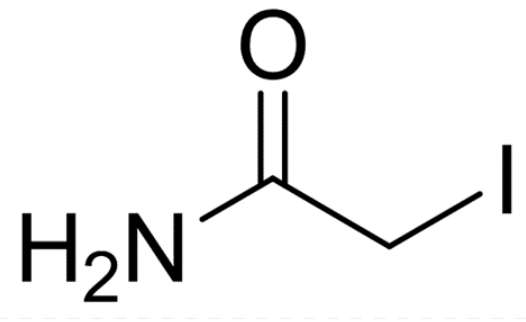
This study investigated the use of fluorescently-labeled glutathiones to quantify cysteine reactivity of GAPDH and LDH with FGSSG, FGSSGF, DGSSG, and DGSSGD under various cellular conditions. It demonstrates that reactivity of protein cysteines with fluorescently-labeled glutathiones through thiol-disulfide exchange can be monitored through UV/Vis spectroscopy. Absorbance peak values can then be used to determine the percentage of cysteine that reacted, which can be used to predict cysteine reactivity.

VIII. Supplementary Data

Properties of GAPDH and LDH		
Protein	**Molecular Weight (kDa)**	**Number of Reduced Cysteines**
LDH	36	5
GAPDH	36	4

Relevant Abbreviations	
Name	**Abbreviation**
Glyceraldehyde-3-Phosphate Dehydrogenase	GAPDH
Lactate Dehydrogenase	LDH
FGSSG	F1
FGSSGF	F2
DGSSG	D1
DGSSGD	D2
ROS	reactive oxygen species
dithiothreitol	DTT
Iodoacetamide	IAM

Chemical Index	
	Structure of glutathione. The amino group is modified in the synthesis of fluorescent glutathiones by either FITCe isothiocyanate or dansyl
	Structure of fluorescent molecule fluorescein isothiocyanate A=498 nm ε=77000 $M^{-1}cm^{-1}$
	Structure of fluorescent molecule dansyl A=340 nm ε=4300 $M^{-1}cm^{-1}$
	Structure of fluorescent molecule 7-methoxy-4-methylcoumarin-3-carboxylic acid succinimidyl ester (mCSE) A=358 nm ε=25600 $M^{-1}cm^{-1}$

	Structure of cysteine chelating agent iodoacetamide

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