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Connecting Energy Metabolism and Neurodegeneration: Pyruvate Kinase and Oxidative Stress

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Connecting Energy Metabolism and Neurodegeneration:
Pyruvate Kinase and Oxidative Stress

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by

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ABSTRACT:

Pyruvate kinase (PK), an important glycolytic enzyme that catalyzes the final step of glycolysis through an irreversible mechanism to produce energy for the cell, contains nine cysteines, some of which are highly reactive and critical for function. This research sought to develop a protocol to measure quantitatively the functionality of PK under different experimental conditions. Using a three stage, consistent procedure, it was possible to analyze the effects of thiol modification by Ellman's Reagent (DTNB) and oxidation by hypochlorous acid (HOCl) on PK activity. Results showed that both DTNB and HOCl inhibited activity at high concentrations, which illustrates that some cysteines are essential for PK to catalyze its reaction. Furthermore, analysis of cysteine labeling with 5-iodoacetamidofluorescein on SDS-PAGE demonstrates that not all cysteines must be modified to see inhibitory effects. Additionally, incubation studies with ATP and fructose-1,6-bisphosphate, known regulators with different binding sites in PK, located important cysteines in the enzyme and explored their reactivity and significance. This characterization of the enzyme has notable implications *in vivo* in that PK could potentially serve a protective role for other cysteine-containing proteins necessary for cellular function that might be more sensitive to the effects of oxidative damage, such as tubulin, tau, or other glycolytic enzymes, accumulated damage to which could lead to neurodegenerative diseases, such as Alzheimer's disease (AD).

PART I: INTRODUCTION:

A. <u>Reactivity of Cysteines and their Oxidation:</u>

Despite their relatively low abundance when compared to other amino acids, cysteines (see Figure 1) are highly conserved within functional sites in proteins due to their distinctive chemistry (Poole, L.B., 2015). The lone pairs of electrons on the sulfur atoms make cysteines highly nucleophilic. Furthermore, the ionization of the thiol group (R-SH) to a thiolate ion (R-S⁻) can increase the reactivity of the cysteine and facilitate the formation of disulfide linkages, especially since cysteines tend to cluster, or be found together in groupings. Cysteine has a pK_a of approximately 8.3; however, this value can change based on the specific protein environment in which the residue is found. The unique reactive characteristics of cysteine have led to an "extreme pattern of conservation at both ends of the spectrum," meaning that cysteines can be "irreplaceable" and highly conserved (>90%) in certain locations but not (<10%) in others based on their functional importance (Poole, L.B., 2015). Conserved residues tend to be found on the interior of the protein since interactions with the solvent could affect reactivity and conformation. Lastly, cysteines are subject to oxidation by reactive oxygen and nitrogen species (ROS and RNS), which can drastically affect the protein's function.
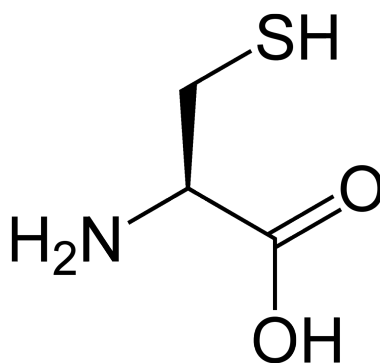
Figure 1: Molecular structure of cysteine, one of the twenty naturally occurring amino acids

Some degree of oxidative stress is normal in a cell, and antioxidants such as superoxide dismutase and glutathione ensure that ROS concentrations are maintained at non-toxic levels. Unfortunately, oxidants are an "unavoidable consequence of metabolism" and generally produce superoxide radicals ($O_2^{\bullet -}$), hydroxyl radicals, or peroxides, like hydrogen peroxide (H_2O_2), a byproduct of monooxygenases (Poole, L.B., 2015). Oxidation can lead to reversible and irreversible cellular damage to proteins, lipids, and DNA (Lyras, L. *et al.*, 1997). Accumulation of this damage over time can lead to cancer or neurodegenerative diseases, such as Alzheimer's disease (AD) and Parkinson's disease.
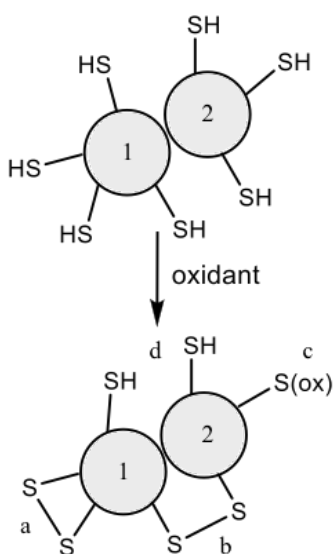
Figure 2: Possible thiol modifications in response to oxidation by a reactive oxygen species (ROS):
  a) *disulfide bonds between cysteines within the same protein unit*
  b) *disulfide bonds between cysteines between different protein units*
  c) *formation of a sulfoxide (SO$_X$)*
  d) *no effect (reduced thiols maintained)*

When cysteines are oxidized, they have the potential to form disulfide bonds (see Figure 2). While this could serve a post-translational regulatory purpose for some proteins, disulfide bonds that form where reduced thiols are required for function can have functional consequences. These linkages can occur between cysteines of the same protein, which could alter the conformation based on the distance between the residues (see Figure 2a). For example, a cluster of cysteines that form a disulfide bond could see minimal structural changes; however, a bond between two cysteines that are further away on the protein could induce a severe

conformational change that inhibits function. Additionally, cysteines between two separate

proteins could form disulfide bonds (see Figure 2b), which could lead to a loss of function and/or

the formation of protein aggregates, a common hallmark of neurodegeneration (Aksenov, M.Y.

et al., 2001). Oxidized cysteines also have the potential to form sulfoxides (SO_X) that have a

high reactivity (see Figure 2c). It is important to note that oxidation does not guarantee all

cysteines will be modified (see Figure 2d). In fact, some research has shown that a low degree of

oxidation can have a slight activating effect on protein function (Lain, T.K., 2015). The

functional effects of cysteine oxidation depend on the many factors, including the type of oxidant

and its concentration, the location of the residues and the targets for disulfide linkages, and the

extent modification has on the protein's conformation.

B. <u>Linking Microtubules, Oxidative Damage, and Glycolytic Enzymes:</u>

There is a strong correlation between oxidative damage and neurodegeneration (Aksenov,

M.Y. *et al*., 2001). Alzheimer's disease, for example, is delineated by neurofibrillary tangles and

amyloid plaques, both of which could be caused in part by oxidation of cysteines within the

proteins (Dent, E.W. & Baas, P.W. 2014; Green, P.S. *et al*. 2004). The tangles are made from

improperly formed networks of microtubules made of the protein tubulin. Each tubulin

heterodimer contains an α- and β-subunit, each of which is approximately 50 kDa in size (Green,

P.S. *et al*. 2004). Associations between key cysteines of tubulin molecules allow for microtubule

elongation. Microtubule-associated proteins (MAPs), such as tau, increase the efficiency of

microtubule formation by stabilizing the structure through electrostatic interactions (Serrano, L.,

Avila, J., & Maccioni, R.B. 1984). These cytoskeletal elements are in a flux between cycles of

polymerization and depolymerization, which aids in their function of maintaining neuronal cell

shape, facilitating movement of signaling molecules, and acting as a scaffold to recruit certain

proteins (Dent, E.W. & Baas, P.W. 2014). Thus, damage to tubulin and MAPs could have far-reaching cellular consequences. In fact, researchers have shown *in vitro* that cysteine oxidation of tubulin and microtubule-associated protein-2 (MAP-2) by physiologically relevant oxidants inhibits microtubule polymerization (Landino, L.M. *et al*. 2002). Over time, oxidative damage to microtubules can amass, leading to conglomerates of the microtubule-associated protein tau, or neurofibrillary tangles, a common hallmark of neurodegenerative diseases.

Furthermore, damage to tubulin can affect other cellular processes that are dependent on cytoskeletal networks. Due to their basic pI values, it is likely that glycolytic enzymes, among other molecules, interact with acidic tubulin molecules in such a way as to provide a possible means of regulation of the complex pathway of metabolism (Landino, L.M., Hagedorn, T., & Kennett, K.L., 2014). Therefore, tubulin under oxidative stress could affect cellular energetics, which could restrict the availability of energy-rich molecules to fuel normal cell function.

Additionally, because glycolytic enzymes, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), lactate dehydrogenase (LDH), and pyruvate kinase (PK), are nearly ubiquitous and contain reduced cysteines, researchers have identified these proteins as targets for cysteine oxidation (Foley, T.D. *et al*. 2014). The effects of oxidative damage to these enzymes are not well characterized, however. It is likely that loss of function could occur, but researchers have yet to determine the extent and at what concentration of oxidant. Glycolytic enzymes may play additional roles in cellular processes, such as the link between pyruvate kinase and cell growth and cancer and immunity (Gau, X., *et al*., 2013; Uzan, J, *et al*., 2011). Therefore, damage to these enzymes could have consequences beyond inhibited cellular energetics. Taken together, glycolytic enzymes under oxidative stress are an interesting target of research due to the potential

for both loss of regulation from microtubule destabilization and oxidative inhibition of enzymatic function.

C. <u>Pyruvate Kinase:</u>

Pyruvate kinase (PK) is a key enzyme in glycolysis, which catalyzes the conversion of phosphoenolpyruvate (PEP) and ADP to pyruvate and ATP illustrated in Figure 3. Due to the highly exergonic nature of this reaction, PK mediates an irreversible step, making the enzyme an important target for metabolic regulation. Producing 2 moles of ATP and 2 moles of pyruvate per 1 mole of glucose, PK paves the way for further metabolic processes and energy harvesting, both aerobically and anaerobically. The highly specific structure of pyruvate kinase amplifies small molecular changes, which has profound effects on the enzyme's function. PK has two main binding sites, an active site and a regulatory site, each of which contain important cysteines. The location of these cysteines and their interactions with monovalent and divalent metal cations affect the extent to which oxidative damage occurs for rabbit muscle PK.

Figure 3: The reaction catalyzed by pyruvate kinase
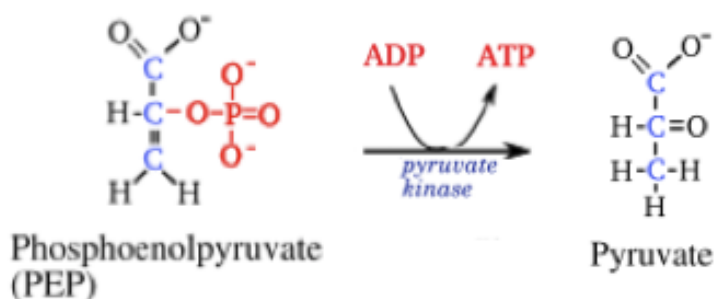
As with all proteins, pyruvate kinase's structure is essential for function. The enzyme is a tetramer weighing approximately 200 kDa (Lee, J.C., & Herman, P., 2010; Valentini, G. *et al*., 2002). Each of the four identical subunits (shown in Figure 4) has approximately 530 residues that form three domains: "a small N-terminal helical domain, a β-α barrel domain surrounding a

β barrel domain, and a three-layer α-β-α sandwich domain" (Lee, J.C., & Herman, P., 2010; UCSD Signaling Gateway, 2014; Valentini, G. *et al*., 2002). Each domain has an activator-binding site, corresponding to the reactants, PEP and ADP, and the allosteric regulator, fructose-1,6-bisphosphate. (Valentini, G. *et al*., 2002). Allosteric regulation can activate or inhibit one of the binding sites, which is located in a cleft between the two β barrel domains, by rotating those domains. Because of the effects of allosteric molecules, the four clefts are important regulatory sites for the enzyme (UCSD Signaling Gateway, 2014; Valentini, G. *et al*., 2002). PK is inhibited by acetylCoA, alanine, or ATP and activated by fructose-1,6-bisphophate (FBP) or AMP. Covalent modification of the protein due to insulin, an activator, or glucagon, an inhibitor, can affect the enzymatic rate. Changes in metabolic efficiency beyond general regulation have been linked to cell growth, cancers, and immunity.

Most research involving pyruvate kinase involves rabbit PK. Rabbits have four forms of PK: L, found in the liver; R, in red blood cells; M1, in muscle, heart, and brain tissue; and M2, in early fetal tissue (UCSD Signaling Gateway, 2014). M1 and M2 stem from the same genetic coding region but differ because of alternative splicing of mRNA transcript (Valentini, G. et al., 2002). Although the isoforms have the same function for energy harvesting and pyruvate production, each differs slightly in primary structure, which translates into larger differences in regulation for the enzyme (Table 1). Because PK-L is involved in maintaining necessary glucose levels, it is the most sensitive to allosteric regulation from fructose-1,6-bisphosphate, ATP, alanine, as well as hormonal regulation from insulin and glucagon. On the other hand, PK-M1 is only inhibited by ATP because brain and muscle tissue need a constant supply of glucose to function properly. Although, research has shown that phenylalanine can significantly reduce the enzymatic capabilities of the M1 isoform and Zn^{2+} can reduce that of both the M1 and M2

isoforms (Li, F., Yu, T., Jiang, H., & Yu, S., 2013; Tamaki, N., Ikeda, T., Kimura, K., & Morioka, S., 1981). The reduction in sensitivity to allosteric molecules in PK-M1 and PK-M2 causes less fluctuating glucose levels than PK-L (UCSD Signaling Gateway, 2014). The different isoforms in rabbit PK give a greater range of sensitivity and specificity to PK experimentation.

Isoform	Activators	Inhibitors
PK-L	Fructose-1,6-bisphosphate (FBP), AMP	ATP, acetylcoA, alanine
PK-R	Fructose-1,6-bisphosphate (FBP), AMP	ATP, acetylcoA, alanine
PK-M1	Fructose-1,6-bisphosphate (FBP), AMP	ATP; phenylalanine; Zn^{2+}
PK-M2	Fructose-1,6-bisphosphate (FBP), AMP	ATP; Zn^{2+}

Table 1: Differences in regulation for the four isoforms of pyruvate kinase

D. <u>Potential Protective Effects of Cations Against Oxidative Damage to Pyruvate Kinase:</u>

The cations K^+ and Mg^{2+} are necessary for pyruvate kinase activity and may have protective effects. The activator-binding site in each subunit interacts with a catalytic center for which both magnesium and potassium ions are necessary (Valentini, G. *et al*., 2002). It has been proposed that the cations are part of the PEP binding site of muscle PK (Brown, C.E., Taylor, J.M., & Chan, L., 1985). Further research supported this idea after finding four total binding sites per PK for monovalent metal cations, divalent metal cations, and PEP (Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972). Potassium ions increase the binding affinity for PEP to increase the maximum velocity of the reaction, and magnesium ions counteract the negative charge of ADP and stabilize the enol-form of pyruvate in the intermediate and transition states (Orla-Hernandez, J. *et al*., 2005). Moreover, PK seems to have a particular defense mechanism against low levels of oxidative stress such that the catalytic ability of reactive cysteines is maintained (Lane, T.K., 2015). When present together with an oxidant, it is likely that Mg^{2+} and K^+ protect PK to maintain activity (Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972). Therefore, it appears that

the ions involved in the PEP binding site act as the defense mechanism against oxidative damage in addition to maximizing enzymatic efficiency.

E. Cysteines in Pyruvate Kinase:
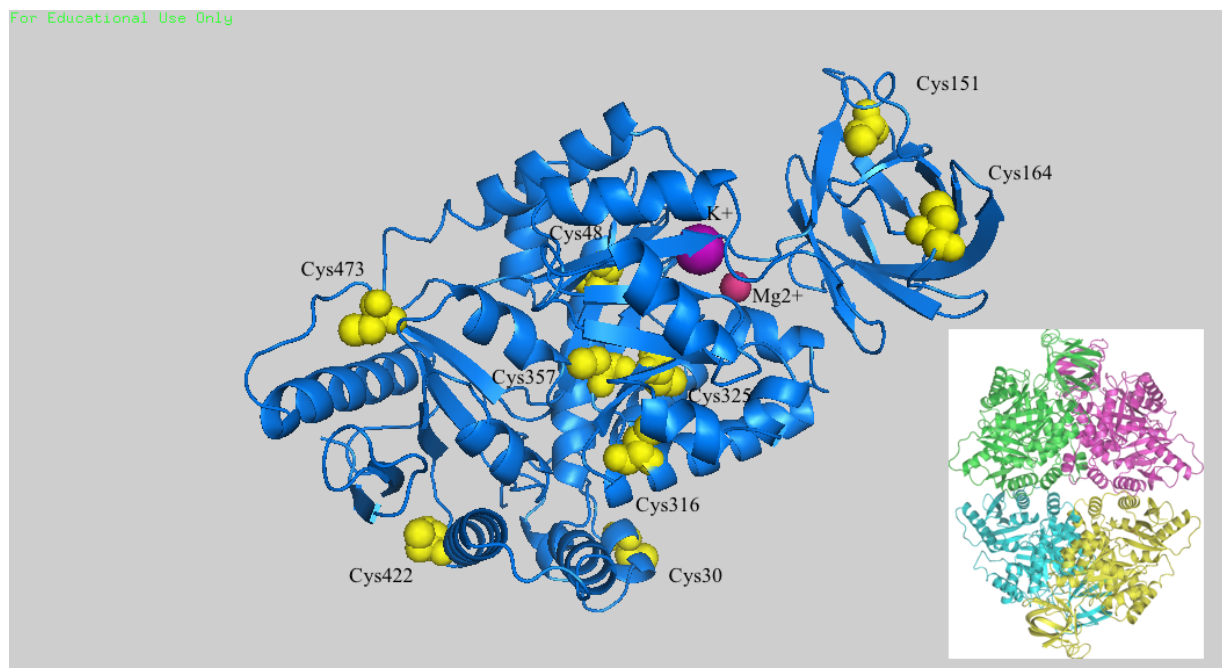
Figure 4: The cysteines in a pyruvate kinase monomer oriented alongside the functional tetramer

Pyruvate kinase has many important thiol groups, which makes the enzyme an intracellular target for oxidative damage. Each of the four subunits has nine cysteine residues as illustrated in Figure 4 (Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972; Rafter, G.W., & Blair, J.B., 1987). One of these cysteines per subunit is minimally reactive but necessary for catalytic function; another is highly reactive but not essential for enzymatic activity. The minimally reactive cysteines are necessary for catalytic function because these cysteines activate and stabilize PK at low pH or in the presence of PEP and metal cations (Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972). The cations Mg^{2+} and K^+ likely shield these cysteines to prevent loss of function (Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972). Because cations are only active in the PEP binding site, it is likely that the PEP binding site of each subunit

contains the less reactive but necessary cysteines (Brown, C.E., Taylor, J.M., & Chan, L., 1985; Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972). Furthermore, because the PEP binding site is the primary active site of the protein and necessary for all PK isoforms, the cysteine in this location is highly conserved, a common result of selective pressure for irreplaceable cysteines in a protein's interior (Poole, L.B., 2015). Based on a 2011 study in which PK was mutated and cysteine residues were replaced with serines, it is possible that the minimally reactive but necessary cysteine in each subunit is Cys 357, a residue located in the β-barrel that "includes residues essential for catalytic activity" and "primarily accounts for ROS-induced inactivation" (Anastasious, D., *et al*., 2011). The quaternary structure of PK minimizes access to Cys 357, which could account for the low reactivity of these residues (Anastasious, D., *et al*., 2011). Thiol reagents, particularly Ellman's Reagent (5,5-dithio-bis-[2-nitrobenzoic acid]), a thiol-specific reagent, and GSSG, an oxidized form of glutathione that serves as an intracellular antioxidant, react with cysteines to change the affinity for PEP but not fructose-1,6-bisphosphate (Rafter, G.W., & Blair, J.B., 1987), which would indicate that these agents inhibit PK's ability to catalyze a reaction. These oxidants could affect the less reactive but catalytically necessary cysteines in PK. If an ROS is present, either after the reaction occurs or as part of the incubation mixture, it is likely that fructose-1,6-bisphosphate can bind in the allosteric site and counteract loss of function by preventing conformational changes to the β-barrel that render the enzyme unable to catalyze reactions (Anastasious, D., *et al*., 2011; Rafter, G.W., & Blair, J.B., 1987). Oxidative damage to these minimally reactive cysteines impedes catalytic function.

The highly reactive cysteines are more accessible to oxidants. Based on the same previously referenced 2011 study, it is possible that these highly reactive cysteines are either Cys30 or Cys422, both of which are highly accessible by the solvent (Anastasious, D., *et al*.,

2011). There is most likely a cysteine in the fructose-1,6-bisphosphate binding site that is not shielded by the cations necessary for PK's function (Brown, C.E., Taylor, J.M., & Chan, L., 1985). While the cysteine in this binding site is found in PK isoforms sensitive to regulation by fructose-1,6-bisphosphate, it is not conserved to the extent of the cysteine in the PEP binding site. Because the cysteines in the fructose-1,6-bisphosphate binding site are accessible and unprotected, they are likely the more reactive but non-essential cysteines. The increased reactivity is likely due to the more exposed location of the cysteine in the fructose-1,6-bisphosphate regulatory site, which, combined with the fact that the sequence tends to be less conserved than that of the PEP binding site, would make sense for the cysteine in the regulatory site to have decreased selective pressure since the residue is not critical to enzymatic function (Poole, L.B., 2015). This idea is further supported by research illustrating that thiol reagents do not affect the fructose-1,6-bisphosphate affinity (Rafter, G.W., & Blair, J.B., 1987). Although the cysteines are modified, there is only an alteration of the enzyme's kinetic properties; the catalytic ability of the enzyme is not impaired (Rafter, G.W., & Blair, J.B., 1987). Thus, the fructose-1,6-bisphosphate binding site likely has the non-essential cysteine. Oxidation of these highly reactive cysteine residues does not affect PK's function.

When oxidized, the cysteines in PK are likely to form disulfide bridges, and the resulting change in structure affects the enzyme's function. In order to successfully modify the catalytically essential cysteines in the PEP binding site, ions cannot be present in the buffer. The potential protective effects could shield the cysteines from oxidative damage, which would lead to unreliable results on enzyme catalysis. It is possible that the oxidation of PK's cysteines could be reversed via reduction processes, which could further restore the enzyme's function. Preliminary studies seem to illustrate that PK's activity can be modulated by cellular redox

conditions; however, the oxidative damage to the enzyme was most likely minimal during the experiments because PK was reacted with oxidants in the presence of cations, which could confound the results (Lain, T.K., 2015). Further experiments will be necessary to understand the full extent of the enzyme's self-regulation and the effects of this capability on other molecules in the cell. If PK is able to prevent oxidative damage by shielding itself through interactions with cations, it may be possible that the enzyme can buffer oxidative damage to other intracellularly targeted molecules. Damage to these molecules, such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tubulin, could have long-lasting cellular effects including neurodegenerative diseases like Alzheimer's and Parkinson's diseases (Landino, L.M., Hagedorn, T., & Kennett, K.L., 2014). Based on the pI values of PK, GAPDH, and tubulin, it is likely that these molecules interact electrostatically, which further illustrates the importance of PK's potential ability to modulate oxidative environments (Landino, L.M., Hagedorn, T., & Kennett, K.L., 2014). Therefore, research on pyruvate kinase and oxidative stress could have important results for understanding cellular regulation.

F. <u>Goals of this Research:</u>

   Taken altogether, pyruvate kinase is an important enzyme in metabolism that warrants further study. Because glycolytic enzymes are ubiquitous *in vivo* and requires reduced cysteines for proper function, thiol modification in response to oxidative stress could have dramatic effects on enzymatic activity. Therefore, it is possible for extensive oxidative damage over time to decrease energy metabolism, induce protein aggregation, and have other negative intracellular consequences associated with neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.

The primary aim of this thesis was to develop a functional protocol for an assay that would give a reliable measure of PK activity for the purposes of analyzing the effects of oxidation. Drawing from assays used by a previous student in the laboratory of Dr. Landino, this protocol sought to control for the potential protective effects of the monovalent and divalent cations, the variability from small sample preparations, and the kinetic rate of lactate dehydrogenase during the coupled assay. By having an established methodology in place, it was possible to investigate PK activity in response to various modifications.

Once the protocol was shown to be consistent, the goals of this thesis expanded to gain a larger understanding of PK in response to oxidative stress. To what extent does oxidation target cysteines? How does PK activity after treatment with Ellman's reagent, a thiol-specific reagent, compare to that after treatment with hypochlorous acid (HOCl), a non-specific oxidant? Do ions protect PK from oxidation, and, if so, to what extent? How does the presence of ligands, such as adenosine triphosphate and fructose-1,6-bisphosphate, affect oxidative damage if present throughout oxidation reactions? Do these molecules give insight into the location of key cysteines in PK?

Because of the association between glycolytic enzymes, microtubules, and oxidative stress, understanding how oxidation affects pyruvate kinase could help researchers better understand early cellular changes in the complex progression of neurodegenerative diseases. As oxidized PK has a reduction in energy metabolism, potential conformational changes from disulfide linkages could alter interactions with tubulin in the microtubule network that might accelerate the formation of tau conglomerates, called neurofibrillary tangles, a characteristic of Alzheimer's disease. Therefore, while the goals of this research are narrow in scope, the results could help add to the larger picture of neurodegeneration.

PART II: METHODS AND MATERIALS:

A. <u>Materials:</u>

Pyruvate kinase from rabbit muscle (PK-M2), HOCl, 5-(Iodoacetamido)fluorescein (IAF),

disodium salt of ATP, trisodium salt of PEP, and 5,5′-Dithiobis(2-nitrobenzoic acid) (DTNB)

were obtained from Sigma-Aldrich. Guanidine hydrochloride, ammonium persulfate, and

TEMED were purchased from Fisher Scientific. FBP was purchased from Alfa Aesar. Ethyl

alcohol was obtained from Pharmco-Aaper.

B. <u>Pyruvate Kinase Purification:</u>

The commercially available rabbit muscle PKM2 samples in the laboratory came in two

varieties: type II and type III. Very little literature could be found on the differences in these

products, but activity assays determined the enzymes to have parallel activity. It is likely that the

only difference in these products is the preparation method. Type II is found in an ammonium

sulfate solution, and type III is a solid lyophilized powder. To control for possible air oxidation

of PK and to ensure all of the enzyme in the sample was fully reduced, PK was treated with DTT

to reduce any disulfides present in the commercial products. Then, to remove the DTT and

collect the reduced enzyme, samples were run on a desalting column. Using absorbance

measurements ($E_{280}^{1\%}$=5.4), the final concentrations of the reduced enzymes were calculated as 1.3

mg/mL and 1.64 mg/mL for PK type II and type III, respectively.

C. <u>Coupled Lactate Dehydrogenase Activity Assays:</u>

This assay design is broken into three stages to streamline the process and to reduce error

as illustrated in Figure 5 below. A major portion of this project was to develop a working assay

to measure pyruvate kinase activity through nicotinamide adenine dinucleotide (NADH) absorbance, and more complete details on this process and each stage can be found in the "Data and Results" section.
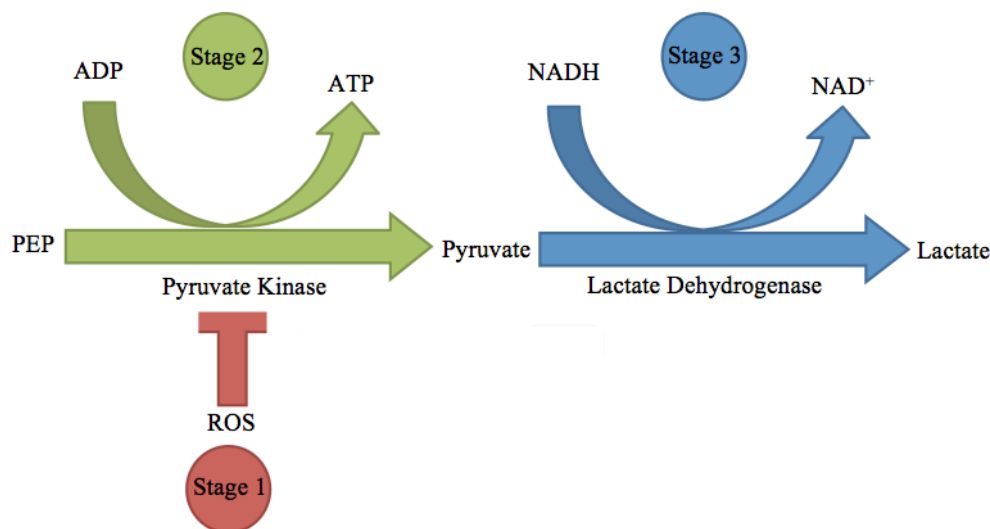

Figure 5: Graphical representation of the stepwise approach to the coupled LDH activity assay

The first stage of the assay oxidized pyruvate kinase in 0.1 mM phosphate buffer pH 7.4 to give a final solution volume of 15 µL. PK was added to give a final concentration of 0.43 mg/mL or 7.5 µM in solution. With each subunit having nine cysteines, the maximum concentration of thiols available for modification would be 67.9 µM, but it is unlikely that all could be reached because many are found in the interior of the protein. Reactions proceeded following the outlined procedure in Table 2. After the addition of the oxidant, the reaction was preceded to react for 10 minutes at room temperature. Modifiers, including hypochlorous acid and Ellman's Reagent (DTNB) were added to give a final concentration of 0, 25, 50, 100, or 250 µM. The concentrations were chosen because they gave simple factors for the ratio of cysteines to oxidant shown in Table 2. As the ratio shifts in favor of the oxidant, it becomes more likely that an oxidant could come in contact with a thiol and modify the residue.

Concentration of Oxidant	0.1 mM PB 7.4	1.3 mg/mL PK	HOCl	Cys:Oxidant
Control	10 µL	5 µL	0 µL	--
25 µM HOCl final	6.25 µL	5 µL	3.75 µL 100 µM	1:10
50 µM HOCl final	8.5 µL	5 µL	1.5 µL 500 µM	1:20
100 µM HOCl final	7 µL	5 µL	3 µL 500 µM	1:40
250 µM HOCl final	5 µL	5 µL	5 µL 750 µM	1:100

Table 2: Oxidation conditions for various samples of hypochlorous acid

Next, the volume of the Stage 1 solution was added to 240 µL of an equimolar Stage 2 solution containing 3 mM phosphoenolpyruvate (PEP) and adenosine diphosphate (ADP). This reaction proceeded for 5 minutes at room temperature. At the end of the 5 minutes, 15 µL $ZnCl_2$ was added to inhibit PK and stop the reaction. This ensured no further reaction products formed.

Lastly, the Stage 2 solution was divided into 50 µL portions in each of 5 wells of a 96-well plate. Each well also contained 150 µL of the Stage 3 solution: final concentrations of 0.5 mM nicotinamide adenine dinucleotide (NADH) and 0.0055 µg/µL lactate dehydrogenase in STris buffer, 50 mM Tris buffer with final concentrations of 100 mM KCl and 10 mM $MgSO_4$. To reduce error, the Stage 3 solution was mixed in sufficient volume for 10 wells then divided. The assay conditions for each individual well are summarized in Table 3 below. The plate was read both before and after the addition of the Stage 2 solution on the BioTek ELx808 absorbance plate reader in order to calculate the percent change in NADH absorbance at 340 nm. Data for each reaction condition was collected from a minimum of three runs with a run consisting of 5 wells of the 96-well plate.

Stage	Reagent	Blank	Control	Sample	Final Concentration
1	PK Solution	0 µL	0 µL	3 µL	0.0075 mM (7.5 µM)
2	3 mM ADP/PEP	0 µL	0 µL	44 µL	0.66 mM
2	20 mM Zn^{2+}	0 µL	0 µL	3 µL	0.03 mM
3	1:5 LDH	0 µL	0 µL	2 µL	0.039 µM
3	10 mM NADH	0 µL	10 µL	10 µL	0.5 mM
3	STris Buffer	200 µL	190 µL	138 µL	---------

Table 3: Assay conditions for each 200 µL well in a 96-well plate

D. <u>Visualization of IAF Tagging by SDS-PAGE:</u>

In order to visualize how oxidation affects the cysteines in pyruvate kinase, samples were tagged with 5-iodoacetamidofluorescein, a fluorescent yellow tag that binds to reduced cysteines. Samples were prepared in the same 15 µL volumes as those used for the coupled lactate dehydrogenase activity assays. Next, 0.9 µL 25 mM IAF was added to each sample (for a final concentration of 1.4 mM IAF) and incubated for 30 minutes to allow the reaction to occur. After incubation, 16 µL of SB (+) was added to each sample, and the total volume was loaded onto a 7.5% SDS-polyacrylamide gel. The gel ran for 90 minutes at 90 V before visualization with the BIO-RAD ChemiDoc XRS+ imaging system.

PART III: DATA AND DISCUSSION:

A. <u>Lactate Dehydrogenase Coupled Assay Development:</u>

The efficiency of the reaction catalyzed by PK cannot be measured directly, so this research relied on a pyruvate kinase-lactate dehydrogenase (LDH) coupled enzyme assay in order to measure PK activity quantitatively. PK converts adenosine diphosphate (ADP) and phosphoenolpyruvate (PEP) into pyruvate and adenosine triphosphate (ATP). The pyruvate produced from the reaction becomes the substrate for lactate dehydrogenase that reduces pyruvate into lactate while oxidizing nicotinamide adenine dinucleotide (NADH). Because NADH absorbs at 340 nm and the oxidized NAD^+ does not, measuring the percent change in absorbance at 340 nm gives a quantitative measure of the efficiency of the PK under different physiological conditions. While previous studies have measured the kinetic rate of the NADH oxidation reaction to determine the effects of oxidation, this form of data collection could be

confounded based on the LDH reaction rate in the coupling. Measuring the percent change in NADH absorbance from the initial and final absorbance readings allowed for a control of the LDH reaction since kinetic rates for the two enzymes were no longer necessary. Although the reactions are coupled, treating each reaction as an independent step streamlined the process and decreased the chance for error. Solutions were made in larger volumes then distributed throughout the wells in a 96-well plate, which reduced the chance for error. The assays were divided into three sequential stages schematically illustrated in Figure 6: 1) PK modification, 2) PK catalysis, and 3) LDH catalysis.
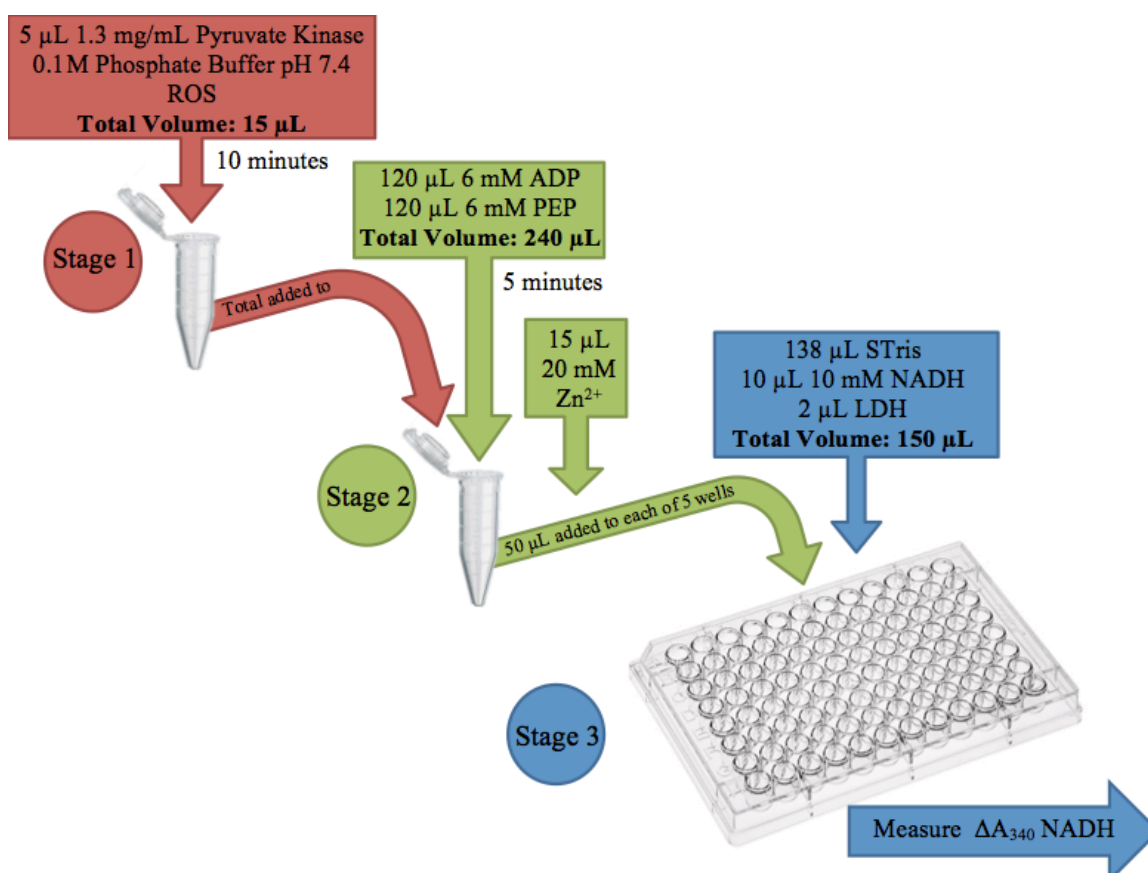

Figure 6: Outline of developed protocol for pyruvate kinase activity assay

Stage 1:

First, PK must be reacted with a physiologically relevant oxidant in 0.1 M phosphate buffer at pH 7.4 lacking ions. Stage 1 occurred at room temperature for 10 minutes at a final volume of 15 µL. Because phosphate is fully oxidized, the oxidant could only react with the enzyme and not with the buffer. Hypochlorous acid was added under varying final concentrations (0-250 µM) to analyze the extent of general oxidative damage on the protein. For comparison, pyruvate kinase was treated with Ellman's Reagent (5,5'-dithio-bis-[2-nitrobenzoic acid]), a cysteine-specific reagent, to observe the changes in activity against the non-specific interactions of hypochlorous acid. Based on a previous student's research in the lab, hydrogen peroxide, a common antioxidant under physiological conditions, did not oxidize PK to enough of a degree to warrant further focus. For this reason, hydrogen peroxide was not used as an oxidant in this project. Later in the research, various chemicals that act as reducing agents were added to the oxidized PK in order to determine if oxidative damage could be reversed under appropriate conditions.

Researchers have debated the role ions (Mg^{2+} and K^+) play in oxidative stress. While some research indicates that the ions serve as a protective mechanism for cysteines critical for PK function and serve as a defense against oxidative stress, other research seems to indicate that ions hold the protein in a conformation that makes cysteines more accessible for oxidants to react. Because more research seems to indicate protective effects of ions, Stage 1 of the assay excluded Mg^{2+} and K^+ but also examined the effects of the ions in further experiments. Reacting PK with oxidants without ions was thought to give a better sense of the capabilities of the oxidants.

Stage 2:

 The next stage of the assay included the PK reaction. In order to produce pyruvate, the 15 µL of the modified PK from Stage 1 was added to 240 µL of a 1.6 mM 1:1 solution of PEP:ADP in 50 mM Tris buffer with final concentrations of 100 mM KCl and 10 mM $MgSO_4$. The buffer with ions, termed "STris," was made from a 1:1 dilution of 0.1 M Tris buffer in deionized water with salts added to achieve the stated final concentrations. STris follows the ion concentration of other prominent research with PK and is necessary because the enzyme requires these ions in order to produce pyruvate. Experimentation illustrated that these ion concentrations were sufficient for PK activity and did not limit functionality of PK. Initial testing was focused on determining controls for reduced conditions: 1) the concentration of PK needed for the reaction to occur, 2) the time the reaction should run before PK has produced all of the pyruvate it can with the given reagent concentrations, and 3) the method of inhibition to provide a mechanism of control.

 When determining the concentration of PK to use in each sample, the main consideration was the final absorbance at 340 nm. An absorbance measurement below 0.1 has questionable reliability due to the detection limits of the spectrophotometer. For that reason, the concentration of PK for the unmodified enzymatic control needed to reduce the NADH absorbance to about 0.100 but not below – approximately a 90% decrease. A final concentration of 0.0065 µg/µL PK in the 200 µL well solution provided the required percent change.

 A system of trial and error was necessary with regard to the time for the reaction to take place. Using an estimated five-minute timeframe, the control reaction saw a 90% decrease in NADH absorbance, which indicated that the reaction was able to produce enough pyruvate for the LDH-coupled reaction to occur within the desired range.

At the end of the five-minute reaction period, there needed to be a mechanism to inhibit

further PK activity to control for the time PK is able to react with its substrates. This ensured that

the reaction was not continuing to produce pyruvate while the LDH assay and data collection

period was underway, which could confound the results by increasing the amount of NADH

oxidation possible due to a longer time to react. Zinc ions have been shown to inhibit this

isoform (M2) of PK by competing with Mg^{2+} in the active site. While the literature suggests that

a final zinc concentration of 0.1 mM in each 200 μL well sample of the 96-well plate would be

sufficient to inhibit PK activity, this research indicates that 0.1 mM Zn^{2+} only inhibited activity

by 19% of the uninhibited control (Tamaki, N., Ikeda, T., Kimura, K., & Morioka, S., 1981). At

this concentration, PK activity was decreased to approximately 80% of the control as

summarized in Figure 7. Although lowered from the initial 10 mM $MgSO_4$ previously contained

in STris from a previous student's research on the topic, it is likely that the 2.5 mM magnesium

ion concentrations used in this STris buffer were high enough in outcompete the zinc ions in the

ADP/ATP binding site in order to maintain enzymatic activity. 2.5 mM was chosen after

examining another paper that used PK-LDH coupled assays with great success at this

concentration of ion (Tamaki, N., Ikeda, T., Kimura, K., & Morioka, S., 1981). For this reason,

additional Zn^{2+} was added to give a final concentration of 0.3 mM in the 200 μL well volume (or

1.1 mM in the reaction mixture with only ADP, PEP, and PK before adding the solution to the

LDH-coupled reaction mixture). Experimentation showed that a final concentration of 0.3 mM

$ZnCl_2$ decreased activity by 44% and decreased the percent change in NADH absorbance from

92% (0 mM Zn^{2+}) to 48% - approximately 52% of the control. From the 20 mM $ZnCl_2$ stock

solution, a final zinc ion concentration of 0.1 mM required 1 μL per 200 μL well while that of

0.3 mM required 3 μL per 200 μL well. Perhaps the concentration of $ZnCl_2$ could have been

increased in an effort to obtain near complete inhibition of PK; however, the degree of inhibition had to be weighed against the volume added since it diluted the pyruvate produced necessary for the LDH coupled assay in Stage 3. Further, data over the length of the LDH kinetics run showed that 0.3 mM Zn Cl$_2$ inhibited sufficiently to maintain a consistent final NADH absorbance for each reaction. Therefore, it was decided that 0.3 mM ZnCl$_2$ met the needs 15 μL of 20 mM Zn^{2+} was added to each 255 μL Stage 2 solution after the 5 minute reaction time before distributing the solution into the wells. Once the final concentration of pyruvate has been reached, the assay can proceed to Stage 3.
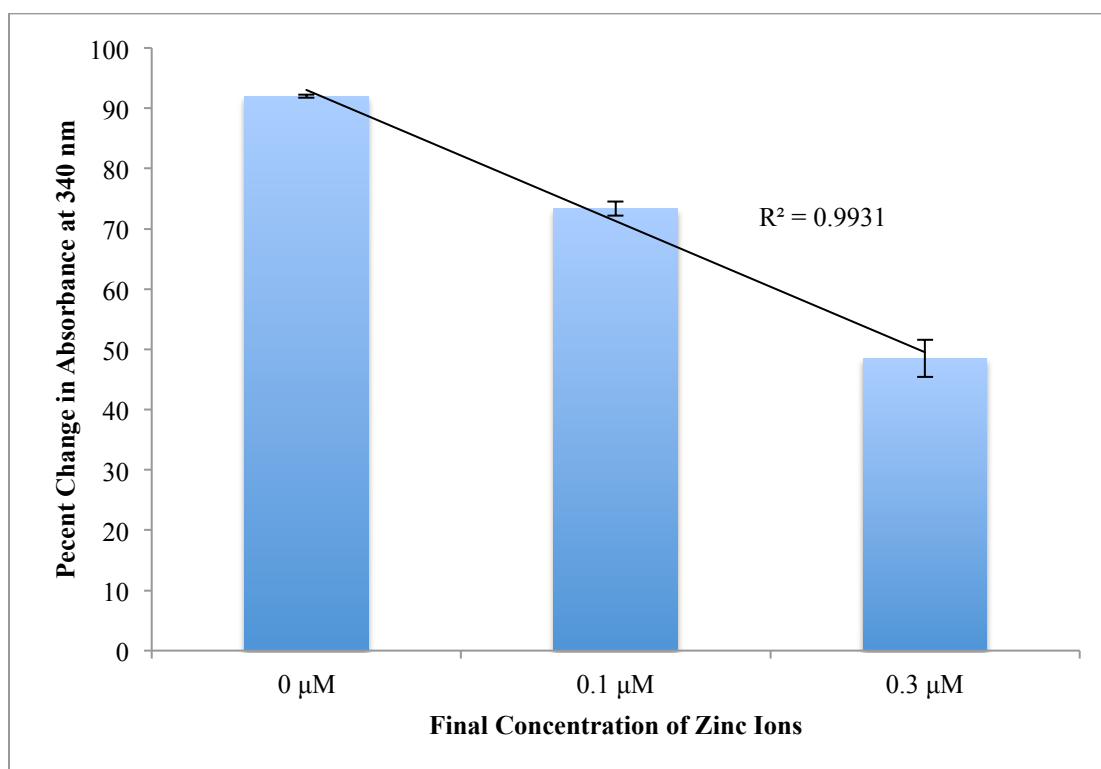

Figure 7: Inhibition of pyruvate kinase activity at various concentrations of Zn^{2+}

Stage 3:

The final stage for the assay revolved around the LDH-catalyzed reaction. In order to analyze the effects of PK modification on its activity, there needed to be a way to analyze the change in concentration of NADH as the coupled reactant is oxidized to NAD+ by LDH. From

Stage 2, the maximum concentration of pyruvate that could have been produced from the reagents was 1.6 mM. Adding 50 µL of this solution to sample well with 150 µL of a pre-mixed solution of LDH, NADH, and STris distributed throughout the sample wells gave a maximum final concentration of pyruvate of 0.4 mM. In order for the effects to be attributed to PK modification rather than mere NADH oxidation by exposure to air, the concentration of pyruvate must be less than that of NADH in the final 200 µL volume. An initial NADH concentration of 0.5 mM gave a starting absorbance (A_{340i}) between 1.5 and 2. It is worth mentioning that the sample wells did not contain the full 200 µL volume when measuring the initial absorbance because the 50 µL of the PEP/ADP/PK solution had not been added. Controlling for this volume, an increase of 50 µL from STris increased the absorbance by about 5%. Therefore, a decrease in NADH absorbance is not due simply to the change in volume. Furthermore, this increase is unlikely to cause a significant change in the quantitative data because it modified percent change in absorbance by less than 1%. By measuring the absorbance of NADH, it is possible to determine the amount of pyruvate produced by modified PK compared to the reduced standard. Similarly to the first step of the coupled reaction, initial experimentation determined the controls: 1) the concentration of LDH necessary for conversion of pyruvate to lactate and 2) the time the reaction should run before all of the NADH has been oxidized.

For the reaction to be successful, LDH needed to be added in excess such that the resulting concentration of pyruvate is the limiting factor rather than the enzymatic rate. Initially, each 200 µL well solution contained 0.00275 µg/µL LDH. The percent decrease in NADH absorbance for the unmodified PK control was approximately 40%. Since the ideal change was closer to 90%, the LDH concentration in each well was doubled to 0.0055 µg/µL. Results of the

updated protocol matched the intended values, which illustrated the necessity of altering the protein amount for catalysis.

Based on the results of absorbance measurements over the span of an hour for the unmodified PK control, all of the NADH is oxidized within seven minutes of adding the Stage 2 solution, which is why the plate needs to be analyzed by the absorbance reader within two minutes of the first sample being added to the well in order to have comparable results across samples at the end of the five minute LDH-coupled activity experiment. This limitation restricted the number of samples per run to two, each of which provided enough solution for five wells.

B. Modification by Ellman's Reagent:

To determine the extent to which an oxidant in solution could access the cysteines in pyruvate kinase, Ellman's Reagent (5,5'-dithio-bis-[2-nitrobenzoic acid]), or DNTB, was used as a control. DTNB is a thiol-specific agent due to its reactive thiol group that can undergo thiol-disulfide exchange to modify its target. In this case, DTNB modified cysteines and likely induced some disulfide linkages between cysteines on the same subunit or on different subunits, which has the potential to cause conformational change that affects enzymatic activity. Through these modifications of the cysteine residues, it was possible to determine that some of the nine cysteines in each PK subunit are accessible for modification, as well as the effect of these modifications on PK function. Additionally, the effects of cations present in solution during modification was examined to see if the ions protect the enzyme from modification as potentially indicated in the research (Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972).

Visualization with IAF Labeling:

In an effort to determine the extent to which cysteines are accessible for modification, PK samples were incubated with different final concentrations of DTNB (0, 440, 590, and 880 μM) in the Stage 1 modification step for 10 minutes at room temperature. After this reaction window, the samples were tagged with IAF and visualized on the SDS-PAGE shown in Figure 8. As the concentration of DTNB increased, the extent of IAF labeling decreased. This is logical because thiol-disulfide exchange between DTNB and the thiol group of PK's cysteines likely formed disulfide bonds between residues that decreased the number of free thiols available for the fluorescent tag. These results indicate that cysteines can be modified, but further analysis was necessary to understand the effects of these modifications on enzymatic activity.
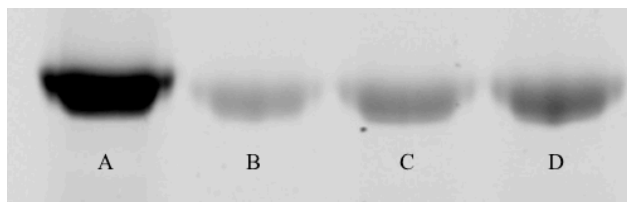

Figure 8: Visualization of pyruvate kinase oxidation by varying final concentrations of DTNB with IAF labeling
Lane A: 0 μM Lane B: 880 μM Lane C: 590 μM Lane D: 440 μM
Figure courtesy of Duff, M.C. & Landino, L.M. (2017).

Effects on Enzymatic Activity:

In order to show that modification by DTNB affected PK activity, samples with a final concentration of 0 and 200 μM DTNB in phosphate buffer without ions were analyzed with activity assays as shown in Figure 9. The control (0 μM DTNB) saw a percent decrease in NADH absorbance at 340 nm of 92% with a spread of 0%. At 200 μM DTNB, activity was impacted such that the percent change in absorbance was only a loss of 69% ranging from a decrease of 64-74%. At least some of the cysteines, then, are necessary for proper enzymatic

function. This information was necessary to understand the effects an oxidant could have on the

enzyme and provided an important control for treatment with HOCl.

Ion Effects:

Since research seemed to indicate the possibility of cations (Mg^{2+} and K^+) serving as a

defense mechanism against damage to the protein, exposure to DNTB in the presence of these

ions could decrease the extent of thiol-disulfide exchange to reduce the loss of function

experienced by the ion-free 200 µM DTNB sample (Flashner, M., Hollenberg, P.F., & Coon,

M.J., 1972). Instead of the 100 mM phosphate buffer pH 7.4 used in the Stage 1 modification,

samples were reacted in SPBS (50 mM phosphate buffer pH 7.4 with 100 mM KCl and 10 mM

$MgSO_4$). Activity assay controls did not show a difference in enzymatic function between the

different buffers.

Again, the PK samples were run with 0 and 200 µM DTNB, and the results are presented

in Figure 9 below. Interestingly, the presence of ions in solution actually increased the loss of

function to the enzyme. No protective effects were observed with modification by DTNB. As

compared to the PBS control at the same level of modification by DTNB that saw a percent

decrease in absorbance at 340 nm of 69%, the SPBS samples decreased absorbance by 51% over

a span of 42-60%. After additional research, it appears that divalent metal ions (i.e. Mg^{2+})

increased DTNB modification of thiol groups in rabbit muscle PK-M2 by inducing different

conformational changes that allow for a greater extent of thiol-disulfide exchange that mimics

allosteric conformational changes (Kwan, C.Y. & Davis, R.C. 1981). Therefore, the ions in

solution actually serve to increase damage from DTNB modification rather than to protect as is

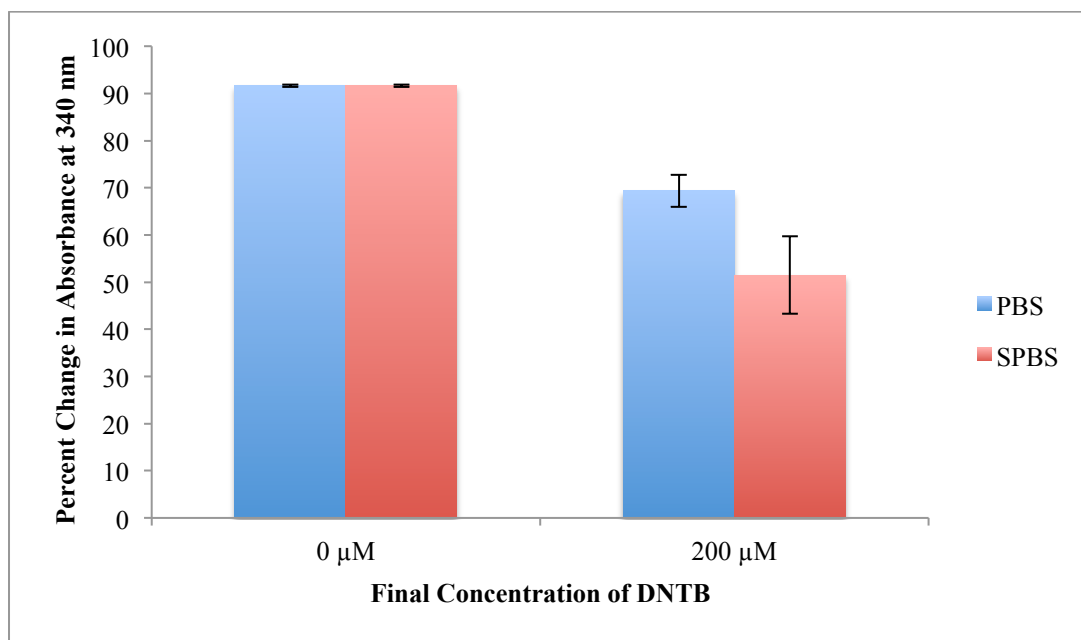the case in literature with exposure to an ROS.

Figure 9: Changes in pyruvate kinase activity at various final concentrations of DTNB in the presence or absence of cations
PBS: *100 mM pH 7.4* **SPBS:** *50 mM PB pH 7.4 50 mM PB pH 7.4 w/ 100 mM KCl + 10 mM MgSO$_4$*

C. Oxidation by Hypochlorous Acid:

While DTNB is a great control to show that oxidation of pyruvate kinase's cysteines

inhibits activity, oxidation in physiological systems is likely to be less specific and have the

potential to affect activity to a larger extent because of the broader range of possible targets. In

order to replicate cellular oxidative conditions, hypochlorous acid (HOCl) was chosen as a

physiologically relevant oxidant for studies on PK activity. In addition to HOCl produced in

other contexts, a 2004 paper reported uncharacteristically high levels of HOCl generated from

increased expression of the enzyme myeloperoxidase (MPO) in neurons in the brain of patients

with Alzheimer's disease (Green, P.S. *et al*. 2004). In an effort to understand how exposure to

HOCl affects PK function, enzyme samples with exposure to increasing concentrations of the

oxidant were visualized with IAF labeling and analyzed with an activity assay (see Table 2 for

experimental conditions). Similarly to studies with DTNB, the effects of cations present in

solution during oxidation were examined in order to determine whether the ions provide a degree

of protection potentially indicated by the research (Flashner, M., Hollenberg, P.F., & Coon, M.J.,

1972).

Visualization with IAF Labeling:

As illustrated in Figure 10, IAF labeling of pyruvate kinase generally decreases as the

concentration of hypochlorous acid increases from the control of 0 µM to the highest treatment

group of 250 µM. Because IAF binds only to free thiols, these results imply that increasing

oxidation decreases the number of free thiols either by oxidizing some of the thiol groups to

sulfoxides, forming disulfide bonds between some cysteines, or changing the conformation of

PK such that fewer cysteines are exposed for tagging in the first place. Interestingly, it is possible

that the PK treated with 50 µM HOCl could have slightly increased IAF labeling when compared

to 25 µM HOCl but still less than the control. This could be due to a conformational change that

exposed another cysteine not previously accessible to the fluorescent tag. From this SDS-PAGE,

it is unclear how thiol availability affects PK activity; however, the image illustrates that HOCl

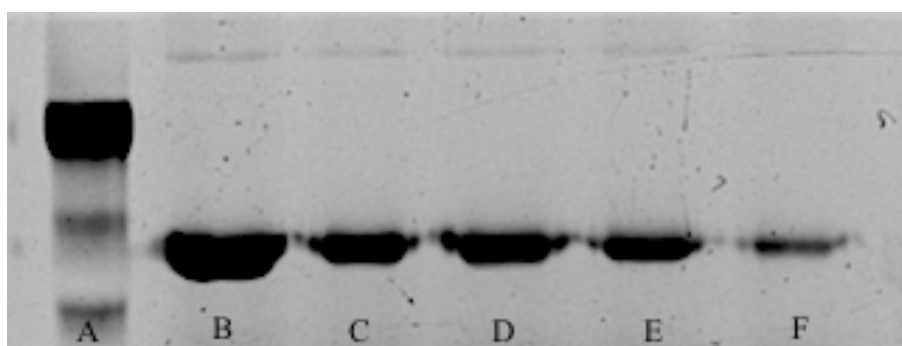oxidizes the protein in a concentration-dependent manner.

Figure 10: Visualization of pyruvate kinase oxidation by varying concentrations of HOCl with IAF labeling
Lane A: Molecular Weight Standard Lane B: 0 µM HOCl Lane C: 25 µM HOCl
Lane D: 50 µM HOCl Lane E: 100 µM HOCl Lane F: 250 µM HOCl

Effects on Enzymatic Activity:

In an effort to understand how HOCl oxidation alters pyruvate kinase, samples of the enzyme treated with a final concentration of 0, 25, 50, 100, and 250 µM HOCl were run with the activity assay. The results are summarized in Figure 11 below. Low concentrations (0-50 µM HOCl) did not affect activity and consistently saw an average percent decrease in NADH absorbance of 92% with a range of 87-92%. As the concentration of HOCl increased to 100 µM, the oxidative damage started altering activity for an average percent decrease of 75% with values ranging from 71-79%. Oxidation with a final concentration of 250 µM HOCl decreased the percent change in NADH absorbance to 49% with a range of 45-57%. As the amount of oxidation increased, the spread seemed to widen. It is possible that assay could benefit in more precise averages from a quenching step added after the ROS reacts with PK. However, the range was not so wide as to warrant the additional risk for error inherent in adding further steps to the reaction protocol. From the period over 50-250 µM HOCl, the percent decrease in NADH absorbance occurred in a linear fashion (R^2=0.9881), which correlates with the concentration-dependence seen from the visualization of HOCl oxidation with IAF labeling. From this data, oxidative damage from high concentrations of HOCl clearly inhibits PK activity.

Hypochlorous acid is not a cysteine-specific oxidant. Because treatment with DTNB showed that modifications to PK's cysteines decreased activity, it is likely that the damage from treatment with HOCl is due at least in part to cysteine oxidation. Furthermore, because the percent decrease in NADH absorbance (340 nm) is approximately equivalent between 200 µM DTNB and 250 µM HOCl, the chances of oxidative damage to other amino acids as the major culprit for enzymatic inhibition are low. Therefore, modifications to cysteines are probably the leading cause of inhibition to PK following exposure to oxidation by HOCl.
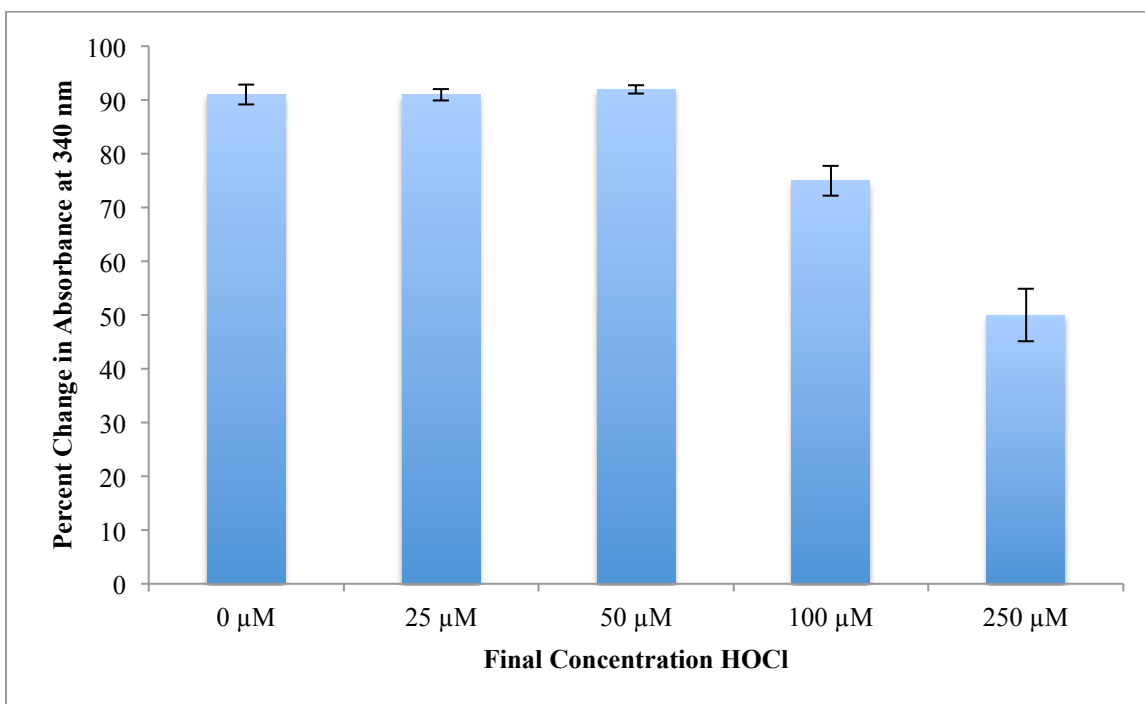
Figure 11:Changes in pyruvate kinase activity as the concentration of HOCl increases

While these results do not definitively illustrate a change in conformation, it is likely that there is some change to the shape of the protein as the concentration of the oxidant increases since function is dependent on structure. The decrease in enzymatic activity in the presence of higher concentrations of HOCl implies the formation of sulfoxides and/or disulfide linkages between or within proteins to at least some cysteines. Further research with tryptophan fluorescence and circular dichroism spectroscopy is necessary to observe the changes in PK's shape and would add to the current understanding of the effects of oxidative damage.

Ions Effects:

Due to literature implicating the monovalent and divalent cations necessary for pyruvate kinase activity (Mg^{2+} and K^+) in protecting the enzyme from oxidative damage, Stage 1 oxidation experiments were carried out in phosphate buffer both with and without ions in order to visualize any effects and determine the extent of the protection on activity (Flashner, M.,

Hollenberg, P.F., & Coon, M.J., 1972). Following the buffer solutions used previously in the lab

for PK oxidation studies, samples with ions were reacted in SPBS (50 mM PB pH 7.4 with 100

mM KCl and 10 mM $MgSO_4$). Samples without ions were reacted in PBS (100 mM PB pH 7.4).

A control for the difference in phosphate buffer concentration showed no disparity in IAF

labeling or enzymatic activity.

The presence or absence of ions drastically affects labeling with IAF as visualized from

the SDS-PAGE illustrated in Figure 12. While tagging continued to decrease as the final

concentration of HOCl increased over 0, 50, 100, and 250 μM regardless of the buffer solution

used (Lanes A-D: PBS; Lanes E-H: SPBS), ions reduced the number of available thiols,

presumably by blocking the cysteines from interactions. This finding is important because ions

are present in normal, cellular conditions, and such stark differences could imply a cellular

impediment against protein damage and aggregation under oxidative stress that could prolong the

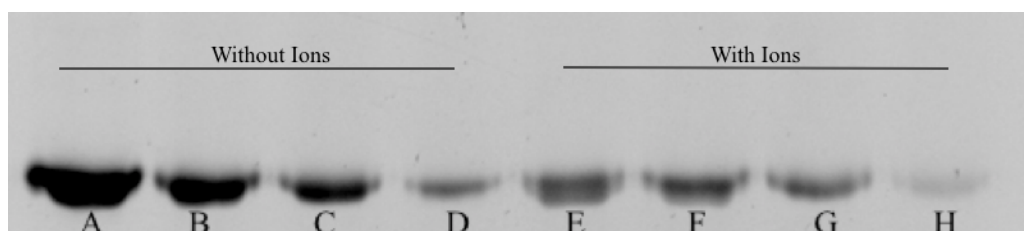maintenance of homeostasis as the concentration of an ROS increases.

Figure 12: Visualization of the effect of cations present in solution at various concentrations of HOCl on
IAF labeling of pyruvate kinase
Lanes A-D: *Reacted in 100 mM PB pH 7.4*
Lanes E-H: *Reacted in 50 mM PB pH 7.4 w/ 100 mM KCl + 10 mM MgSO₄*
Lane A/E: *0 μM HOCl* **Lane B/F:** *50 μM HOCl* **Lane C/G:** *100 μM HOCl* **Lane D/H:** *250 μM HOCl*

Based on the results from IAF labeling with or without ions present for PK oxidation, one

would assume that the activity saw similar protection; the ions would prevent HOCl from

interacting with PK's cysteines and effectually maintain enzymatic function when compared

with the samples reacted in the absence of ions. Unfortunately, this was not the case. After

countless trials, the activity assays did not indicate any change in activity when ions were present during oxidation from the data collected without ions. Nonetheless, it is apparent that the ions reduce accessibility of cysteines, which could have further implications for PK outside of its activity potentially including interactions with other molecules *in vivo*.

D. <u>Effects of Adenosine Triphosphate on Oxidation:</u>

In the active site of pyruvate kinase, the phosphate group on PEP is transferred to ADP, which produces ATP and pyruvate. The cations in the binding site (Mg^{2+} and K^+) stabilize the high-energy intermediate to facilitate this transfer. When the cell has an energetic excess, ATP, the molecule that indicates the energy available in the cell, inhibits PK function since there is not a need for more energy production at that moment. From the literature, it is likely that there is a critical cysteine in the active site that is necessary for function (Anastasious, D., *et al.*, 2011; Brown, C.E., Taylor, J.M., & Chan, L., 1985; Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972; Poole, L.B., 2015). Since activity assays require ADP as a substrate, testing this hypothesis required low concentrations of ATP to understand the effects of blocking the active site from the full extent of exposure to an oxidant. As an additional step to the Stage 1 PK oxidation step of the assay design, PK was incubated with a 75 μM final concentration of ATP for 5 minutes at room temperature before the addition of HOCl at various concentrations to bring the sample to the 15 μL final volume. These samples were visualized with IAF labeling and analyzed catalytically through LDH-coupled assays to understand the enzymatic significance of oxidative damage to the active site.

Visualization with IAF Labeling:

Samples both with and without pre-incubation with ATP were tagged with IAF and run on the visualized SDS-PAGE in Figure 13 below. The presence of ATP appears to have similar yet slightly decreased labeling of cysteines when compared to the corresponding oxidation concentrations (0, 50, 100 µM) without exposure to ATP. This decrease is subtle but of note because it indicates that ATP decreases access of other molecules to the active site.
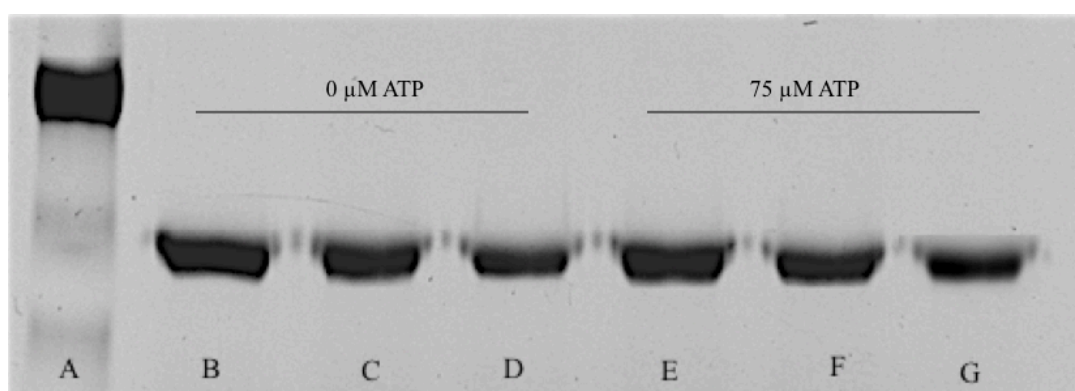

Figure 13: The effect of adenosine triphosphate present in solution at various concentrations of HOCl on IAF labeling of pyruvate kinase
Lane A: *Molecular Weight Standard*
Lanes B-D: *Reacted with 0 µM ATP* **Lanes E-G:** *Reacted with 75 µM ATP final concentration*
Lanes B/E: *0 µM HOCl* **Lanes C/F:** *50 µM HOCl* **Lanes D/G:** *100 µM HOCl*

Effects on Enzymatic Activity:

In order to understand the effects ATP on PK oxidation, samples with and without the 75 µM final concentration of ATP were run on activity assays. Testing showed that this concentration of ATP was too low to exhibit inhibitory effects on the enzyme but still high enough to bind in some active sites and potentially restrict access of the oxidant to the cysteines of interest. As illustrated in Figure 14, the presence of ATP in solution served a protective role on the enzymatic function such that the extent of oxidative damage was less when incubated with ATP. In fact, the presence of ATP completely maintained function at 100 µM HOCl despite a partial loss of catalytic ability for the control.
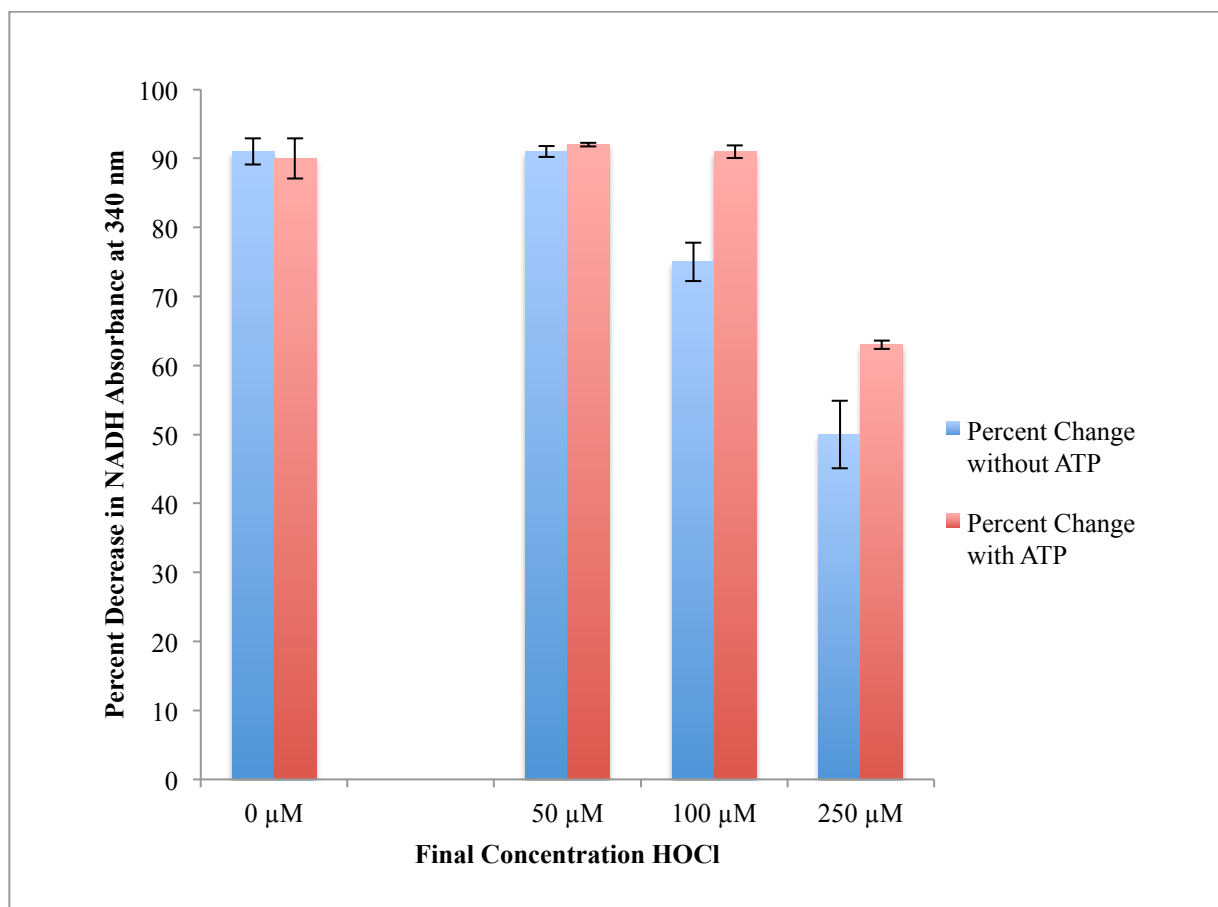
Figure 14: Changes in pyruvate kinase activity as the concentration of HOCl increases in the presence or absence of adenosine triphosphate

These results imply that ATP is binding in the active site and blocking oxidation of a cysteine that is essential for function. Furthermore, because activity is maintained until incubation with higher concentrations of HOCl, it is likely that this cysteine is minimally reactive. While there has been much speculation about the location of this less reactive but necessary cysteine, incubation with ATP seems to support the idea of this residue in the active site. Knowing where the important cysteines in pyruvate kinase can be found can help better understand the effects of oxidative damage *in vivo*.

E. <u>Effects of Fructose-1,6-Bisphosphate on Oxidation:</u>

Fructose-1,6-bisphosphate (FBP) is an allosteric activator of PK that induces the

catalytically active tetramer. The FBP allosteric site contains positively charged residues (Arg-

516, Arg-498, Lys-433) that coordinate with the negatively charged phosphate groups of the

molecule, in addition to hydrogen bonds formed between FBP and the protein backbone (Jurica,

M.S. *et al.* 1998). It is likely that there is a cysteine in this binding site, and this residue is

thought to be highly reactive but non-essential (Anastasious, D., *et al*., 2011; Brown, C.E.,

Taylor, J.M., & Chan, L., 1985; Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972; Poole, L.B.,

2015). In order to show the presence of a cysteine in this binding site and to understand the

effects of oxidation to this residue, PK was incubated with a low concentration of FBP as an

additional step before Stage 1 of the activity assay similar to experimentation with ATP. PK was

exposed to a 75 µM final concentration of FBP for 5 minutes at room temperature before the

addition of HOCl at various concentrations to bring the sample to the 15 µL final volume. These

samples were visualized with IAF labeling and analyzed catalytically through LDH-coupled

assays to understand the enzymatic significance of oxidative damage to the active site.

Visualization with IAF Labeling:

Samples were incubated with different concentrations of FBP (0, 25, 50, 100, 150, 330

µM) before tagging with IAF and analyzing on an SDS-PAGE as shown in Figure 15 courtesy of

M.C. Duff, another researcher in Dr. Landino's laboratory. As the concentration of FBP

increases, the extent of labeling decreased. This finding is interesting because it implies the

presence of a cysteine in the FBP allosteric site such that increasing the concentration of the

regulatory molecule increases the frequency of binding in the site, which decreases access to the

residue. It is unclear whether this decreased access is due specifically to FBP binding in the

allosteric site or whether merely an increased presence of molecules in solution obstructs the path of the fluorescent tag, a similar concern with ATP incubation. Fortunately, the corresponding activity assays provided some insight to the potential issue.
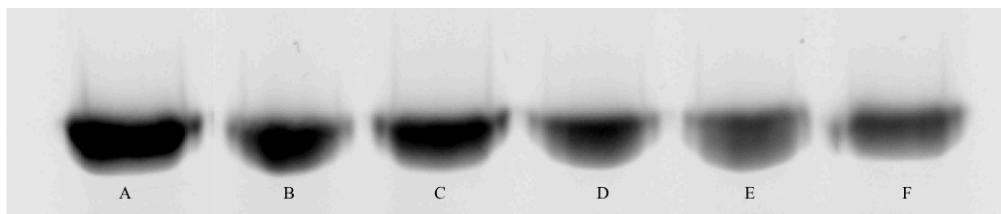

Figure 15: The effect of various final concentrations of fructose-1,6-bisphosphate present in solution on IAF labeling of pyruvate kinase
***Lane A**: 0 μM **Lane B:** 25 μM **Lane C:** 50 μM **Lane D:** 100 μM **Lane E:** 150 μM **Lane F:** 330 μM*
Figure courtesy of Duff, M.C. & Landino, L.M. (2017).

Effects on Enzymatic Activity:

PK incubated with 75 µL FBP before exposure to either 0 or 250 µM HOCl did not see any difference in activity levels as measured through percent change in NADH absorbance at 340 nm, which indicates that the cysteine in the allosteric site is not critical for enzymatic function. The results of these assays are important for two main reasons. First, it illustrates an important control: that the mere presence of molecules in solution is not enough to impede oxidation but rather that FBP and ATP were most likely binding in their respective binding sites throughout the incubation experiments. Secondly, it confirms that the cysteine in the FBP binding site is likely to be the highly reactive but non-essential thiol, which is vital for understanding how the protein works and the impacts oxidative damage could have *in vivo*.

PART IV: CONCLUSIONS:

Overall, this research gave new insight into the effects of oxidative damage to pyruvate kinase and the location of key cysteines in each PK subunit through visualization of IAF labeling by SDS-PAGE and the development of a LDH-coupled activity assay.

One of the major accomplishments of this research was the protocol to analyze PK function. By measuring the percent change in NADH absorbance at 340 nm, it was possible to determine the degree of PK catalytic activity quantitatively in response to various experimental conditions. The protocol relied on a three-step design: 1) PK modification, 2) PK catalysis, and 3) LDH catalysis. By dividing the assay into these stages, it was possible to establish more controls for more accurate results. After the protocol demonstrated its consistency and reliability, analysis of PK functionality expanded to understand the effects of oxidation.

Using the activity assay protocol, it was possible to understand the broader extent of oxidative stress on PK. Non-specific oxidation of PK by hypochlorous acid (HOCl) likely affects cysteines as illustrated through IAF labeling and comparisons with modification of thiol groups by Ellman's Reagent (DTNB), a thiol-specific reagent. Oxidative damage to PK causes a partial loss of function observable at cysteine:oxidant ratios at or exceeding 1:40. Inhibition to PK as a result of oxidation increases as the concentration of HOCl increases. Interestingly, the presence of cations (Mg^{2+} and K^+) in solution increases the frequency of modification by DTNB with corresponding decreased activity. Ions present in solution for HOCl oxidation, on the other hand, dramatically decreased IAF labeling but did not appear to affect catalytic function as indicated by the LDH-coupled activity assays. These results expand the knowledge of PK thiol reactivity and enzymatic ability.

Additionally, the assay facilitated a better understanding of enzymatic structure and the location of key cysteines that are important for cellular function. By incubating PK with a low concentration of ATP before adding the oxidant, it was possible to observe the changes to IAF labeling and enzymatic function. Based on the results, there is likely a cysteine in the active site, where ATP binds, that is critical for function. When ATP was present in solution, there was

decreased access to the cysteine in the binding site, which maintained enzymatic function at higher levels of oxidation and decreased IAF labeling. Based on the research, it is likely that the cysteine in the active site is the minimally reactive but highly essential cysteine, which corresponds with predictions from the literature (Anastasious, D., *et al*., 2011; Brown, C.E., Taylor, J.M., & Chan, L., 1985; Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972; Poole, L.B., 2015). Similarly, incubation with a low concentration of fructose-1,6-bisphosphate before treatment with HOCl illustrates that there is likely a cysteine in the FBP allosteric site as indicated by the decrease in IAF labeling. The presence of FBP in solution, however, did not affect activity in response to oxidative stress, which indicates that this cysteine is likely the highly reactive but non-essential cysteine discussed in the literature (Anastasious, D., *et al*., 2011; Brown, C.E., Taylor, J.M., & Chan, L., 1985; Flashner, M., Hollenberg, P.F., & Coon, M.J., 1972; Poole, L.B., 2015). The data from this research confirms speculation of key cysteines in addition to giving insight to PK structure and function.

From this data, there are numerous avenues for immediate further research. It may be possible that the loss of PK activity after treatment with an oxidant can be restored when treated with a reducing agent. Therefore, further research is necessary to understand the extent to which PK can be modulated to restore catalytic function. It may be that low levels of oxidative stress *in vivo* can occur without severe consequences to cellular metabolism since intracellular conditions favor reduced molecules. On the other hand, because it takes a ratio of cysteine:oxidant greater than 1:40 to see a decrease in enzymatic function, the amount of reducing agent necessary to undo oxidative damage, if this reverse is even possible, may exceed reasonable physiologically relevant concentrations. Another potential route for future research on PK oxidation studies is conformational changes in response to treatment with an ROS. While the results of the project
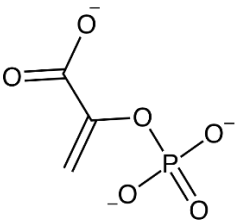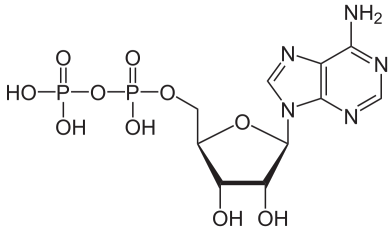
imply a change in protein shape based on the impaired function, more definitive methods of observation should be applied in order fully to understand the effects of oxidative damage on PK. Analysis of the effects of PK oxidation on tryptophan fluorescence and circular dichroism spectroscopy would aid in understanding conformational changes that may occur as the thiol groups are oxidized to form sulfoxides and/or disulfide linkages that could have distorted the overall structure. The data collected for this project adds to the overall picture of PK oxidation, but there is still more to explore.
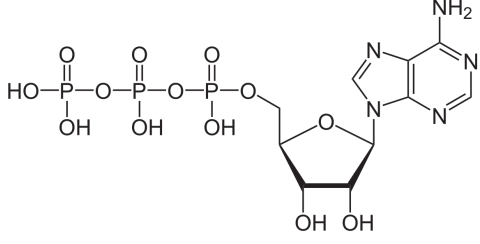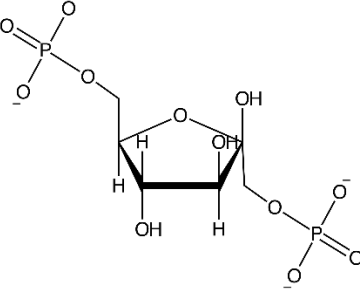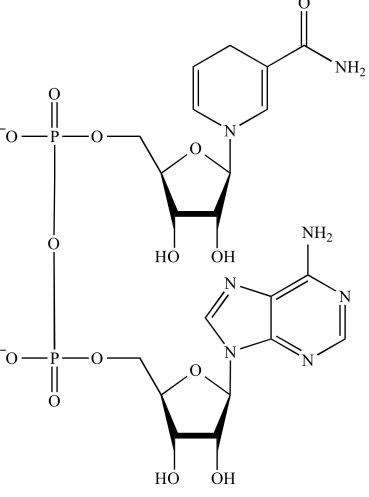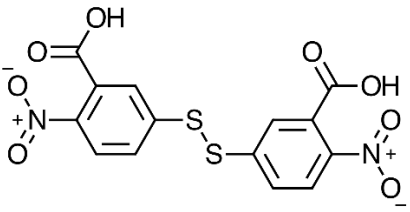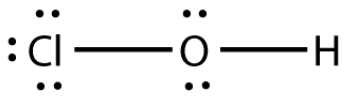
Taken together, this research provides more insight into the role of PK under oxidative stress, as well as a protocol for further research on the topic. It may be possible that PK can modulate function under low levels of oxidation through the modification of the cysteines' thiol groups. This modulation could serve a protective role *in vivo* to maintain metabolic and cytoskeletal function through interactions with other glycolytic enzymes and tubulin. If possible, this defense mechanism could potentially prevent the onset of some neurodegenerative symptoms, such as those characteristic of Alzheimer's disease.
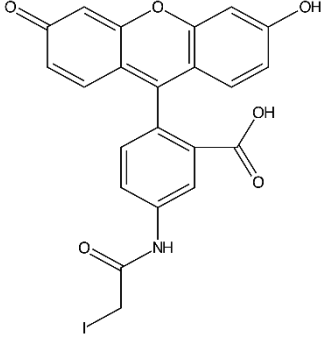
Part V: APPENDIX:

A. Tables of Abbreviations and Chemical Index:

Enzymes	Abbreviation
Pyruvate kinase	PK
Lactate dehydrogenase	LDH

Buffer Solutions	Abbreviation
50 mM Phosphate buffer pH 7.4	PBS
50 mM Phosphate buffer pH 7.4 with 100 mM KCl and 10 mM $MgSO_4$	SPBS
50 mM Tris buffer with of 100 mM KCl and 10 mM $MgSO_4$	STris

Molecules	Structures	Abbreviations
Phosphoenolpyruvate		PEP
Adenosine diphosphate		ADP

Adenosine triphosphate		ATP
Fructose-1,6-bisphosphate		FBP
Nicotinamide adenine dinucleotide		NADH
Ellman's Reagent: 5,5-dithio-bis-[2-nitrobenzoic acid])		DTNB
Hypochlorous Acid		HOCl

| 5-iodoacetamidofluorescein | | IAF |

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