Metabolism and Oxidative Stress: Understanding the Role of Reactive Cysteines in Pyruvate Kinase

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Metabolism and Oxidative Stress: Understanding the Role of Reactive Cysteines in Pyruvate Kinase

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

by

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Abstract

Reactive oxygen species (ROS) play a critical role in intracellular signaling mechanisms. These small molecules initiate reversible, post-translational modifications of redox-sensitive proteins. Pyruvate kinase (PK) is a key glycolytic enzyme whose activity is modulated by ROS; oxidation inhibits its catalytic function through a reaction at a critical cysteine near the active site. PK’s central role in moderating glycolytic flux makes it highly regulated through both structural and allosteric effectors. This research explores the extent of cysteine modification of PK under various models of intracellular conditions. PK was treated with oxidants and allosteric regulators, and changes in the enzyme’s activity and cysteine reactivity were monitored through spectrophotometric and SDS-PAGE assays. Addition of oxidants decreased both PK activity and cysteine accessibility. Addition of ATP and fructose-1,6-bisphosphate (FBP) protected cysteines from tagging and oxidation. Other allosteric effectors like PEP had no effect on cysteine accessibility at low concentrations. Quantifying the reactivity of PK’s cysteines allows researchers to further understand the nuanced relationship that exists between intracellular redox conditions and protein activity. This research has been completed in tandem with Julia Zuercher.
Introduction

Reactive oxygen species (ROS) are produced at low levels during metabolism. These short-lived endogenous species contain oxygen and interact with or damage all classes of biochemical macromolecules. Indeed, ROS can be toxic and even promote cell death. Understanding the role of ROS in oxidative stress has been a critical goal of biochemists since Denham Harman advanced the free radical theory of aging in the 1950s. Oxidative stress has been implicated in a number of pathologies from diabetes (Paravicini and Touyz, 2006) to cancer (Trachootham et al., 2009) to neurodegenerative diseases (Moreira et al., 2005). Recent work on the subject, however, has unveiled a more nuanced relationship; while too much ROS damages cellular components, a minimum level of ROS is required for intracellular signaling pathways. This dual nature of ROS reflects the complexity and balance inherent in biochemistry.

Cells have developed myriad methods to regulate intracellular ROS levels. Maintenance of the cell’s redox balance is critical in homeostasis; thiols are often implicated in this equilibrium due to their unique chemistry. Oxidation of cysteine residues in proteins results in varied responses, from activation to multimerization. Low molecular weight thiols are ubiquitous in cellular environments and can form mixed disulfide bridges with redox-sensitive proteins to protect against irreversible oxidation. The dynamic regulation of protein activity via thiol chemistry is of particular interest to biochemists.

Glycolysis is a central metabolic pathway, transforming glucose into energy rich molecules like pyruvate. Pyruvate kinase (PK) is the enzyme that catalyzes the final step of glycolysis, the direct transfer of a phosphate group from phosphoenolpyruvate (PEP) to adenosine diphosphate (ADP). PK operates at a critical biochemical juncture and thus modulates the relative rates of catabolism versus anabolism; its properties and function will be explored further below.
ROS

ROS refers to superoxide anion, hydrogen peroxide, and other radical and non-radical species that can be generated from them such as OH• (Berg et al., 2012). The mitochondria are the key sites for ROS generation (Chen et al., 2003). These products are created by an incomplete reduction of molecular oxygen at complex III of the electron transport chain (ETC) (Wellen and Thompson, 2010). Instead of four, only one or two electrons are transferred to O₂, yielding partially reduced oxygen in the form of superoxide anion (O₂•⁻) and peroxide (O₂²⁻) respectively.

Ground-state molecular oxygen is a paramagnetic biradical. It is unable to oxidize biological macromolecules because of spin restriction; its two unpaired electrons have parallel spin. Any other reactant must therefore have parallel spin electrons, but in the opposite direction of oxygen’s spin (Krumova and Cosa, 2016). Most organic species are diamagnetic and thus have electron pairs with opposite spins. The diamagnetism precludes orbital overlap and bond formation. However, certain ROS species such as singlet oxygen (¹O₂) and peroxide (O₂²⁻) are diamagnetic and do not have spin restriction, so they are able to rapidly oxidize and damage intracellular species (Krumova and Cosa, 2016).

ROS Modulation

ROS are inevitably produced in aerobic metabolism (Berg et al., 2012). To combat the potentially damaging effects of oxidation, biological systems have developed strategies to reduce these species. For example, upon its generation, superoxide anion is rapidly reduced to H₂O₂ by superoxide dismutases (SODs) (Berg et al., 2012). Antioxidant enzymes like catalase, glutathione peroxidases (Gpx), and peroxiredoxins (Prx) then scavenge H₂O₂ (Krengel and Tömroth-Horsefield, 2015). These enzymes work to detoxify the intracellular environment, often using glutathione (GSH) as a substrate for reduction. Antioxidant enzymes have high rate constants and high abundance in vivo to halt the
formation of ever more dangerous ROS (Sena and Chandel, 2012). For instance, if H₂O₂ is not promptly scavenged, it can react with ferrous or cuprous ions, which are abundant in cells, leading to the formation of highly reactive OH• radicals (Hulea, 2016). A careful balance must be maintained between endogenous redox elements. Cells have numerous enzymatic tools to modulate ROS levels, such as abundant SODs and high GSH levels that maintain Gpx and Prx activity.

Inevitably, cellular defenses against oxidation are diminished in a process referred to as aging. According to Harman’s free radical theory, aging is the progressive accumulation of diverse, deleterious changes that increase the chance of disease and death with advancing age (Harman, 2006). ROS are the key damaging agents. Indeed, Ku et al. demonstrated that mammalian life spans are inversely related to the rate of mitochondrial superoxide formation (1993). Recently, the free radical theory of aging has come under question. It is yet unclear whether ROS are the cause or the consequence of age-associated diseases. Excessive levels of ROS engender pathologies, but it appears that a low level of ROS is necessary for cell function.

**ROS Signaling**

Highly reactive ROS such as ozone or hydroxyl radical oxidize indiscriminately. Therefore, these species are not implicated in ROS signaling. Less reactive ROS like H₂O₂ oxidize specific residues, initiating reversible, post-translational modifications on certain redox-sensitive proteins. More often than not, ROS signaling is propagated by the oxidation of cysteine thiol groups (Sena and Chandel, 2012). H₂O₂ diffuses through membranes and participates in signaling cascades, even in the nanomolar range (Glasauer and Chandel, 2013).

Recent studies have shown that ROS are critical to signal-transduction pathways. Sundaresan et al. demonstrated that platelet-derived growth factor stimulates production of ROS upon ligand binding, and these ROS are responsible for inducing appropriate cellular responses (1995). Binding of another
peptide, epidermal growth factor (EGF), increased intracellular ROS concentrations. Addition of catalase, a H₂O₂ scavenger, inhibited the cell’s downstream responses to EGF binding (Bae et al., 1997). It is postulated that ROS signaling evolved as a mechanism for communication between metabolism and other cellular processes (Sena and Chandel, 2012).

Oxidation of sulphhydryl (−SH) groups to sulphenic acid (−SOH) is followed by two distinct pathways. Further oxidation can irreversibly modify the sulphenic acid to a sulfenic (−SO₂H) or sulfonic (−SO₃H) acid. The oxidized cysteine could instead form a disulfide bridge (−SS−) or a sulfenyl amide (−SN−) with nearby thiols or nitrogens. These new covalent bonds cause conformational changes that may affect the activity of the protein. However, disulfide bridges protect cysteines from irreversible oxidation; thioredoxins (Trx) are able to reduce disulfides and regenerate reduced dimers (Janssen-Heininger et al., 2008). Restoring protein structure and function via formation of reversible disulfide linkages is a key mechanism of ROS signaling.

![Figure 1. Oxidation of sulphhydryls. Cysteines (A) are initially oxidized to a sulphenic acid (B). The formation of a mixed disulfide (C) with another thiol allows for the regeneration of the reduced cysteine and protects the thiol from irreversible modification to a sulfenic (D) or sulfonic (E) acid.](image)

Changing the redox state of a protein affects activity (Giles et al., 2003). As the sulfur of cysteine can occur in 10 different oxidation states (Jacob et al., 2003), cysteine oxidation can result in unique functional outcomes. Oxidation can activate or inactivate a signaling protein, as with Ras and
protein tyrosine phosphatase respectively (Janssen-Heininger et al., 2008; Kwon et al., 2004). Heat shock proteins multimerize upon thiol oxidation, whereas glucokinase dissociates (Ahn and Thiele, 2003; Rizzo and Piston, 2003). Thus, all of these factors illustrate that ROS-mediated signaling pathways are regulated and specific processes.

**Redox Environment**

The intracellular redox environment is maintained by low molecular weight thiols. Chief among these small molecules is glutathione (GSH), a tripeptide of glutamic acid, cysteine, and glycine with an atypical, gamma peptide linkage between the carboxyl of Glu and the amine of Cys (Figure 2). Glutathione is the most abundant free thiol in eukaryotic cells (Poole, 2015). Reduced GSH modulates oxidative stress through the formation of disulfide linkages (GSSG). Indeed, the GSH:GSSG is indicative of intracellular redox state.

Careful regulation of intracellular redox conditions is critical for homeostasis. Different cellular components have different redox needs; glutathione is able to accommodate a range of redox environments. GSH has distinct concentrations and redox states for different cellular compartments. Circu and Aw define four major regions of unique glutathione conditions: the cytosol, the mitochondria, the nucleus, and the endoplasmic reticulum (2010). In the cytosol, a reducing environment is key for proper protein function. Therefore, glutathione concentrations are high, ranging from 2-10 mM. The GSH:GSSG ratio is dynamic, rapidly responding to changes in the oxidative environment, however, it usually exceeds 100 to 1. As the mitochondria are the major endogenous source of ROS, more GSH is necessary to form protective disulfides and insulate proteins from ROS. The mitochondria, therefore,
have a higher concentration of glutathione, usually ranging from 5-10 mM. Nuclear glutathione concentrations are less understood, but are considered more dynamic because nuclear GSH fluctuates with the cell cycle (Markovic et al., 2007). The endoplasmic reticulum has total GSH concentrations similar to the cytosol, yet GSSG is more abundant. Disulfide bonds are often key to proper protein folding, and GSSG encourages the formation of these linkages. The ratio of reduced glutathione to oxidized glutathione is considered to be about 3 to 1 (Bass et al., 2004), though this finding has recently come under question (Dixon et al., 2008). Each cellular compartment has a unique function that coincides with a unique redox environment, and the GSH/GSSG redox couple is a central buffer for the maintenance of homeostasis. Glutathione and other small thiols modulate the redox balance by forming protective disulfides with other peptides or proteins.

![Glutathione oxidation and reduction](image)

Glutathione works in tandem with other redox proteins. Thioredoxins (Trx) are small, ubiquitous proteins with two catalytic, redox-active cysteines in a conserved sequence: Cys-Gly-Pro-Cys (Nakamura et al., 1997). As previously mentioned, Trxs catalyze the reduction of protein disulfide bonds, thus restoring protein structure and activity. When one cysteine is oxidized to a sulfenic acid, the other cysteine reacts and forms a disulfide bond, thus resolving the oxidation. Peroxiredoxins (Prxs), another antioxidant enzyme, also has two catalytic cysteines that function similarly. Prx and glutathione
peroxidases (Gpx) detoxify H$_2$O$_2$. A synergistic relationship exists between these two enzymes in which, at high H$_2$O$_2$ concentrations, a portion of each antioxidant enzyme remains in the oxidized form, and the reactivity of both Gpx and Prx is increased (Molavian et al., 2015). The fact that two seemingly independent proteins would be affected by the concentration of another enzyme reflects the intricacy of cellular redox mechanisms.

**Cysteines as Reactive Species**

Though cysteines have a low abundance in protein structures, they often play a critical role in modulating enzyme function and binding properties (Poole, 2015). Cysteine residues engage in unique reactions due to their innate chemical properties. The amino acid’s nucleophilicity, redox activity, and metal binding properties make cysteines a critical regulatory component of many proteins (Giles et al., 2003).

Thiol groups are vulnerable to deprotonation. The relative ratio of thiol (−SH) to thiolate anion (−S$^-$) varies according to the pH. In proteins, cysteine has an average pKa of 8.3 (Berg et al., 2012). However, the local microenvironment can greatly influence an amino acid’s pKa. Stabilization of the thiolate anion with nearby positive residues can lower the pKa, whereas hydrophobic environments favor the amino acid’s neutral form and can thus increase the pKa. Indeed, protein cysteine pKa values in the literature vary from 2.5 to 11.1 (Pace et al., 2009).

Certain cysteine residues have a pKa of 4 or lower, meaning the amino acid exists primarily as a thiolate anion at physiological pH. Indeed, many enzymes have a catalytic cysteine that functions as a protease. This mechanism involves deprotonation of a cysteine by a nearby basic residue, typically histidine. The thiol anion initiates a nucleophilic attack on the carbonyl carbon of the peptide backbone, resulting in its cleavage (Berg et al., 2012). Their critical role in an enzyme’s catalytic behavior makes cysteines a highly conserved residue. In areas where cysteines are conserved, the degree of conservation
often exceeds 90% (Marino and Gladyshev, 2010). Highly conserved cysteines are typically buried in
the protein’s hydrophobic core (Rose et al., 1985). The degree of burial varies; however, these thiol
groups are insulated from solvent interactions (Janin, 1979) and are more likely to be found near other
cysteines (Marino and Gladyshev, 2010). It is likely that these proximal cysteines are able to form
disulfide bridges to modulate protein activity in response to ROS or to associate with metals in sulfur
clusters. Clustered cysteines are associated with redox sensitivity and metal binding (Marino and
Gladyshev, 2010).

On the other hand, areas of poor cysteine conservation usually have less than a 10% rate of
conservation (Marino and Gladyshev, 2010). These cysteines tend to be near the protein’s surface and
interact directly with the solvent (Marino and Gladyshev, 2010). Indeed, there seems to be an
evolutionary bias against surface-exposed, unpaired cysteines (Beeby et al., 2005). Given the inherent
reactivity of sulfur, this finding is not surprising.

Cysteines have low redox potential, reported as a range from -270 mV to -125 mV (Giles et al.,
2003). This allows cysteines to function as an electron donor and to facilitate electron transfer. For
example, glutathione reductase (GR) functions by transferring two electrons from NADPH to oxidized
 glutathione (Berg et al., 2012). This electron transfer reduces GSSG to two equivalents of GSH.
Cysteine’s low redox potential allows proteins to modulate diverse cellular conditions through the
transfer of electrons and the formation of disulfide bridges.

Cysteines have unique metal binding properties. Because of the range of oxidation states it can
assume, Giles et al. refer to sulfur as a storage facility for metal ions (Giles et al., 2003). Indeed, sulfur
can accommodate a large number of bonds by changing its geometry. Iron-sulfur clusters are
particularly important in biochemistry. They are often implicated in electron transfer reactions as both
Fe and S are able to adapt numerous electronic configurations thus “accepting, donating, shifting, and
storing electrons” according to Beinert et al. (1997). Fe-S clusters even serve as redox sensors; oxidation of [Fe₂S₂]⁺⁺ to [Fe₂S₂]²⁺ results in the activation of intracellular detoxifying mechanisms in E. coli (Gaudu and Weiss, 1996). Beyond redox functions, iron-sulfur clusters have been found to stabilize radicals, initiate dimerization, stabilize binding sites, and protect against protease attack (Beinert et al., 1997). Iron-sulfur clusters are critical to many biochemical reactions. The unique properties of cysteines, coupled with transition metals like iron, allow its participation in a wide array of critical pathways.

**Glycolysis**

Glycolysis is one of the oldest biochemical processes. It is the central metabolic pathway in prokaryotes and eukaryotes. Through glycolysis, one molecule of glucose is converted into two molecules of pyruvate with the concomitant net production of two molecules of ATP. Pyruvate can then be funneled into other biochemical pathways such as the Citric Acid Cycle for large-scale ATP production or to fermentation to regenerate glycolytic cofactors. ATP production in glycolysis, though minimal, is independent of oxygen, making glycolysis an important biochemical pathway in anaerobic environments.

Glycolysis consists of ten reactions and can be divided into two major stages. First, glucose is broken down. Then, ATP is harvested through the oxidation of glucose fragments to pyruvate. Pyruvate kinase (PK) is a key glycolytic enzyme that catalyzes the final, rate-limiting step in glycolysis, the conversion of phosphoenolpyruvate (PEP) to pyruvate with the concurrent generation of one molecule of ATP. The ninth step of glycolysis generates the thermodynamically unstable PEP. PK catalyzes the nucleophilic attack of the β-phosphoryl oxygen of ADP on PEP. This reaction generates ATP and enolpyruvate; enolpyruvate then tautomerizes to form the more thermodynamically stable pyruvate. Lys-269 in PK’s active site stabilizes the intermediate as the phosphoryl group of PEP is
directly transferred to ADP (Iqbal et al., 2014). Enolpyruvate tautomerizes after receiving a proton from a water molecule bound to Thr-327 and Ser-361 (Israelsen and Vander Heiden, 2015). The driving force of the reaction is the tautomerization; its high free energy loss allows for the coupling of a more unfavorable reaction, in this case, the generation of ATP from ADP (McKee and McKee, 2012). Because of the highly negative ΔG, this reaction is irreversible and highly regulated.

**Pyruvate Kinase**

Pyruvate kinase (PK) is a homotetramer of 237 kDa; each identical subunit is about 57 kDa and consists of 530 amino acid residues (Cottam et al., 1969). Each monomer has one active site and consists of four major domains: the N-terminus, Domain A, Domain B, and Domain C (Ou et al., 2010). Larsen et al. defined the N-terminus’s structure as a helix-turn-helix, and it represents residues 1-42 (1997). Domain A, consisting of residues 43-115 and 219-387, is the most highly conserved (Muirhead et al., 1986). It is arranged in a (β/α)8-barrel, a common enzymatic motif. In this structure, eight parallel β-strands form a cylinder that is encased in a wheel of eight α-helices (Vega et al., 2003). Domain B (residues 116-218) is the least conserved. It is a nine-stranded β-barrel that protrudes into the solvent (Larsen et al., 1994). Residues 388-530 arranged...
in five α-helices and five β-sheets form Domain C. This domain is implicated in the enzyme’s tetramerization.

PK Activity

PK’s active site lies at the cleft between Domains A and B. A flexible hinge allows PK to modulate its activity. In its inactive T-state, the cleft is open; activation of the enzyme closes the cleft (Consler and Lee, 1988). X-ray crystallography studies by Larsen et al. reveal that Domain B’s backbone rotates more than 20º with respect to Domain A to open or close the active site (1997). Furthermore, Larsen et al. warn that assuming PK only has two states - open or closed - is a little simplistic. Indeed, subunits adopt different orientations with varied activities in response to allosteric effectors.

Cations are required for PK’s activity. Two equivalents of Mg$^{2+}$ and one equivalent of K$^+$ are typically coordinated to the active enzyme. Binding of cations engenders conformational changes; Ou et al. specify that the coordinated enzyme-metal complex has a different conformation than free enzyme (2010). Divalent magnesium ions assist in the orientation of PEP and ADP (Muirhead et al., 1986). Their positive charges minimize the electrostatic repulsion resulting from three highly charged phosphoryl groups being held in close proximity. One magnesium cation binds PK and the nucleotide
substrate (ATP or ADP), while the other Mg\(^{2+}\) holds PK and PEP (Larsen et al., 1994). Critical residues for Mg\(^{2+}\) coordination are the carboxyl groups of Glu-271, Ala-292, Arg-293, and Asp-295 (Muirhead et al., 1986). The γ-phosphate group of ATP forms a bridge between the two divalent cations (Lodato and Reed, 1987).

The monovalent cation is also essential for maximum enzymatic activity (Kachmar and Boyer, 1953). Without K\(^+\), PEP must be bound first. Binding of K\(^+\) allows the enzyme to proceed in a random order mechanism (Oria-Hernández et al., 2005). Monovalent potassium allows PEP and ADP to bind independently, thus making the \(V_{\text{max}}\) of the K\(^+\)-PK-complex 400 times higher than wild type PK (Oria-Hernández et al., 2005).

The cation changes the orientation of the residues that bind the substrates, namely Glu-363 and Gln-328. Monovalent potassium is coordinated to the carbonyl of Thr-113 and the oxygens of Ser-76, Asn-74, and Asp-112 (Larsen et al., 1997)

PK’s active site has unique properties and is finely tuned to labilize γ-phosphate groups and stabilize enolpyruvate. PK has been implicated in other, similar reactions including the decarboxylation of oxaloacetate, the enolization of pyruvate, and the ATP-dependent phosphorylation of α-hydroy or α-thio carboxylates (Creighton and Rose, 1976; Rose, 1960; Ash et al., 1984). Key residues in the active site create favorable interactions with these high-energy substrates. Coordinated mono- and divalent
ions further mitigate destabilizing electrostatic forces.

**PK Isozymes**

Mammals have two genes for PK that encode four isoforms: L, R, M1, and M2. The expression of a specific isozyme is dependent on the energetic needs of the surrounding tissue; tissues preferentially express one PK isoform (Urich, 2013). In sites of gluconeogenesis, such as the liver, the L isozyme is expressed. The R isozyme is found only in red blood cells. Brain and heart cells express PK-M1, and fetal and proliferating tissues express PK-M2.

Alternative splicing of the PKM gene leads to the formation of M1 or M2 (Noguchi *et al*., 1986). Though these isoforms differ by only 22 of 531 amino acids, their activity is quite different. PK-M1 is locked in the catalytically active R-state, and it exists as a stable tetramer (Corder *et al*., 1989; Israelsen and Vander Heiden, 2015). PK-M1 undergoes hyperbolic kinetics and is not allosterically regulated (Ikeda and Noguchi, 1998). On the other hand, PK-M2 requires fructose-1,6-bisphosphate (FBP), an allosteric activator, to transition from a dimer to a tetramer. Additionally, PK-M2 undergoes sigmoidal kinetics and is allosterically regulated (Berg *et al*., 2012). As microorganisms do not express PK-M1, it is believed that this isoform is an evolutionary adaptation for specialized energy production in specific tissues like the heart and brain (Ikeda *et al*., 1997). These tissues are critical for an organism’s survival and have a constant need for ATP. Therefore, expressing a form of PK that is always active provides a continuous stream of energy to perform essential life functions.

The differences in the primary sequence of PK-M1 and PK-M2 lie at the inter-subunit junction, located in Domain C. This area controls the association of inactive PK dimers into catalytically active PK tetramers. Several residues have been identified as researchers seek an explanation of the differential allosteric properties between the isoforms. PK-M1 has a highly conserved glutamic acid at residue 432, whereas PK-M2 has either a threonine or a lysine (Ikeda *et al*., 2000). The negative charge
of the glutamic acid creates a repulsive electrostatic interaction with the negative phosphate groups of FBP. This repulsion destabilizes FBP’s binding and is critical in PK-M1’s lack of allosteric regulation. Cys-423 in PK-M2 has been found to destabilize the R-state of the protein (Ikeda and Noguchi, 1998). It is deprotonated and thus disrupts the hydrophobic interactions of the enzyme’s core. PK-M1 has a hydrophobic leucine at this position; the hydrophobic stabilization at the inter-subunit contact allows the M1 isozyme to exist as a stable tetramer (R-state). Indeed, when Leu-423 is replaced with cysteine, the mutated PK-M1 exhibits allosteric regulation similar to wild-type PK-M2 (Ikeda and Noguchi, 1998). Different amino acids along the inter-subunit domain affect the electrostatic profiles of PK-M1 and M2 and account for the isozymes’s dissimilar activity and regulation.

**PK Regulation**

Due to its placement at a key biochemical branching point, PK modulates the glycolytic flux. When PK is abundant and active in a cellular milieu, aerobic metabolism is promoted. Glycolytic intermediates are funneled towards the production of ATP. Conversely, when PK is scarce or inactivated, glucose, the key intracellular carbon source, is used to construct larger biological molecules like nucleic acids, lipids, and amino acids. PK’s activity thus controls the relative rates of metabolism and anabolism. Because this ratio is critical to cellular homeostasis, PK is highly regulated through its quaternary structure and allosteric regulators.

**Structural Regulation**

The organization of PK’s subunits into either a tetramer or a dimer affords a dynamic regulation of its activity. As a tetramer, PK has a high PEP affinity and is active; it promotes energy metabolism through the catalysis of the final step in glycolysis. Conversely, as a dimer, PK has a low affinity for PEP and is catalytically inactive. The dimer form shunts glucose towards macromolecule synthesis.
Dimerized PK may not participate in glycolysis, but it does function as an active protein kinase. This form of PK is implicated in signaling and gene transcription processes. Dimerized PK phosphorylates key proteins involved in proliferation processes. For example, this enzyme interacts with and phosphorylates histone H3 at Thr-11. Phosphorylated H3 T11 is implicated in cell proliferation and tumorigenesis (Yang et al., 2012).

The ratio between tetramerized and dimerized PK is controlled by numerous factors including post-translational modifications and allosteric effectors. Post-translational modifications include the addition of phosphate or lipid groups to amino acid residues. For example, in cancerous cells, oncogenic tyrosine kinases phosphorylate Tyr-105. The addition of a phosphate group at this residue causes the release of FBP, which leads to the formation of the inactive dimer (Iqbal et al., 2014).

Anastasiou et al. (2011) found that oxidation of Cys-358 also results in PK inactivation and the re-routing of glycolytic intermediates to the pentose phosphate pathway. The pentose phosphate pathway stimulates the formation of NADPH, an important cofactor for the maintenance of the cellular redox environment. Higher concentrations of NADPH allow for glutathione reductase (GR) to catalytically reduce glutathione. Reduction of glutathione increases the reducing potential of the cell and alleviates the oxidative environment that caused the initial thiol oxidation. Therefore, Cys-358 oxidation initiates a cascade that results in the generation of reductive species like GSH and the restoration of the cell’s reducing environment. This process is particularly important in cancerous cells because of their inherent oxidative state; oncogenic cells generate high levels of ROS because of hypoxia, matrix detachment, mitochondrial dysfunction, and inflammation (Anastasiou et al., 2011). These cells are often starved for nutrients as tumor growth outpaces angiogenesis (Wellen and Thompson, 2010). However, detoxification with GSH is critical for all cells. PK acts as a redox-switch; its inactivation stimulates the production of NADPH and other potent reducing factors to maintain the
intracellular redox environment.

*Allosteric Regulation*

PK-M2 is regulated by FBP, an upstream metabolite. As previously discussed, FBP promotes PK-M2 tetramerization and stabilizes the protein’s active state. FBP generation is highly regulated, so an abundance of FBP indicates that the cell has committed to glycolysis (McKee and McKee, 2012). PK-M2 activity is increased to funnel these metabolites towards ATP production. Low levels of FBP indicate that the cell is not promoting glycolysis, so PK-M2 activity is diminished. FBP binds about 40 Å away from the active site (Jurica et al., 1998). However, its binding invokes significant changes in the protein’s structure. Allosteric binding of FBP causes PK-M2 to transition from its inactive T-state to its active R-state. This transition results from the concerted rotation of each of the 12 domains of the tetramer, modifying the geometry of the allosteric, FBP binding site and the active site (Mattevi et al., 1996).

In addition to changing PK-M2’s quaternary structure, FBP increases PEP’s binding affinity, thus increasing catalysis. Intracellular levels of FBP assist in determining the glycolytic flux.

The FBP binding pocket is located in Domain C. When FBP is bound, the ligand is almost completely buried in the pocket created by residues 402-407. Jurica et al. analyzed FBP binding in *S. cerevisiae* and determined its coordination to the protein’s primary structure (1998). Each monomer of PK-M2 binds one FBP, and there is a
strong, stabilizing salt bridge formed between Arg-429 and a negatively charged phosphoryl group of FBP (Valentini et al., 2000). Hydrogen bonds from polar amino acids (Ser-402, Ser-404, and Thr-407) and π-stacking with Trp-452 further stabilize FBP’s binding to PK-M2.

ATP is a downstream allosteric inhibitor of PK activity (Berg et al., 2012). High intracellular ATP levels indicate that the cell does not need to use glucose for catabolism because the cell already has excess energy. ATP acts as an allosteric inhibitor by competing with ADP for access to PK’s shared active site. Reynard et al. found that ATP inhibited binding of both ADP and PEP (1960). The enzyme proceeds via a direct phosphoryl transfer between the two substrates, so the presence of ATP in the active site precludes binding of other species. Indeed, ATP decreases V_{max} and has a low K_{i}, indicating competitive inhibition (Holmsen and Storm, 1968). The structure of PK’s active site and its catalytic mechanism allow for dynamic modulation of the enzyme’s activity using downstream products like ATP.

Other notable allosteric effectors include phenylalanine, alanine, and serine. Phenylalanine is unique in that it affects both PK-M1 and M2 (Israelsen and Vander Heiden, 2015). Phenylalanine binds at a location distinct from the active site and decreases the enzyme’s affinity for PEP by loosening the overall quaternary structure (Yu et al., 2003). PK becomes more asymmetric as interatomic distances expand up to 110 Å (Yu et al., 2003). However, despite this expansion, the protein remains a tetramer
Alanine also inhibits PK-M2 activity. It binds in the same location as phenylalanine (Domain C: Ile-468 and His-463), but it does not engender dramatic conformational changes (Williams et al., 2006). Instead, alanine favors PK’s inactive dimer. Alanine is one of the first amino acids produced as proteins are degraded; indeed, alanine can be synthesized from pyruvate in one step (Berg et al., 2012). Therefore, an abundance of alanine signals a shift in the cell’s glycolytic flux from metabolism to anabolism; alanine binding adjusts PK’s activity to accommodate this trend. Serine acts as an allosteric activator of PK. This amino acid is implicated in biosynthesis of macromolecules for proliferation; high levels of serine indicate that glycolytic intermediates have been funneled towards anabolism (Ye et al., 2012). Therefore, serine allosterically binds and activates PK in a feedback loop that ensures the continued production of metabolic molecules to fuel cell processes. Interestingly, Yang et al. proposed a synergistic allosteric mechanism for FBP and serine binding; like FBP, serine increases the binding affinity of PK-M2 for PEP (2016). Each allosteric activator engages in significant hydrogen bonding with PK-M2, and the binding free energy of FBP/Ser/PK-M2 is lower than their individual binding free energies of FBP/PK-M2 and Ser/PK-M2 (Yang et al., 2016).

<table>
<thead>
<tr>
<th>Activators</th>
<th>Inhibitors</th>
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<tbody>
<tr>
<td>FBP</td>
<td>ATP</td>
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<tr>
<td>Serine</td>
<td>Phenylalanine</td>
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<tr>
<td></td>
<td>Alanine</td>
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</tbody>
</table>

Table 1. Allosteric Regulators of PK.
factors represent dynamic, rapid, and reversible methods to modulate PK activity in response to diverse stimuli.

**Research Goals**

The goal of this research is to elucidate the extent of cysteine modification of PK under various models of intracellular conditions. Notably, this research is focused on understanding the effects of oxidant treatment on PK’s activity and conformation. This research has been completed in tandem with Julia Zuercher to enhance the breadth of realized research goals.

Julia focused on activity assays. Using an Absorbance Microplate Reader (BioTek Instruments, Winooski, VT), Julia determined PK activity by monitoring changes in absorbance at 340 nm, the absorbance wavelength of NADH. She coupled PK’s phosphoryl transfer from PEP to ADP with homolactic fermentation. Lactate dehydrogenase (LDH) catalyzes the regeneration of NAD\(^+\) via the reduction of pyruvate to lactate. Julia was able to determine PK activity by monitoring the decrease in absorbance at 340 nm as NADH was oxidized to NAD\(^+\).

![PK-LDH Coupled Reaction](image)

Figure 10. PK-LDH Coupled Reaction.

Julia’s activity assays were coupled with spectrophotometric determinations of the effects of various treatments on cysteine reactivity and accessibility within PK. Cysteines are critical residues in proteins that affect catalytic activity and protein conformation. 5-Iodoacetamidofluorescein (IAF) reacts
with reduced thiol groups and binds a fluorescent tag that can be detected using SDS-PAGE and absorbance assays. The thioether bond formed between the cysteine and IAF is stable at physiological pH and at room temperature. The stability of the fluorescent tag and the physiologically relevant reaction conditions make IAF labeling a potent technique for quantifying cysteine reactivity (Landino et al., 2011).

After PK was subjected to different treatments, IAF in DMF was added to the sample to quantify the number of accessible sulfhydryls remaining in the protein. Treatments included the addition of oxidants and allosteric regulators, both of which affect the ability of IAF to tag cysteines. Oxidation can result in the formation of disulfide bridges, rendering the cysteine inaccessible to IAF tags. The binding of allosteric regulators may block cysteines. Therefore, measuring the extent of IAF tagging via SDS-PAGE or absorbance measurements allows researchers to determine and quantify the fate of reactive cysteines in various intracellular conditions.

Figure 11. Structure of 5-Iodoacetamidofluorescein (IAF).

Figure 12. IAF Tagging of Cysteines.
Methods and Materials

Materials

Pyruvate kinase from rabbit muscle (PK-M2), HOCl, 5-(lodoacetamido)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.

Protein Purification

Pyruvate kinase from rabbit muscle (PK-M2) was obtained as a suspension in an ammonium sulfate solution (3.2 M (NH₄)₂SO₄ solution, pH 6) (Type II) or as a lyophilized powder (Type III). PK-M2 aliquots of known concentration were created to ensure reproducibility of assays.

PK suspended in ammonium sulfate solution was purified using a desalting column. Econo-Pac 10DG columns (Bio-Rad Laboratories, Munich, Germany) were used with a buffer solution. This column’s polyacrylamide gel matrix excluded molecules larger than 6,000 daltons. PK, with a subunit weight of 57 kDa, was excluded, while salt impurities from the suspension were trapped in the matrix. PK was added to the column in small volumes after the sodium azide preservative buffer was poured off and the column was equilibrated to the buffer. Fractional volumes were collected of the species of interest.

Concentrations of purified PK-M2 were determined using extinction coefficients. Proteins and peptides have a unique UV-light absorptivity owing to their primary structure; aromatic amino acids absorb around 280 nm. Manipulation of Beer’s Law allows for the determination of a sample’s protein concentration.
\[ c = \frac{A_{280}}{\varepsilon} \]

where \( c \) is the concentration of the protein sample, \( A_{280} \) is the solution’s absorbance measured at 280 nm, and \( \varepsilon \) is the protein’s extinction coefficient, established as 0.54 mL/(mg*cm) from the literature (Boyer, 1962).

Oxidation of Samples

Protein samples were removed from the -80° C freezer and kept on ice during sample preparation. HOCl was prepared using aliquots from a stock solution. PK (7.6 µM) was added to 0.1 M Phosphate buffer pH 7.4. Samples were incubated for 10 minutes at room temperature with a concentration of HOCl prepared from the stock. The total reaction volume was 15 µL. After 10 minutes, 1.4 mM IAF was added to the solution. Samples were incubated for 30 minutes at 37° C (Fisher Scientific, Dubuque, IA).

Addition of Allosteric Effectors

Allosteric effectors were added to the samples prior to oxidation. The samples were incubated with the substrates for 10-15 minutes at room temperature, then HOCl concentrations were added. The remainder of the procedure followed the oxidation protocol.

Absorbance Measurements

To measure absorbance, 120 µL (four times the reaction volume) of 100% EtOH was added to each sample after incubation with IAF. Samples were then frozen for at least 2 hours, sometimes overnight, at -20°C.

Samples were removed from the freezer and centrifuged at 12,000 rpm for 15 minutes. The supernatant was removed, and the samples were washed with 150 µL of 80% EtOH. Samples were left
for 10 minutes on ice, then re-centrifuged. This washing process was repeated until the supernatant was no longer fluorescent, as verified by a black light (UVP, Upland, CA). The non-fluorescent supernatant was removed and the samples were allowed to dry. The protein pellet was resuspended in 6M guanidine HCl pH 8.8.

Protein absorbance was measured using a UV/VIS spectrophotometer (OceanOptics, USB2000). Quartz cuvettes (Starna Cells, Atascadero, CA) were used, and guanidine HCl was used as the blank for the absorbance measures. Absorbance values were recorded at the wavelength of interest, and the peaks were stored for comparison among samples.

**SDS-PAGE**

Gels were prepared using Mini-PROTEAN Tetra Cell equipment (Bio-Rad Laboratories, Munich, Germany). A 7.5% separating gel was prepared using deionized water, 1.5 M Tris buffer pH 8.8, and 30% acrylamide. This solution was degased under vacuum and thoroughly mixed. Addition of 10% SDS, ammonium persulfate, and TEMED initiated polymerization. The mixture was added to the assembled gel cassette and overlaid with a small amount of water-saturated butanol.

After polymerization (20-30 minutes), the butanol was rinsed off, the gel was dried, the well combs were positioned, and the stacking gel was added. The stacking gel was prepared using deionized water, 0.5 M Tris buffer pH 6.8, 30% acrylamide, 10% SDS, ammonium persulfate, and TEMED. Standard mixtures used for gel preparation are found in the appendix. The stacking gel polymerized (15-20 minutes), and the well combs were removed. Wells were rinsed with deionized water, and the gel plates were mounted in the Mini-PROTEAN Tetra Cell assembly. Running buffer was added to the mini tank.

To perform gel electrophoresis, 16 µL of SB (+) was added to each microcentrifuge tube after incubation with IAF. Solutions were then loaded into wells. Gels were run for 90 minutes at 90 V.
The gels were removed from the mounts and rinsed. Fluorescence measures were taken on ChemiDoc XRS+ Imaging System (Bio-Rad Laboratories, Munich, Germany). Integration software provided by ImageLab was used to quantify fluorescence.

**DTNB Assay**

DTNB was used to verify that PK-M2’s cysteines are accessible to small molecule labeling. DTNB reacts with reduced thiols to form a mixed disulfide and TNB$^{2-}$, a molecule that absorbs at 412 nm (Figure 13). PK (14.4 µM) was added to 0.1 M Phosphate buffer pH 7.4. The concentration of cysteines was calculated (~130 µM cysteines), and ten times this concentration of DTNB (1.3 mM) was added to the solution. The total reaction volume was about 20 µL. After reaction, the sample was diluted to 100 µL with buffer. The diluted sample was pipetted into a quartz cuvette and absorbance measures were taken at 325 nm (absorbance of DTNB) and 412 nm (absorbance of TNB$^{2-}$) on a UV/VIS spectrophotometer (OceanOptics, USB2000) to determine the extent of reaction.

![Reaction of DTNB with thiol (R-S).](image)

*Figure 13. Reaction of DTNB with thiol (R-S). The reaction can be monitored at 412 nm to determine the extent of TNB$^{2-}$ generation. The formation of TNB$^{2-}$ causes a color change; the clear solution becomes pale yellow as TNB$^{2-}$ is generated.*
Results

Oxidation Inhibits PK-M2 Activity and IAF Labeling

Julia measured PK-M2 activity in response to oxidant treatment. HOCl was added to the reaction mixture after the addition of PK-M2. PK-M2 activity was measured as a percent change in absorbance at 340 nm over 5 minutes. At 0 µM HOCl, the absorbance at 340 nm decreases by about 90%, representing the full extent of PK activity in this system. The addition of HOCl results in less generation of NAD⁺, as determined by less of a total change in absorbance at 340 nm. Lower concentrations of HOCl do not have an appreciable effect on enzymatic function. However, as the concentration of oxidant in the reaction mixture exceeds 100 µM, the protein becomes inactive. PK activity continues to decrease as the concentration of oxidant increases past 100 µM.

![Figure 14. Effects of Oxidation on PK-M2 Activity (Zuercher, 2017). PK-M2 activity is measured as a decrease in absorbance at 340 nm as NADH is oxidized to NAD⁺. Oxidation by HOCl decreases PK-M2’s activity.](image-url)
Oxidation also decreases the extent of IAF tagging of PK-M2. SDS-PAGE shows a clear decrease in fluorescence as the concentration of HOCl increases. At 0 µM HOCl, maximum IAF tagging is attained for this system. Addition of increasing concentrations of HOCl decreases the extent of IAF labeling of PK-M2’s cysteines.

Figure 15. Oxidation Inhibits IAF Tagging: Gel. IAF reacts with reduced thiols to form a stable fluorescent tag. Oxidation of PK with HOCl reduces the number of accessible sulfhydryl residues and inhibits IAF tagging.

Absorbance measurements allow for a more quantitative determination of cysteine reactivity. At 498 nm, HOCl concentration has an inverse relationship with absorbance intensity.

Figure 16. Oxidation Inhibits IAF Tagging: Absorbance Spectra. IAF reacts with reduced thiols to form a stable tag that absorbs at 498 nm. Oxidation of PK-M2 with HOCl reduces the number of accessible sulfhydryl residues and inhibits IAF tagging.
Ions Protect PK-M2 from IAF Tagging

PK-M2 requires ions for activity, and these ions have been shown to exert a protective effect against oxidation and IAF tagging. Ions (100 mM KCl and 10 mM MgSO₄) were added to the 0.1 M Phosphate buffer pH 7.4. SDS-PAGE show a decrease in the extent of IAF labeling with the addition of ions. IAF tagging was lower for samples with ions than samples without ions at 0, 50, and 100 µM HOCl.

Figure 17. Ions Inhibit IAF Tagging: Gel. IAF reacts with reduced thiols to form a stable fluorescent tag. Addition of ions reduces the extent of IAF labeling across oxidative environments.

Figure 18. Ions Inhibit IAF Tagging: Gel Summary. Average fluorescent intensity obtained from SDS-PAGE was converted to a percent of the control. Values displayed above represent the average of four trials. Addition of ions reduces the extent of IAF tagging across oxidative environments.
Samples with ions have a lower absorbance at 498 nm than samples without ions, indicating that ions decrease the extent of IAF labeling of PK-M2. At 0 µM HOCl, the extent of IAF labeling was similar for samples with and without ions. However, as HOCl concentration increased, the protective effects of ions became more apparent. 50 µM HOCl with ions has a much lower absorbance than 50 µM HOCl without ions; indeed, its absorbance is even lower than 100 µM HOCl without ions. 100 µM HOCl with ions also had a lower absorbance than 100 µM HOCl without ions.

![Absorbance Spectra](image)

Figure 19. Ions Inhibit IAF Tagging: Absorbance Spectra. IAF reacts with reduced thiols to form a stable tag that absorbs at 498 nm. Oxidation of PK-M2 and the presence of ions reduces the number of accessible sulfhydryl residues and inhibits IAF tagging.

**ATP Protects PK-M2 from Oxidation and IAF Tagging**

ATP binds in PK-M2’s active site. Activity assays performed by Julia show that moderate concentrations of ATP exhibit protective effects against oxidation by HOCl. PK-M2 activity is retained at 100 µM HOCl with the addition of 75 µM ATP.
Figure 20. Effects of Oxidation on PK-M2 Activity (Zuercher, 2017). PK-M2 activity is measured as a decrease in absorbance at 340 nm as NADH is oxidized to NAD$^+$. Oxidation decreases PK-M2’s activity, but ATP (75µM) protects against inactivation by HOCl.

SDS-PAGE results shown below demonstrate a clear decrease in the extent of IAF tagging as the concentration of ATP increases. Interestingly, 37.5 and 75 µM ATP have about the same extent of IAF tagging. As ATP concentration increases, however, IAF labeling decreases more consistently. Addition of ATP inhibits IAF labeling of cysteines.

Figure 21. Effects of ATP on IAF Tagging: Gel. IAF reacts with reduced thiols to form a stable fluorescent tag. Increasing concentrations of ATP decrease the extent of IAF labeling of PK-M2.
Samples of PK-M2 incubated with 75 µM ATP for 10 minutes had a lower absorbance at 498 nm than samples of PK-M2 without ATP.

Figure 22. Effects of ATP on IAF Tagging: Gel Summary. Average fluorescent intensity obtained from SDS-PAGE was converted to a percent of the control. Values displayed above represent the average of four trials. Increasing concentrations of ATP decrease the accessibility of cysteines within the protein and inhibit IAF tagging.

Figure 23. Effects of ATP on IAF Tagging: Absorbance Spectra. IAF reacts with reduced thiols to form a stable tag that absorbs at 498 nm. ATP absorbs at 262 nm. Addition of ATP reduces the accessibility of cysteine residues and inhibits IAF tagging.
PEP Has No Effect on PK Oxidation or IAF Tagging

PEP is a substrate in PK’s glycolytic reaction. SDS-PAGE analysis shows almost no difference between the extent of IAF labeling after oxidation with and without the addition of 75 μM PEP.

Figure 24. Effects of PEP on PK-M2 Oxidation: Gel. IAF reacts with reduced thiols to form a stable fluorescent tag. Oxidation of PK-M2 with HOCl reduces the number of accessible sulfhydryl residues and inhibits IAF tagging. Addition of PEP exerted no effect on cysteine accessibility to HOCl or IAF.

Figure 25. Effects of PEP on PK-M2 Oxidation: Gel Summary. Average fluorescent intensity obtained from SDS-PAGE was converted to a percent of the control. Values displayed above represent the average of three trials. Oxidation of PK-M2 reduces the number of accessible sulfhydryl residues and inhibits IAF tagging. Addition of PEP elicited no protective effect against oxidation or IAF labeling.

Absorbance measures show that the differences in the extent of IAF labeling between samples with and without PEP are minimal.
DTNB Inhibits PK-M2 Activity and IAF Tagging

DTNB is a thiol-specific reagent used to quantify sulfhydryls in proteins because it forms stable disulfide linkages with cysteines. Julia found that 200 µM DTNB inhibits PK-M2 activity.

Figure 27. Effects of DTNB on PK-M2 Activity (Zuercher, 2017). PK-M2 activity is measured as a decrease in absorbance at 340 nm as NADH is oxidized to NAD⁷. Addition of DTNB decreases PK-M2 activity.
DTNB binds to PK-M2’s cysteine residues and precludes IAF labeling. Indeed, addition of DTNB to samples results in about 70% less IAF tagging as determined by SDS-PAGE. Increasing DTNB concentrations result in decreased labeling by IAF.

Figure 29. Effects of DTNB of IAF Tagging: Gel Summary. Average fluorescent intensity obtained from SDS-PAGE was converted to a percent of the control. Values displayed above represent the average of three trials. Addition of DTNB reduces the number of accessible sulfhydryl residues and inhibits IAF tagging.

DTNB binds to PK-M2’s cysteine residues and precludes IAF labeling. Indeed, addition of DTNB to samples results in about 70% less IAF tagging as determined by SDS-PAGE. Increasing DTNB concentrations result in decreased labeling by IAF.

Absorbance measures show an increased generation of TNB$^{2-}$ with the addition of PK. TNB$^{2-}$ is a yellow product, and Figure 30 below shows the extent of its formation at various concentrations of HOCl. No TNB$^{2-}$ is formed when there are no available thiol groups to react with. The addition of HOCl decreases the number of cysteines available to react with DTNB, which decreases the extent of
TNB\(^2\) formation. TNB\(^2\) formation is inversely related to HOCl concentration.

![Figure 30. DTNB Reacts with Cysteines and Forms TNB\(^2\). Control contains only buffer, DTNB, and IAF and shows no generation of yellow TNB\(^2\) product. TNB\(^2\) formation is visible in slight coloration of samples 6, 7, and 8 (each contain PK, buffer, DTNB, and IAF). DTNB reacts with cysteines of PK to form mixed disulfides and TNB\(^2\). Oxidation decreases the number of cysteines available to react with DTNB and decreases of TNB\(^2\) formation.](image)

**FBP Protects PK-M2 from IAF Tagging**

FBP is an allosteric activator of PK-M2. Possible protective effects of FBP on IAF labeling were assessed using SDS-PAGE. Increasing FBP concentrations decreased the extent of IAF tagging, and this effect increased with time.

![Figure 31. Effects of FBP on IAF Tagging: Gel. IAF reacts with reduced thiols to form a stable fluorescent tag. Increasing concentrations of FBP decrease the extent of IAF labeling of PK-M2.](image)
Absorbance measures at 498 nm show a decrease in IAF tagging as FBP concentration increases. This decrease proceeded in a stepwise manner, with 37.5 and 75 µM FBP absorbing with approximately the same intensity. 100 and 150 µM FBP are also similar in absorbance intensity at 498 nm. 330 µM FBP has the lowest absorbance, indicating that increasing FBP concentrations inhibits PK-M2 IAF labeling.

![Graph showing absorbance measures at 498 nm](image)

Figure 32. Effects of FBP on IAF Tagging: Gel Summary. Average fluorescent intensity obtained from SDS-PAGE was converted to a percent of the control. Values displayed above represent the average of two trials. An additional 10 minutes of incubation with FBP further decreases the extent of IAF labeling. Increasing concentrations of FBP decrease the accessibility of cysteines within the protein and inhibit IAF labeling.

Addition of FBP reduces the number of accessible sulfhydryl residues and inhibits IAF tagging.

![Absorbance spectra](image)

Figure 33. Effects of FBP on IAF Tagging: Absorbance Spectra. IAF reacts with reduced thiols to form a stable tag that absorbs at 498 nm. Figure 33.A shows the entire peak at 498 nm. Figure 33.B is zoomed in on the peak’s maximum to show the similar absorption profiles between certain concentrations of FBP. Addition of FBP reduces the number of accessible sulfhydryl residues and inhibits IAF tagging.
Discussion

The accessibility of cysteines within proteins in ligand-dependent (Oberfelder et al., 1984). This research concentrated on understanding the effects of certain ligands and oxidizing environments on the accessibility of sulfhydryl residues in PK-M2.

Oxidation Inhibits PK-M2 Activity and IAF Tagging

Oxidation has been shown to affect the PK-M2’s reactivity and function. Treatment of PK-M2 with HOCl results in oxidation of cysteine residues and the formation of disulfide bridges. These covalent bonds can induce conformational changes in the protein’s tertiary structure. Cys-357, located near the active site, is critical for PK-M2’s enzymatic function. Anastasiou et al. showed that oxidation of this residue results in inactivation of the enzyme (2011). Because of the importance of Cys-357 in catalysis, it is buried in the (β/α)₈-barrel of Domain A.

Figure 34. Structure of pyruvate kinase tetramer (PK) with cysteines. FBP binding site (blue), active site (red), cysteines (green). Drawn from 1A49.pdb

Figure 35. Oxidation Inhibits IAF Labeling. Addition of HOCl causes the formation of disulfide bridges. IAF can only react with reduced thiols, so oxidation decreases the extent of IAF reaction of PK.
It is postulated that 100 µM HOCl oxidizes Cys-357, which results in the protein’s inactivation. Prior to the 100 µM mark, however, other, more exposed cysteines are oxidized. Indeed, at 50 µM, the extent of IAF labeling is already only 32% of theoretical $A_{498}$. The theoretical absorbance of IAF-labeled PK is a function of the concentration of cysteines in the system and the molar extinction coefficient ($\varepsilon$) of IAF (80,000-85,000 M$^{-1}$cm$^{-1}$).

$$A_{theoretical} = [\text{Cys}] \times \varepsilon_{IAF}$$

If all cysteines were tagged, PK-M2 would have an absorbance of 1.68. However, many cysteines in PK-M2 are buried within the tetrameric structure and are thus unlikely to be tagged by IAF without the addition of a detergent. Indeed, the maximum recorded absorbance for PK-M2 is 0.827, representing 49% of all cysteines being tagged. Likely candidates for oxidation or IAF labeling are shown in Figure 37. These cysteines are located near the enzyme’s interface with the solvent and are thus more reactive. Sixteen of 36 total cysteines per PK-M2 tetramer have been identified as particularly susceptible to reaction.

IAF can only react with reduced thiols; PK-M2 oxidation, with its formation of disulfide bridges, inhibits IAF tagging. Increasing concentrations of oxidant in the reaction mixture decrease the
extent of IAF labeling of PK-M2 through a reduction in the number of reduced thiols available for tagging. Addition of 50 and 100 μM HOCl decreases the recorded $A_{498}$ to 32% and 20% of theoretical $A_{498}$ respectively.

Oxidation with HOCl decreases PK-M2 activity by oxidizing a critical residue, Cys-357. Low concentrations of HOCl do not affect PK-M2 activity because this cysteine is buried in the active site. Low concentrations of HOCl do affect IAF tagging because oxidation promotes the formation of disulfide bridges which inhibit IAF tagging ability. As the concentration of HOCl increases, the extent of IAF labeling decreases because more cysteines are oxidized and become inaccessible. HOCl oxidation inhibits PK-M2 activity and IAF labeling via the formation of disulfide bonds.

Ions Protect PK-M2 from IAF Tagging

The addition of ions affected IAF tagging. This inhibition may have resulted from an altered SDS-binding profile in SDS-PAGE, varied labeling affinities for IAF, or protective effects of ions against HOCl oxidation. Further research is necessary to investigate this relationship.
Ions decreased the recorded absorbance at 498 nm across oxidative environments. The absorbance at 0 µM HOCl was quite similar between samples with and without ions. At 50 µM HOCl, ions decreased the recorded absorbance from 32% (without ions) to 17% (with ions) of theoretical \( A_{498} \). At 100 µM HOCl, ions decreased the recorded absorbance from 19% (without ions) to 11% (with ions) of theoretical \( A_{498} \). Addition of 100 mM KCl and 10 mM MgSO₄ to the reaction caused significant changes to the extent of IAF labeling in PK-M2.

![Figure 38. Ions Inhibit IAF Tagging: Theoretical Comparison. IAF reacts with reduced thiols to form a stable tag that absorbs at 498 nm. Oxidation decreases realized absorbance to 49%, 32%, and 20% of theoretical \( A_{498} \) for 0, 50, and 100 µM HOCl respectively. Addition of ions results further diminishes the realized absorbances to 47%, 17%, and 11% of theoretical \( A_{498} \). Addition of ions affects the extent of IAF labeling.](image)

**ATP Protects PK-M2 from Oxidation and IAF Tagging**

Addition of 75 µM ATP was shown to protect PK-M2 activity against oxidation by HOCl. At low concentrations, ATP likely blocks access to Cys-357, a critical residue located near the enzyme’s active site. ATP binds near this residue and blocks both HOCl and IAF from accessing this cysteine. Low concentrations of ATP block access to a few cysteines. At moderate ATP concentrations, this ligand still only inhibits IAF tagging of specific residues, shown as a flattening of the curve in Figure 22. However, when ATP concentrations exceed 150µM, ATP binds less specifically. These high
concentrations result in less specific blocking of cysteines; other cysteines located near the active site include Cys-48, Cys-316, and Cys-325.

![Diagram of PK active site](image.png)

**Figure 39.** Active site of PK. Domain B has been removed, so the viewer is looking down the (β/α)_8-barrel of Domain A. ATP binds in the active site and specifically blocks access to Cys-357 at 75 μM. As ATP concentration increases, the ligand blocks less specifically. Cysteines (green), ions (red), key residues (yellow and blue), pyruvate (magenta). Drawn from 1PKN.pdb

Absorbance measures show than instead of 47% of theoretical A_498, the addition of 75 μM ATP results in 35% tagging of cysteines. This supports the idea that critical cysteines (four per tetramer or 11%) are blocked by ATP. Increasing concentrations of ATP in the reaction mixture
decrease the extent of IAF labeling of PK-M2 by blocking access to cysteines near ATP’s binding site.

Each monomer of PK has an active site with Cys-357; this residue is selectively blocked by moderate concentrations of ATP as the ligand binds in the active site. This selective binding results in the protective effects of ATP. Indeed, binding of ATP blocks access to Cys-357, which prevents oxidation and IAF labeling of this critical residue.

**PEP Has No Effect on PK-M2 Oxidation or IAF Tagging**

At 2 mM, PEP has been shown to make sulfhydryl groups less accessible by increasing the compactness of PK (Oberfelder et al., 1984; Ou et al., 2010). At 75 µM, PEP does not affect the extent of IAF labeling of PK-M2. Though PEP does bind in the protein’s active site like ATP, it did not exert any protective effects against IAF or HOCl accessing Cys-357 or other cysteines near the \((\beta/\alpha)_{8}\)-barrel. Further research could be undertaken to investigate at what concentration PEP begins to affect PK-M2 conformation.

![Figure 40. Effects of ATP on IAF Tagging: Theoretical Comparison. IAF reacts with reduced thiols to form a stable tag that absorbs at 498 nm. Without ATP, \(A_{498}\) nm is about 47% of theoretical \(A_{498}\). With ATP, \(A_{498}\) nm is about 35% theoretical \(A_{498}\). Addition of ATP reduces the accessibility of cysteine residues and inhibits IAF tagging.](image)

![Figure 41. Effects of PEP on IAF Tagging: Theoretical Comparison. IAF reacts with reduced thiols to form a stable tag that absorbs at 498 nm. Oxidation decreases realized absorbance to 37%, 20%, and 7% of theoretical \(A_{498}\) for 0, 50, and 100 µM HOCl respectively. Addition of PEP decreases the realized absorbances to 35%, 16%, and 7% of theoretical \(A_{498}\). Addition of 75 µM PEP has no appreciable effect on the extent of IAF labeling at 0 and 100 µM HOCl and has a small diminishing effect at 50 µM HOCl.](image)
Comparison to the theoretical absorbance at 498 nm yields similar results. At 0 µM HOCl, both samples have about 35% of theoretical $A_{498}$, representing IAF tags on about 13 cysteines per PK tetramer. At 50 µM HOCl, samples without PEP have 20% theoretical $A_{498}$, whereas samples with PEP have 16% theoretical $A_{498}$. This difference may be due to one additional cysteine tag on samples without PEP. At 100 µM HOCl, both samples have about 7% of cysteines tagged with IAF. In sum, 75 µM PEP does not exert substantial protective effects against oxidation or IAF labeling.

**DTNB Inhibits PK-M2 Activity and IAF Tagging**

DTNB inhibits both PK-M2 activity and IAF tagging. DTNB reacts with cysteines, so at 200 µM DTMB, the substrate likely reacts with Cys-357 in PK's active site. The creation of a mixed disulfide at this residue inactivates the protein.

DTNB was used in 10x excess to assess its effects on IAF labeling. DTNB inhibited IAF tagging because it also reacts with reduced thiols. Reaction with DTNB occurred first and replaced many accessible reduced cysteines with mixed disulfides with the concurrent generation of $\text{TNB}^2^-$. $\text{TNB}^2-$ formation was verified using absorbance measurements at 412 nm and by visual comparison. Samples without PK remained clear, whereas samples with PK turned yellow. The addition of PK provided a thiol for the mixed disulfide reaction, which results in the formation of $\text{TNB}^2-$.

DTNB was used to ensure that IAF was tagging cysteines within

![Figure 42. DTNB Inhibits IAF Labeling. DTNB reacts with cysteines (as thiolate anions) to form a mixed disulfide and $\text{TNB}^2-$. $\text{TNB}^2-$ absorbs at 412 nm and its formation causes the solution to turn yellow. IAF is unable to tag the mixed disulfides, so there is no absorbance at 498 nm.](image)
PK. Since DTNB greatly inhibited IAF tagging, we can conclude that IAF also targets PK's cysteine residues.

**FBP Protects PK-M2 from IAF Tagging**

FBP is a potent modulator of PK-M2 activity. Binding of FBP favors the active, tetrameric form of PK. The FBP binding site is removed from the active site, but it is located near Cys-422 (Cys-418 in *S. cerevisiae*). Increasing concentrations of FBP resulted in decreased IAF tagging.

Interestingly, this decrease proceeded in a stepwise manner. The maximum absorbance recorded for this system was 37% theoretical $A_{498}$. Both 37.5 and 75 µM FBP resulted in about 35% of theoretical $A_{498}$. The difference between these two $A_{498}$ is about 2%, which corresponds to a tag on one cysteine residue. An additional cysteine is blocked between 100-150 µM FBP, and another cysteine is blocked at 330 µM FBP. FBP binding limits the extent of IAF tagging.

Figure 43. FBP Binding Protects Cysteines. Cys-422 in rabbit muscle (above Cys-418 in *S. cerevisiae*) is thought to be protected by FBP binding. Cysteines (green), FBP (magenta), coordinated residues (blue). Drawn from 1A3W.pdb.
Figure 4. Effects of FBP on IAF Tagging: Theoretical Comparison. IAF reacts with reduced thiols to form a stable tag that absorbs at 498 nm. Without FBP, $A_{498}$ nm is about 37% of theoretical $A_{498}$. With low concentrations of FBP, $A_{498}$ nm is about 35% theoretical $A_{498}$. Moderate concentrations of FBP result in 34% theoretical $A_{498}$. High concentrations of FBP result in 32% of theoretical $A_{498}$. Addition of FBP reduces the accessibility of cysteine residues and inhibits IAF tagging.
Conclusion

The purpose of this research is to understand the cysteine modifications of PK under physiologically relevant conditions. Cysteines are unique residues with myriad functional properties. However, the chemistry that makes cysteines versatile agents in biochemical processes also makes them vulnerable to oxidation. Within proteins, cysteines can be modified by oxidation and blocked by allosteric regulators.

Oxidation of cysteines can be reversible. A sulfenic acid can form a disulfide bond with a proximal thiol group, which can then be reduced to the two initial sulfhydryls by redox proteins. However, the absence of nearby reduced thiols can result in irreversible oxidation. Both reversible and irreversible oxidation alters protein cysteines.

Allosteric effectors can also modify cysteines. Binding of these ligands can induce conformational changes. For example, PEP has been shown to decrease the accessibility of sulfhydryls in PK by affecting the protein’s global conformation (Oberfelder et al., 1984). In addition to inducing structural changes, allosteric regulators may bind to the enzyme in a location that blocks other species from interacting with cysteines. We postulate that ATP’s binding in the active site precludes IAF tagging or HOCl oxidation of Cys-357. The allosteric regulator—in this case ATP—creates a barrier that protects a critical cysteine. Allosteric effectors modify cysteine accessibility by perturbing protein structure and creating physical shields through their binding.

Our research only touched on the possibilities of the study of cysteine reactivity in glycolytic enzymes. In the future, researchers could investigate the roles of other small molecule allosteric effectors such as alanine, phenylalanine, or serine. These downstream metabolites affect PK activity and bind in a location disparate from PEP/ADP and FBP binding sites. Additionally, it could be interesting to investigate the synergistic effects of allosteric regulators. Oberfelder et al. showed that the
presence of FBP and ions leads to larger conformational changes upon PEP binding in the active site (1984). Our research did not show that PEP affected IAF labeling at 75 μM; however, the addition of FBP or ions to the system may cause PEP to exert protective effects on cysteines. Serine and FBP create synergies for PK activity, but it is unknown what effects these pairings have on cysteine reactivity. Many questions remain to be explored in furthering our understanding of the effects of allosteric regulators on PK and other glycolytic proteins.

Studying cysteine modifications allows us to further our understanding of ROS. Oxidative stress is implicated in many pathologies, from cancer to Alzheimer’s. Cysteine reactivity may be critical to unraveling the consequences of oxidative stress on key proteins like glycolytic enzymes. Furthering our understanding of the effects of ROS on specific proteins could have significant implications in the study, diagnosis, and treatment of ROS-mediated diseases.
## Appendix

### Chemical Index

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<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Structure</th>
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<tr>
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<td>Cys</td>
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<tr>
<td>EGF</td>
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<td>Peroxiredoxins</td>
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<td>Reactive oxygen species</td>
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<td>Superoxide dismutases</td>
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<tr>
<td>Trx</td>
<td>Thioredoxins</td>
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Standard Mixtures for Gel Electrophoresis

*For two 1.5 mm separating gels*

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<tr>
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*For two 1.5 mm stacking gels (4%)*
Bibliography


Pierce Biotechnology. Extinction Coefficients.


