**Front line antioxidant defenses in the freeze
tolerant wood frog, *Rana sylvatica*:
An in-depth analysis of mechanisms of enzyme
regulation.**

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A Thesis Submitted to the Faculty of Graduate Studies and Research in partial fulfillment
of the requirements for the degree of

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The undersigned hereby recommend to the Faculty of Graduate Studies and Research
acceptance of this thesis

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ABSTRACT

The wood frog, *Rana sylvatica*, is one of few species that can survive whole-body freezing during overwintering. Frogs endure freezing of up to 70% of their total body water, and demonstrate a complete lack of respiration, heart beat and brain activity. Freezing imposes multiple stresses including anoxia/ischemia, cellular dehydration when water is lost to extracellular ice masses, wide temperature changes, and potential physical damage by ice. One crucial adaptation for freezing survival is well-developed antioxidant defenses to protect tissues from abiotic stress while frozen and deal with rapid changes in the generation of reactive oxygen species associated with anoxia and reoxygenation over freeze/thaw cycles.

This thesis explores the properties and regulation of key antioxidant enzymes, purified via novel schemes, from frog muscle – both Cu/Zn- and Mn-dependent isoforms of superoxide dismutase (SOD), glutathione reductase (GR), and catalase (CAT). The studies show that changes in activity, stability, and substrate affinity of antioxidant enzymes during the frozen state may be significant preparatory mechanisms employed by *R. sylvatica* to support the transition from frozen to thawed states and deal effectively with oxidative stress accompanying reperfusion. Moreover, reversible protein phosphorylation plays a central role in regulating the activity of these enzymes to suit physiological needs throughout freeze-thaw cycles. For example, CuZnSOD from muscle of frozen frogs showed a significantly higher $V_{\max}$ compared to the control enzyme. Muscle MnSOD from frozen frogs showed a significantly lower K_m for O_2^- , higher phosphorylation, and increased enzyme stability compared to control MnSOD. GR from

frog muscle showed a significantly lower K_m for GSSG in the face of physiological levels of glucose encountered during freezing, as well as the potential for phosphorylation via endogenous kinases. CAT from muscle of frozen frogs showed a significantly lower K_m for H_2O_2 and a higher level of phosphorylation; furthermore, stimulation of endogenous kinases decreased K_m H_2O_2 similar to what occurred in muscle of frozen animals. This thesis provides compelling evidence for regulation of antioxidant enzymes via reversible protein phosphorylation and augmentation of key antioxidant enzymes during freezing of the frog, likely in preparation to endure oxidative stress encountered during reperfusion over winter freeze-thaw cycles.

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LIST OF ABBREVIATIONS

AMP	adenosine monophosphate
AMPK	5' adenosine monophosphate-activated protein kinase
BSA	bovine serum albumin
CAT	catalase
cDNA	complementary deoxyribonucleic acid
C_m	concentration at which half of the protein is unfolded
CuZnSOD	copper and zinc containing SOD
DEAE⁺	diethylaminoethyl cellulose
DEPC	diethylpyrocarbonate
dNTP	deoxyribonucleotide triphosphate
DSF	differential scanning fluorimetry
DTT	dithiothreitol
E_a	activation energy
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
ETS	electron transport system
FeSOD	iron containing superoxide dismutase
GMP	guanosine monophosphate
GPx	glutathione peroxidase
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GST	glutathione-s-transferase
I₅₀	half maximal inhibitory concentration
IMAC	immobilized metal ion affinity chromatography
IP₃	inositol trisphosphate
K_a	half maximal activation concentration

K_m	Michaelis-Menten constant
KPi	potassium phosphate
MAPK	mitogen activated protein kinase
MnSOD	manganese containing superoxide dismutase
mRNA	messenger ribonucleic acid
NAD(P)H	reduced form of nicotinamide adenine dinucleotide (phosphate)
NBT	nitrotetrazolium blue
NiSOD	nickel containing superoxide dismutase
PCR	polymerase chain reaction
PKA	protein kinase A
PKC	protein kinase C
PKG	protein kinase G
PMSF	phenylmethylsulfonyl fluoride
PP1	protein phosphatase 1
PP2A	protein phosphatase 2A
PP2C	protein phosphatase 2C
PVDF	polyvinylidene difluoride
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBARS	thiobarbituric acid reactive substances
TBST	tris buffered saline with tween
T_m	the melting point
V_{max}	maximal velocity
XO	xanthine oxidase
XDH	xanthine dehydrogenase

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Chapter 1:

General Introduction

1. Introduction

1.1. *Oxygen and Oxygen Radicals*

Oxygen has been present in the earth's atmosphere for an estimated 2 billion years, and is believed to have had a biological origin (Embley and Martin, 2006). Specifically, the production of oxygen is thought to be due to the photosynthesis reaction of primitive plant life, likely cyanobacteria. It was only after this event that early eukaryotes would have acquired mitochondria and evolved to use oxygen as the final electron acceptor for the electron transport system (Embley and Martin, 2006). Oxygen has a high redox potential which facilitates the transfer of electrons from reduced molecules, making it an excellent participant in energy production. The use of oxygen as the terminal electron acceptor is vital for complex life forms as it allows for a greater extraction of energy from organic molecules by using oxygen-linked substrate catabolism and ATP production in mitochondria. As a consequence of this oxygen-dependent life, many animals then became highly sensitive to oxygen deprivation. Animal organ systems (particularly mammals) have evolved to function under relatively high oxygen levels, and often remain functional only within a narrow range of oxygen levels. Prolonged situations of hypoxia or anoxia can lead to rapid tissue damage and if extended further, the outcome is death for hypoxia/anoxia intolerant organisms (Brierley, 1977).

Under normal oxygen levels, another consequence of oxygen dependence is the formation of harmful oxygen radicals, or reactive oxygen species (ROS). Gerschmann *et al.* (1954) first proposed that ROS may be responsible for the toxic effects of oxygen. It is now generally accepted that ROS are produced in all aerobic systems in a process where

oxygen is reduced by one electron at a time (univalent reduction) as opposed to the tetravalent reduction of oxygen by cytochrome oxidase. Single electron reduction of oxygen results in the production of ROS intermediates as seen in Figure 1.1 (Sies, 1983).

Figure 1.1. Single electron reduction of oxygen and the ROS intermediates that arise as a result.

One of the prominent sources of ROS formation is the electron transport system (ETS) (Yu, 1994). Typically >95% of oxygen in cells is used by cytochrome oxidase but oxygen is also consumed by a variety of oxygenase enzymes and a small percentage (about 1-3%) also escapes the ETS in the form of ROS (Reiter, 1995). Complex I

(NADH-ubiquinone oxidoreductase) and III (ubiquinol-cytochrome *c* oxidoreductase) of the ETS are capable of one or two electron reductions of oxygen, and can therefore generate superoxide (O_2^-) and hydrogen peroxide (H_2O_2). These are also produced enzymatically by various oxidases including xanthine oxidase (XO), glucose oxidase, NADPH oxidase, and some metabolites such as catecholamines and flavins which are susceptible to auto-oxidation (Yu, 1994; Reiter, 1995). Although O_2^- and H_2O_2 are potentially damaging to cells, they are most damaging when either H_2O_2 alone or O_2^- and H_2O_2 are converted to form hydroxyl radicals ($OH^\bullet$) (Figure 1.1) through catalysis by transition metals (iron, copper) in the Fenton and Haber-Weiss reactions (Basaga, 1990).

Although mitochondrial production of O_2^- is considered the major source of production, many studies have shown that a substantial conversion of xanthine dehydrogenase (XDH) to XO can occur under ischemic conditions (Mink *et al.*, 1990; Hossain *et al.*, 1995; Korthius and Granger, 1986). During ischemic stress, increased use of ATP leads to a degradation of ATP to hypoxanthine (Dorion *et al.*, 1993). XO/XDH can lead to the formation of O_2^- and H_2O_2 by converting hypoxanthine to xanthine or urate (one and two electron reduction, respectively). However, XDH achieves this using either one or two molecules of NAD^+ , whereas XO simply uses O_2 , generating O_2^- and H_2O_2 . Therefore, a conversion of XDH to XO potentially acts as a predictor of ROS formation and ROS-induced tissue damage. This coupled with the fact that iron and copper can be mobilized during ischemic reperfusion can lead to a rapid increase in $OH^\bullet$ formation (Chevion *et al.*, 1993). The activity of XO, however, was not significantly higher in frozen frogs in comparison to control frogs, and the ratio of XO to XDH remained unchanged (Joanisse and Storey, 1996). Other species that withstand reduced

oxygen or blood flow have exhibited changes in either XO activity or the XO/XDH ratio when in the stressed state ((Hermes-Lima and Storey, 1998; Grundy and Storey, 1998). In addition to XO/XDH ratios, tests used to measure oxidative damage reported that oxidative damage was noticeably absent in frozen and thawed frogs, as assessed using the TBARS test (which measures the terminal breakdown of organic hydroperoxide products) and the xylenol orange test (which measures lipid hydroperoxide content of tissues) (Joanisse and Storey, 1996). Taken together, this suggests an increased level of control over oxidative damage, possibly due to augmented antioxidant enzyme regulation.

To minimize ROS damage, cells maintain enzymatic and non-enzymatic antioxidant defenses that are constantly scavenging free radicals and converting them to non-toxic species. One of the ways to prevent ROS formation is to control the concentrations and locations of free transition metals by using ion binding proteins such as transferrin, desferrioxamine and ferritin to sequester them in nonreactive forms (Gutteridge and Quinlan, 1992; Gutteridge and Halliwell, 1990). ROS formation is also regulated by the subcellular localization of the enzymes of the ETS in the mitochondria, as well as selected antioxidant enzymes themselves to detoxify ROS before they can escape the mitochondria.

1.2. Antioxidant Enzymes

The primary antioxidant enzymes involved directly with ROS detoxification are superoxide dismutase (SOD) that converts O_2^- to H_2O_2 , and catalase (CAT) and several

peroxidases that process hydrogen peroxide to non-radical species. Peroxidases are also linked to the detoxification of lipid hydroperoxides, and whereas CAT has also been shown to exhibit similar activity, this is still largely not understood (Fridovich, 1976; Palcic and Dunford, 1980). Another important enzyme family in ROS management is the glutathione-*S*-transferases (GSTs) that have a primary role in the detoxification of xenobiotics via coupling with GSH (Salinas and Wong, 1999). GSTs catalyse the conjugation of glutathione (GSH) to electrophiles; however, GSH can also spontaneously conjugate with electrophiles and directly detoxify oxygen free radicals (Salinas and Wong, 1999). The secondary antioxidant enzyme, glutathione reductase (GR), is charged with the regeneration of reduced GSH from the oxidized (GSSG) form via a NADPH-dependent reaction; this replenishes the GSH pool and the cell's free radical buffering ability (Mullineaux and Creissen, 1997). Figure 1.2, shows a simplified schematic of the relationship of the main players in the antioxidant mitigating pathway.

Figure 1.2. A simple overview of the primary antioxidant pathways and free radical producing reactions.

Changes in antioxidant enzyme activity, specifically those addressed in this thesis, have been observed during low oxygen stress in a wide variety of animals. Organisms experiencing changes in antioxidant enzyme regulation under oxygen deprived states or experiencing reduced blood flow include: the red eared slider turtle (*Trachemys scripta elegans*) which shows fluctuations in GR activity as well as decreased GSH levels in the liver, heart and muscle, decreases in SOD activity in the liver and brain, and decreased CAT activity in the heart kidney and brain during anoxia (Willmore and Storey, 1997); the leopard frog (*Rana pipiens*) which shows a reduction in GR and SOD activity in

muscle tissue during severe dehydration, and shows an increase in CAT activity in muscle tissue during severe dehydration and anoxic conditions (Hermes-Lima and Storey, 1998); the ground squirrel (*Spermophilus citellus*) which shows increased CAT expression and a reduction in CuZnSOD expression during hibernation (Vucetic et al., 2013); the African clawed frog (*Xenopus laevis*) which exhibits increased SOD and CAT expression in both the muscle and liver in response to whole body dehydration (Malik and Storey, 2011); the common carp (*Cyprinus carpio*) which shows increases in GR activity in the brain, increases in SOD activity in kidney and skeletal muscle, and increased CAT activity in the brain and kidneys during hypoxia (Lushchak *et al.*, 2005); the garter snake (*Thamnophis sirtalis parietalis*) which shows an increase in SOD activity during freezing (Hermes-Lima and Storey, 1993); the spadefoot toad (*Scaphiopus couchii*) where GR activity in the liver, heart and kidney was significantly lower during estivation while CAT activity in the liver and heart were significantly higher, while being lower in the kidney during estivation, and SOD activity decreased upon exit from estivation (Grundy and Storey, 1998); the marine periwinkle (*Littorina littorea*) where GR and SOD activity decreased in the hepatopancreas during anoxia as well as foot muscle upon exit from anoxia, and where CAT activity increased in the hepatopancreas during anoxia as well as in hepatopancreas and foot muscle upon exit from anoxia (Pannunzio and Storey, 1998); and the land snail (*Otala lactea*) where SOD activity rose in hepatopancreas and foot muscle, and where CAT activity decreases in foot muscle during estivation during estivation (Hermes-Lima and Storey, 1995).

1.3. *Seasonal Freezing and the Wood Frog*

Throughout the history of our planet subzero temperatures have been an unforgiving and often deadly force claiming the lives of individual animals, and often whole species, that are not adapted to endure these harsh conditions. However, some animals have adapted to live in seasonally cold environments and have developed many different methods to survive prolonged exposure to subzero temperatures. Endothermic animals (mammals, birds) generally use one of three methods; remaining active, migration to warmer climates, or hibernating through the winter. Ectotherms also have three general methods of their own for survival; hibernation in locations that do not experience subzero temperatures (under water or deep underground), or two methods that deal with subzero exposure: freeze-avoidance and freeze-tolerance. Marine fish and many terrestrial invertebrates use freeze-avoidance by amassing high concentrations of antifreeze metabolites and proteins in their internal fluids which allow them to suppress their freezing and supercooling points below environmental temperatures (Storey, 2004). Freeze-tolerance involves a delicate process in which organisms allow ice crystals to form in extracellular spaces while maintaining an intracellular liquid state. This latter method of cold hardiness is used by many insects, intertidal molluscs, and some amphibians and reptiles (Storey and Storey, 2004).

Subzero temperatures and freezing are highly damaging and often lethal for most animals, including humans. To understand the reasons for freezing injury, the stresses that accompany freezing of an organism must be fully explored. These include: (a) freezing causes muscle-based physiological processes such as movement, breathing, and heart beat to grind to a halt; (b) ice crystals can cause severe physical damage to delicate

tissues, particularly rupturing delicate capillaries; (c) ice formation in extracellular spaces sets up an osmotic stress that leads to severe cellular dehydration due to the outflow of water into the extracellular spaces; (d) freezing of body fluids halts blood flow which cuts off of oxygen supply, nutrient flow, waste removal, and the transmission of blood-borne signal hormones/signals to tissues (Storey, 1990; Rubinsky *et al.*, 1987). Furthermore, thawing adds another set of problems such as how to reactivate vital organs, reestablishment of osmotic balance (so as to not swell or burst cells), and dealing with a sudden increase in reactive oxygen species (ROS) formation that is a consequence of the rapid reperfusion of oxygenated blood into tissues.

One organism that has demonstrated an ability to survive these complications of freeze/thaw is the wood frog, *Rana sylvatica*. Previous studies from our lab have demonstrated high survival rates (~100%) of frogs during freezing exposures to temperatures as low as about -5°C (Storey and Storey, 1984) and with ice accumulation in extracellular spaces of up to 65-70% of total body water (Costanzo *et al.*, 1993). Indeed, control over ice nucleation seems to be one of the most important adaptations for freezing survival. By taking control over nucleation and initiating ice formation at a relatively high subzero temperature (e.g. around -2°C), frogs can minimize the speed of ice formation and allow adequate time for their tissues to adapt to coming stressors. Ice nucleation can be triggered by contact with environmental ice which can trigger ice nucleation across the skin, by ice nucleating bacteria found on the skin and in the gut, or by endogenous ice nucleating agents (INAs) found in the plasma (Layne *et al.*, 1990; Lee *et al.*, 1995; Storey, 1990). Figure 1.3 shows the freezing process for *R. sylvatica* highlighting ice nucleation, and the subsequent exotherm that accompanies controlled ice

formation in *R. sylvatica* (Storey and Storey, 1985).

Figure 1.3. Composite freezing curve for *R. sylvatica*. * - Indicates ice nucleation event. (Storey and Storey, 1985).

Ice formation in the extracellular spaces of the frog's body during freezing leads to osmotic stress and dehydration of cells. However, the wood frog limits the amount of dehydration and quells subsequent damage to cell shape and membrane integrity by accumulating and distributing low molecular weight carbohydrate cryoprotectants, primarily glucose, to reduce these osmotic pressures (Storey and Storey, 1984). The production of glucose, among other cryoprotectants, is achieved rapidly during the initial stages of freezing (first few hours) before blood circulation is halted.

1.4. *State of Protein Kinases and Protein Phosphatases in the Wood Frog*

The biosynthesis of glucose as a cryoprotectant is initiated within minutes of freezing exposure in wood frogs, whereas catabolic pathways including glycolysis are suppressed (Storey and Storey, 1986; Cowan and Storey, 2001; Storey, 1987). Glucose production in the liver begins with activation of β -adrenergic membrane receptors which then trigger the production of cyclic AMP that in turn activates cyclic AMP-dependent protein kinase (PKA). PKA then activates phosphorylase kinase which converts glycogen phosphorylase to its active form and initiates the breakdown of glycogen in the liver. Activation of phosphorylase along with inhibitory controls on other enzymes contributes to channelling carbon flow from glucose-1-phosphate to glucose-6-phosphate to glucose which is then exported to the blood (Storey, 1987; Holden and Storey, 1996). Other protein kinases including the mitogen-activated protein kinases (MAPKs), the AMP-activated protein kinase (AMPK) and cyclic GMP-dependent protein kinase (PKG) also show differential regulation in wood frog tissues during freezing, implicating protein kinase regulation in the implementation of multiple survival mechanisms in wood frogs (Cowan and Storey, 2003; Holden and Storey, 1996; Rider *et al.*, 2006). The second messenger of protein kinase C (PKC), D-*myo*-inositol 1,4,5-trisphosphate (IP₃), was also observed to continuously increase (measured between 2 minutes and 24 hours) during freezing in the wood frog, implicating PKC involvement in freeze tolerance (Holden and Storey, 1996). This pattern of PKC second messenger changes was markedly different from that of cyclic AMP which rises immediately upon freezing exposure, before falling gradually over time to as little as 55% of the control value after 24 hours in frozen muscle. Therefore, whereas PKA has an immediate role in activating cryoprotectant

production, PKC seems to have a role in regulating events that occur later in the freezing episode and they are both reasonable candidate protein kinases for controlling antioxidant enzymes during freezing.

1.5. Hypothesis

Key antioxidant enzymes are regulated, likely via post-translational control, over freeze-thaw cycles in tissues of *R. sylvatica*. This control will allow for rapid changes in activity, kinetic properties, or structural stabilization of the enzymes to support fine-tuned rapid changes in antioxidant buffering capacity in response to freezing stress on the organism. The proposed mechanism underlying this is protein phosphorylation mediated by specific serine/threonine protein kinases and protein phosphatases.

To address this hypothesis, I investigated the properties and regulation of specific antioxidant enzymes involved in the detoxification of oxygen radicals and their byproducts, as listed in the following objectives:

Objective 1: Detoxification of superoxide by superoxide dismutase

Although studies have previously demonstrated increased antioxidant capacity in *R. sylvatica* tissues in comparison to freeze intolerant frogs, including increased overall SOD activity, it is not clear how CuZnSOD or MnSOD are independently affected. Since the mitochondria are a major site of ROS production, it would make sense that the

activity of MnSOD would be modified in order to bolster O_2^- detoxification capacity at the main site of its production. In this regard, I expect there to be different forms of regulation governing the activities of each form of SOD that are independent and unique.

Specific Hypothesis 1: SOD will be modified in order to increase the ability of *R. sylvatica* muscle to disproportionate O_2^- molecules as they are formed, with specific attention to the mitochondrial SOD form, since O_2^- production is most concentrated in the mitochondria.

Objective 2: Recycling of glutathione by glutathione reductase

It has been previously reported that GSH levels in *R. sylvatica* are elevated in comparison to freeze intolerant frogs, and that oxidized GSH levels remain largely unchanged across freeze-thaw episodes. This discovery suggests that glutathione recycling could possibly be elevated during freeze-thaw in order to sustain constant GSSG levels, and to provide the capacity to deal with increased GSSG formation due to rapid ROS formation experienced during freeze thaw cycles.

Specific Hypothesis 2: GR will be modified in order to increase the ability of *R. sylvatica* muscle to recycle GSH levels that are needed for detoxification of globally produced reactive oxygen species.

Objective 3: Breakdown of hydrogen peroxide by catalase

CAT is often considered to work in conjunction with SOD to completely detoxify ROS production. In this thesis, catalase function is assumed to be linked with increases in SOD activity to act in tandem, breaking down excess H_2O_2 formed from the dismutation of O_2^- in the mitochondria. In this regard, I predict that there will be regulation of CAT that likely parallels any changes in SOD activity.

Specific Hypothesis 3: Catalase will be modified in order to increase the ability of *R. sylvatica* muscle to breakdown high levels of H_2O_2 , likely produced by an elevated production of H_2O_2 in the mitochondria due to increased SOD activity.

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Chapter 2:

Free-radical first responders: Characterization of CuZnSOD and MnSOD regulation during freezing of the freeze-tolerant North American wood frog, *Rana sylvatica*.

2. Introduction

The wood frog withstands ischemic stress imposed on cells and tissues due to the freezing of blood plasma. The importance of antioxidant enzymes for freeze tolerance stems from the fact that ischemic events are accompanied by the buildup of ROS during, and upon exit from ischemia (Sinclair et al., 2013). Although ROS are generated in several ways, one of the most prominent sources is the electron transport system (ETS) (Yu, 1994). Typically >95% of oxygen in cells is used by cytochrome oxidase but oxygen is also consumed by a variety of oxygenase enzymes and a small percentage also escapes the ETS in the form of ROS (Reiter, 1995). Complexes I (NADH-ubiquinone oxidoreductase) and III (ubiquinol-cytochrome *c* oxidoreductase) are capable of one or two electron reductions of oxygen, and can therefore generate superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). It is estimated that about 1-4% of consumed oxygen is converted to $O_2^{\cdot-}$ due to electron leaks from the mitochondria (Turrens et al., 1982; Konstantinov et al., 1987). $O_2^{\cdot-}$ and H_2O_2 are also produced enzymatically by various oxidases including xanthine oxidase (XO), glucose oxidase, NADPH oxidase, and some metabolites such as catecholamines and flavins which are susceptible to auto-oxidation (Yu, 1994; Reiter, 1995). Although $O_2^{\cdot-}$ and H_2O_2 are potentially damaging to cells, they are most damaging when either H_2O_2 alone or $O_2^{\cdot-}$ and H_2O_2 are converted to form hydroxyl radicals ($OH^{\cdot}$) through catalysis by transition metals (iron, copper) in the Fenton and Haber-Weiss reactions (Basaga, 1990).

Superoxide dismutases (SOD)(E.C. 1.15.1.1) are crucial enzymes involved in the mitigation of oxidative damage by catalyzing the disproportionation of superoxide ($O_2^{\cdot-}$) into oxygen and hydrogen peroxide. Specifically, SOD catalyzes the following reactions:

$O_2^{\cdot-}$ is one of the main reactive oxygen species found ubiquitously across tissues and organisms. SOD enzymes play critical antioxidant roles in all cells and in every known organism. There are several forms of SOD found in nature, these unique metalloproteins are identified through their metal cofactors: 1) copper and zinc containing SOD (CuZnSOD), are typically found in the cytosol of nearly all eukaryotic cells; 2) manganese SOD (MnSOD) is found in the mitochondria or peroxisomes, or 3) nickel (NiSOD) or iron (FeSOD) containing SODs are typically found in prokaryotes, protists and select eukaryotes (Ryan et al., 2010; Bannister et al., 1987; Fridovich, 1975; Ahmad et al., 1988). Of the many forms of SOD, only CuZnSOD and MnSOD are known to exist in frogs (Schinina et al., 1989; Klein et al., 2002; Hudson et al., 2005; Purrello et al., 2005). It has been suggested that the cytoplasmic form, CuZnSOD, acts like a buffer against the buildup of intracellular $O_2^{\cdot-}$, whereas the mitochondrial form, MnSOD, plays a pivotal role in the disproportionation of $O_2^{\cdot-}$ at the major site of production, the electron transport system (Kokoszka et al., 2001). CuZnSOD and MnSOD have been sequenced from the African clawed frog, *Xenopus laevis*, and exist as homodimers with a molecular weights of approximately 32 kDa and 46 kDa, respectively (Schinina et al., 1989; Purrello et al., 2005). The CuZnSOD and MnSOD proteins bind their respective catalytic metal cofactors with only N/O-donors. For MnSODs, the active-site environments include three histidine (His) residues, one aspartate (Asp) side chain, and a H_2O/OH^- ligand, whereas the Cu center in CuZnSOD is ligated by three imidazole ligands, an

imidazolate, and a water molecule (Bordo et al., 1999; Borgstahl et al., 1992; Lah et al., 1995; Tierney et al., 1995). Studies have shown the importance of functional CuZnSOD, since many disease states can be attributed to mutations in the *sod1* gene (Noor et al., 2002). The role of MnSOD in disease is less understood but knockout experiments involving *sod2* show that oxidative stress quickly rises to lethal amounts in knockout animals (Scott et al., 2010; Li et al, 1995, Kokoszka et al., 2001, Duttaroy et al, 2003).

SOD has been widely studied and characterized from traditional animal models (human, rat mouse), however, less is known about its role in aiding survival in the freeze tolerant frog. There is evidence to support the posttranslational modification of enzymes via reversible phosphorylation as a potential control mechanism for altering enzymatic activity in *R. sylvatica* in response to freezing in muscle. This includes changes in the activity and kinetic parameters of protein kinases (AMPK, PKA, PKC, PKG and MAPK) and phosphatases (PP1, PP2A and PP2C) during freezing (Holden and Storey, 1996; Rider et al., 2006; Greenway and Storey, 2000; Macdonald and Storey, 1999). In addition to changes in kinases and phosphatases, freezing-induced changes in the phosphorylation state of various metabolic enzymes have been observed including: lactate dehydrogenase (Abboud and Storey, 2013), hexokinase (Dieni and Storey, 2011), glucose-6-phosphate dehydrogenase (Dieni and Storey, 2010), creatine kinase (Dieni and Storey, 2009), and 5'-adenosine monophosphate deaminase (Dieni and Storey, 2008). This chapter presents the first investigation of the potential regulation CuZnSOD and MnSOD in the leg muscle of *R. sylvatica* comparing control and frozen states and provides evidence for post-translational modification as the mechanism of enzyme regulation.

3. Materials and Methods

3.1. Chemicals

All biochemicals were from BioShop (Burlington, ON, Canada) with a few exceptions; xanthine and xanthine oxidase were from Sigma (St. Louis, MO), immobilized metal ion affinity chromatography (IMAC) chelating fast flow column was from (Pharmacia, Uppsala, SE), and potassium phosphate, monobasic was from J.T. Baker Chemical Company (London, UK).

3.2. Animals

Male wood frogs were obtained from the Ottawa area and were washed in a tetracycline bath before being placed in plastic containers with damp sphagnum moss at 5°C for one week. Control frogs were sampled from this condition. For freezing exposure, frogs were placed in closed plastic boxes with damp paper toweling on the bottom, and put in an incubator set at -3°C. A 45 min cooling period was allowed during which body temperature of the frogs cooled to below -0.5°C, and nucleation triggered due to skin contact with ice crystals formed on the wet toweling. Frogs were exposed to freezing conditions for 24 hours. Control and frozen frogs were euthanized by pithing. Muscle tissues were quickly excised and flash frozen in liquid N₂. All tissue samples were stored at -80°C until use. The Carleton University Animal Care Committee, in accordance with the Canadian Council on Animal Care guidelines, approved all animal handling protocols used during this study.

3.3. *Preparation of muscle tissue lysates for protein purification*

For protein purification, samples of frozen muscle tissue were homogenized 1:5 w:v in ice-cold homogenizing buffer A [20 mM potassium phosphate (KPi) buffer, pH 7.2, containing 15 mM β -glycerophosphate, 1 mM EGTA, 1 mM EDTA, 5% v:v glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were then centrifuged at $13,500 \times g$ at 4 °C and the supernatant was collected for use in protein purification.

3.4. *Preparation of muscle tissue lysates for determination of relative protein levels*

Samples of muscle tissue were crushed under liquid nitrogen and then homogenized 1:2.5 w:v in homogenizing buffer B (20 mM HEPES, pH 7.5, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4 , 10 mM β -glycerophosphate) with a few crystals of PMSF and 1 $\mu\text{L}/\text{mL}$ Sigma protease inhibitor cocktail (104 mM AEBSF, 80 μM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, 1.5 mM Pepstatin A). Samples were homogenized on ice with a Polytron PT1000 homogenizer and centrifuged at 4 °C for 15 min at $10,000 \times g$. Soluble protein concentrations were assessed using the BioRad protein assay with bovine serum albumin (BSA) as the standard according to manufacturer's instructions. All samples were adjusted to 10 $\mu\text{g}/\mu\text{L}$ by adding a calculated small volume of homogenizing buffer B. Aliquots of samples were then mixed 1:1 v:v with 2 $\times$ SDS loading buffer (100 mM Tris-base, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercaptoethanol). Final sample concentrations

were 5 $\mu\text{g}/\mu\text{L}$. Proteins were denatured by placing the tubes in boiling water for 5 min. Samples were stored at $-80\text{ }^{\circ}\text{C}$ until use.

3.5. *Purification of CuZnSOD and MnSOD*

A 3 mL aliquot of crude supernatant was applied to a DEAE⁺ column (1.5 cm $\times$ 20 cm), previously equilibrated with 50 mL of homogenization buffer A. The DEAE⁺ column was then washed with 50 mL of buffer A to remove unbound protein. SOD was eluted from the DEAE⁺ column with a linear gradient of 0-1 M KCl in homogenization buffer A. Fractions of 1.24 mL were collected and 5 μL from each fraction was assayed to detect SOD activity (see kinetic assays section below for methodology). The fractions of peak SOD activity were pooled and applied to Sephadex G-50 gel buffer exchange columns equilibrated with buffer C (20 mM KPi buffer, pH 7.2 containing 15 mM β -glycerophosphate, 5% v:v glycerol). A 5 cm column of Sephadex G-50 in a syringe barrel was equilibrated in buffer C and centrifuged at $500 \times g$ in a bench-top centrifuge for 2 min to remove excess buffer. Then a 500 μL aliquot of SOD eluant was applied to each buffer exchange column and centrifuged again. The resulting eluant was collected and applied to an IMAC column pre-charged with 1 mg/mL $\text{CuSO}_4 \cdot \text{H}_2\text{O}$ (1.5 cm $\times$ 10 cm), equilibrated in homogenization buffer C. The copper chelate affinity column was then washed with 50 mL of homogenization buffer C to remove unbound protein. Bound proteins were eluted with a linear gradient of 0-1 M KCl in homogenization buffer A. Fractions of 1.24 mL were collected and 10 μL from each fraction was assayed to detect SOD activity. Fractions containing the highest SOD activity from the copper chelate

affinity column were pooled and used for further study. The purity of SOD was determined by combining samples with equal volume of 2× SDS loading buffer, boiling for 5 min, and then running 20 µL of sample purified from each successive purification on SDS-PAGE (see below gel electrophoresis and Western Blotting section). Protein banding was visualized with Coomassie blue.

3.6. *Kinetic assays*

SOD activity was assayed based on the generation of superoxide radicals formed by xanthine and xanthine oxidase: 100 mM KPi buffer (pH 7.4), 1 mM xanthine, 2 U xanthine oxidase, 2 mM nitrotriazolium blue (NBT). This assay mixture generates $O_2^{\cdot-}$, which oxidizes NBT to form formazan dye. SOD (contained within the tissue homogenate supernatant) inhibits the oxidation of NBT by catalyzing the dismutation of $O_2^{\cdot-}$ generated in the xanthine oxidase reaction. One unit of enzyme activity is the amount of SOD that inhibits the reduction of nitrotriazolium blue by 50% at 25°C. The formation of formazan was measured at 545 nm in a Thermo Labsystems Multiskan spectrophotometer (Thermo Scientific, Waltham, MA, USA). Data were analyzed using the Kinetics v.3.5.1 program (Brooks 1992).

3.7. *Gel electrophoresis and Western blotting*

Protein separation was achieved by running samples on 12% (samples from protein purification steps) or 15% (samples from protein stability assays or from tissue lysates) SDS-PAGE gels. Sample aliquots were loaded onto polyacrylamide gels together

with pre-stained molecular weight standards (FroggaBio; Cat# PM005-0500) and separated using a discontinuous buffer system. Electrophoresis was carried out at 185 V for 45 min using the BioRad Mini-Protean 3 system with 1x Tris-glycine running buffer. Proteins on the gel were then electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using a BioRad mini Trans-Blot cell. The transfer was carried out at 160 mA for 1.5 h. Following the transfer, membranes were washed in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) for 3×5 min. The membranes were blocked using 2.5% skim milk in TBST for 30 min. After blocking, the membranes were probed with primary antibody diluted 1:1000 v:v for SOD1 or 1:2000 v:v for SOD2 in TBST for 3 h at room temperature (RT). Primary antibodies were rabbit anti-SOD1 (GenScript, A01005) and rabbit anti-SOD2 (Stress Marq BioSciences, SPC-118C). The membranes were washed 3×5 min with TBST at RT and probed with goat anti-rabbit-peroxidase secondary antibody for 30 min. Membranes were washed again 3×5 min in TBST at RT and were then developed using enhanced chemiluminescence.

Sod1 and *sod2* antibodies cross-reacted with bands corresponding to molecular weights of ~15 kDa and ~26 kDa, respectively. Blots were developed with enhanced chemiluminescence reagents. Images were captured using a ChemiGenius Bio-Imaging system with GeneSnap software and densitometry analysis performed using GeneTools software (Syngene, Frederick, MD). The intensity of the immunoreactive bands were standardized against a group of at least 3 bands, not located close to the band of interest, in the corresponding Coomassie blue stained membrane. The intensity of the immunoreactive bands from control muscle tissue was set to a reference value of 1 to

which the intensity of standardized immunoreactive bands from frozen muscle tissue was expressed as a fold change.

3.8. *Determination of protein stability*

Frog muscle tissue was homogenized as previously described in section 2.2, without the use of PMSF. To assess the possibility of structural/conformational changes in MnSOD or CuZnSOD between the control and 24 h frozen conditions, the susceptibility of both forms of SOD to denaturation by urea (0-7 M) was evaluated. Four separate preparations of crude muscle extracts were incubated with different concentrations of urea for 24 h at 4°C in buffer A without the addition of PMSF. After incubation, each sample was subjected to pulse proteolysis as outlined by Park and Marqusee (2005) to cleave unfolded, denatured enzyme. To do this, thermolysin (0.40 mg/mL in 50 mM Tris, pH 8.0, 2.5 M NaCl, 10 mM CaCl₂) was added to initiate proteolysis. Various incubation times with thermolysin were explored to discover the optimum amount of digestion time required, resulting in the use of a 30 min incubation optimum. Following a 30 min pulse reaction time at 4°C, the reaction was quenched by mixing samples 1:1 v:v with 6x SDS loading buffer (375 mM Tris buffer, pH 6.8, 9% w/v SDS, 50% v/v glycerol, 0.03% w/v bromophenol blue, 10% v/v 2-mercapotethanol) and subsequently boiled for 5 minutes and stored at -20°C until used. Western blotting was then performed as above and MnSOD and CuZnSOD band intensities were quantified to reveal the amount of undigested enzyme remaining. From this, C_m values were calculated, representing the amount of urea that reduced the amount of folded

protein by one-half.

3.9. *ProQ Diamond Phosphoprotein Staining*

Enzyme extracts from both control and frozen frog muscle for MnSOD and CuZnSOD were purified as described in section 2.4. The top three fractions based on activity were pooled, and protein levels in the pooled fractions were quantified using the Coomassie blue dye-binding method. Aliquots of the pooled fractions were then mixed 1:1 v:v with SDS loading buffer (100 mM Tris buffer, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercapotethanol) and subsequently boiled for 5 minutes and stored at -20°C until used.

Equal volumes of each sample were loaded on a 12% SDS-PAGE gel. The gel was run at 185 V for 45 min in running buffer (0.5 M Tris, 5 M glycine, 0.5% w/v SDS). The gel was removed and washed in fixing solution (50% v/v methanol, 10% v/v acetic acid) twice for 10 min, then left in fixing solution overnight at 4°C followed by 3 washes with ddH₂O for 10 min. The gel was then stained with ProQ Diamond Phosphoprotein stain (Invitrogen, Eugene, OR) for 90 min and washed 3 times with ddH₂O for 10 min. The gel was covered during staining (and for the remainder of the protocol) with aluminum foil to prevent the photosensitive stain from interacting with light. To minimize non-specific background, the gel was washed in ProQ Diamond destaining solution (20% v/v acetonitrile, 50 mM sodium acetate, pH 4) for 45 min, and washed 3 times in ddH₂O for 10 min. Bands on the gel were then visualized and quantified using the Chemi-Genius.

3.10. *Dot blotting*

The phosphorylation of MnSOD was further assessed by applying equal amounts of purified enzyme from the muscle of control and frozen frogs onto nitrocellulose membranes (Bio-Rad, Hercules, CA, USA) using a Bio-Dot micro-filtration apparatus (Bio-Rad, Hercules, CA, USA), as previously described (Dawson et al., 2013). Primary antibodies (diluted 1:1000 v:v) were then applied to different wells and membranes were allowed to incubate overnight at 4°C with gentle rocking. Primary antibodies used were: (1) rabbit anti-phosphoserine (Cat #618100, Invitrogen, Carlsbad, CA, USA); (2) rabbit anti-phosphothreonine (Cat. # 718200, Invitrogen, Carlsbad, CA, USA); or (3) rabbit anti-phosphotyrosine (Cat # 615800, Invitrogen, Carlsbad, CA, USA).

3.11. *RNA isolation*

Total RNA from frog muscle tissues was extracted using Trizol™ reagent (Invitrogen) according to manufacturer's instructions and as described previously (Katzenback et al., 2014). The RNA concentration of each sample was determined on a GeneQuant Pro spectrophotometer (Pharmacia, Markham, Ontario, Canada) at 260 nm. RNA purity was assessed using a ratio of absorbance at 260/280 nm, while RNA quality was examined by observing the integrity of 18S and 28S ribosomal RNA (rRNA) bands on a native agarose gel following electrophoresis with SYBR green staining. Total RNA was isolated from the muscle of 4-5 control frogs and 4-5 frozen frogs for use in cDNA synthesis.

3.12. *cDNA synthesis*

Five µg of total RNA in a volume of 10 µL was used for first strand cDNA synthesis. One µl of oligo-dT (200 ng/µL; 5'-TTTTTTTTTTTTTTTTTTTTTTTTTV-3'; V = A or G or C) (Sigma Genosys) was added to each sample before samples were incubated at 65°C for 5 min in a thermocycler (Mastercycler Eppendorf) and then chilled on ice for 5 min. Four µL of 5× first strand buffer (Invitrogen), 2 µL 100 mM DTT (Invitrogen), 1 µL 10 mmol/L dNTPs (Bio Basic), and 1 µL Superscript II reverse transcriptase (Invitrogen) were added to each reaction and further incubated at 42°C for 45 min in an Eppendorf thermocycler (Mississauga, Ontario, Canada). Synthesized cDNA samples were stored at -20°C until use.

3.13. *RT-PCR*

Synthesized cDNA samples were checked for potential genomic contamination using the α -tubulin primer set that was designed to span an intron. All cDNA samples were negative for genomic contamination. For gene expression studies, serial dilutions of cDNA (10^{-1} and 10^{-2}) were made to identify the dilution that produced non-saturating yet visible bands for the gene of interest following PCR amplification for quantification purposes. The primer sequences for target genes were (1) *sod1* sense 5'-tgaaggcvgtgtgtgtgghtgaaggga-3', antisense 5'-ccaatnacccacaagccaracg-3', (2) *sod2* sense 5'-ctggacaaacctctctccta-3', antisense 5'-cttgtaagcggttagattcttg-3', and (3) *α -tubulin* sense 5'-aaggaagatgctgccataa-3', antisense 5'-ggtcacatttcaccatctg-3'.

PCR reactions consisted of 5 μ L of diluted cDNA, 15 μ L of DEPC treated water, 1.25 μ L of primer mixture (0.03 nmol/ μ L), 0.75 μ L of 10 $\times$ PCR buffer (Invitrogen), 1.5 μ L of 50 mmol/L MgCl₂, 0.5 μ L of 10 mmol/L dNTPs, and 1 μ L of *Taq* polymerase. Thermocycling conditions were: (1) 10 min at 94°C, 20 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 45s for *sod1*, (2) 10 min at 94°C, 24 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 45s for *sod2*, or (3) 10 min at 94°C, 22 cycles of 94°C for 30 s, 53.2°C for 30 s, 72°C for 45s for *α -tubulin*. All thermocycling protocols included a final elongation of 72°C for 7 min. Following amplification, 3 μ L of DNA loading dye (1 μ L 2x SYBR[®] Green I nucleic acid gel stain (Invitrogen) + 2 μ L of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, and 40% sucrose in ddH₂O) was added to each PCR reaction. A 14 μ L aliquot of each sample was loaded into 1.5% (*α -tubulin* and *sod1*) or 2.0% (*sod2*) agarose gels and electrophoresed in 1 $\times$ TAE buffer (2 mol/L Tris base, 1.1 mL acetic acid/L, 1 mmol/L EDTA, pH 8.5) for 25-30 min. PCR-amplified products were visualized using the Chemi-Genius BioImaging system. The intensity of the *sod1* or *sod2* PCR products were expressed as a ratio against the corresponding band intensities of *α -tubulin* amplified from the same cDNA sample to correct for any minor variations in sample loading. The ratio of target gene to the reference gene (*α -tubulin*) from the muscle of control frogs was set to a reference value of 1, to which the relative mRNA levels of the target gene in the muscle of frozen frogs was expressed as a fold change. Each data bar represents the means $\pm$ s.e.m. derived from n = 4-5 different animals per group.

The PCR products were isolated from gel slices using the freeze/squeeze method and sequenced at Bio Basic (Markham, ON, Canada) as previously described

(Katzenback et al., 2014). Sequence identities were confirmed by sequence comparison in BLASTn.

3.14. *Statistical analysis*

Comparison of enzyme kinetics, relative protein and mRNA levels were performed using a Student's t test, two-tailed, assuming unequal variances. A probability of $P < 0.05$ was considered significant.

4. **Results**

4.1. *Purification of CuZnSOD and MnSOD from the muscle of control and frozen frogs*

The purification schemes used for wood frog muscle CuZnSOD and MnSOD are shown in Table 2.1 and Table 2.2, respectively. The procedure used ion exchange chromatography on DEAE Sephadex (Figure 2.1A), a buffer exchange step to remove EDTA/EGTA, and copper chelate affinity chromatography. The buffer exchange step was crucial to this purification procedure, since the EGTA and EDTA that were in homogenization buffer A caused the copper to dissociate from the copper chelate column. CuZnSOD eluted from the copper chelate affinity column at approximately 200 mM KCl whereas MnSOD eluted at approximately 400 mM KCl (Figure 2.1B). Frog muscle CuZnSOD was purified 209 fold with an overall yield of activity of 10.8% (Table 2.1). The final specific activity of CuZnSOD was 1385 mU/mg protein (Table 2.1). Frog

muscle MnSOD was purified 234 fold with an overall yield of activity of 20.1% (Table 2.2). The final specific activity of MnSOD was 1550.69 mU/mg protein (Table 2.2). The success of the purification process was assessed using SDS-PAGE gel stained with Coomassie blue staining in conjunction with western blotting. This showed that both CuZnSOD and MnSOD enzymes were purified to near homogeneity as there was one band corresponding to correct molecular weight of ~15 kDa for CuZnSOD and ~26 kDa for MnSOD (Figure 2.3).

4.2. *Kinetic characterization of CuZnSOD and MnSOD*

Kinetic parameters of purified muscle SOD were assessed to determine if the enzyme's properties differed between control and frozen animals. For both CuZnSOD and MnSOD, kinetic parameters changed during freezing in comparison to the control enzyme ($p < 0.05$) (Table 2.3). The frozen form of MnSOD had an apparent K_m for $O_2^{\cdot -}$ that was 34% lower than the control enzyme. In the case of CuZnSOD, however, there was no significant change in the apparent K_m of $O_2^{\cdot -}$ between control and frozen groups. The V_{max} did not change significantly for MnSOD in frozen muscle in comparison to control muscle tissue; however, there was a significant 1.52 fold increase in the V_{max} for CuZnSOD during freezing.

4.3. *mRNA levels of sod1 and sod2*

Using homology based primers, partial sequences for *sod1* and *sod2* in *R. sylvatica* were obtained. The partial sequences of *R. sylvatica* *sod1* and *sod2* were

submitted to NCBI Genbank, accession numbers KJ847420 and KJ87421, respectively. The *R. sylvatica sod1* partial open reading frame was 392 bp, corresponding to 130 amino acids (representing 84.4% of the 154 aa of human CuZnSOD, 86.6% of 150 aa of *Rana catesbeiana* CuZnSOD, 86.1% of 151 aa of *X. tropicalis* CuZnSOD, or 86.1% of 151 aa of *X. laevis* CuZnSOD), as predicted by the Translate Tool (ExPASy, <http://web.expasy.org/translate/>). The 130 aa obtained for *R. sylvatica* CuZnSOD correspond with aa 3-134 of human CuZnSOD. *R. sylvatica sod2* partial open reading frame was 393 bp, corresponding to 130 amino acids (representing 58.6% of 222 aa of human MnSOD, 58.0% of 224 aa of *X. tropicalis* MnSOD, or 58.0% of 224 aa of *X. laevis* MnSOD), as predicted by the Translate Tool (ExPASy, <http://web.expasy.org/translate/>). The 130 aa obtained for *R. sylvatica* MnSOD correspond with aa 61-190 of human MnSOD.

A blastp search of the partial *R. sylvatica* CuZnSOD predicted protein sequence against the NCBI database showed the sequence to have 94% identity to *Rana catesbeiana* CuZnSOD (Genbank accession number AC051906) and 69% identity to *Xenopus tropicalis* CuZnSOD (Genbank accession number XP_007247311). Similarly, a blastp search of the partial *R. sylvatica* MnSOD predicted protein sequence showed the highest similarity to *X. tropicalis* MnSOD (Genbank accession number NP_001005694) with 90% identity.

Primers were designed based on the *R. sylvatica sod1* and *soda* sequences for use in relative quantitation of mRNA levels in the muscle of control and frozen frogs. *Alpha-tubulin* was amplified from the same samples as an endogenous control. Transcript levels

of *sod1* or *sod1* in the muscle of 24 h frozen frogs were not significantly different from those in the muscle of control frogs (Fig. 4A, $p > 0.05$).

4.4. *Relative protein levels of CuZnSOD and MnSOD*

Western blotting was used to evaluate the relative amount of CuZnSOD and MnSOD protein in muscle from control versus frozen frogs. The antibodies for CuZnSOD or MnSOD each cross-reacted with one band corresponding to the known molecular weights of ~15 kDa and ~26 kDa, for CuZnSOD and MnSOD, respectively (Figure 2.3). No significant differences were detected between the protein levels in muscle of control versus frozen frogs for either CuZnSOD or MnSOD (Figure 2.4B).

4.5. *Post-translational modification of CuZnSOD and MnSOD*

To test whether or not reversible phosphorylation was the mechanism by which SOD kinetic parameters changed during anoxia, the top activity fractions from the purification profiles for the control and frozen forms of CuZnSOD or MnSOD were combined, run on an SDS-PAGE gel, and stained with ProQ Diamond phosphoprotein stain. CuZnSOD did not react with the ProQ Diamond phosphoprotein stain, resulting in no apparent banding (data not shown). MnSOD was easily identified as a strong band found at ~23 kDa. When band densities were quantified, they showed a 2.87 fold higher band intensity ($p < 0.05$) for MnSOD from muscle of frozen frogs when compared to the enzyme from control frogs (Figure 2.5).

Immunoblotting using a dot blot apparatus was used to assess the possible differences in the amino acid-specific phosphorylation of muscle MnSOD between control and frozen states. Phosphorylation of serine residues of MnSOD from frozen frogs was 2.37 ± 0.37 fold higher compared to control SOD (1 ± 0.14 , $P < 0.05$; Figure 2.5). Phosphorylation via tyrosine residues of frozen SOD was 1.27 ± 0.05 fold higher compared to control MnSOD (1 ± 0.06 , $P < 0.05$; Figure 2.5). The phosphorylation levels of threonine residues between control MnSOD and frozen MnSOD did not differ ($P > 0.05$; Figure 2.5).

4.6. *Scansite Prediction of Phosphosites*

The Scansite application from the Massachusetts Institute of Technology (<http://scansite.mit.edu/>) was used to analyze the obtained partial protein sequences for CuZnSOD and MnSOD for putative phosphorylation sites. Putative consensus sequences for phosphorylation sites of Erk1 kinase (threonine) and protein kinase C delta (PKC delta) (serine) were predicted for CuZnSOD (Figure 2.6A, 2.6B). Putative consensus sequences for phosphorylation sites of Akt kinase (threonine), calmodulin dependent kinase 2 (threonine), casein kinase 1 (serine), Erk1 kinase (serine), PKC alpha (serine), PKC beta (serine), PKC gamma (serine), PKC delta (serine, threonine), PKC epsilon (serine, threonine), PKC zeta (serine), and PKC mu (serine, threonine) were predicted for MnSOD (Figure 2.6C, 2.6D).

Human CuZnSOD was analyzed for comparison using Scansite, resulting in the following putative consensus sequences for phosphorylation sites: casein kinase 1

(threonine), PKC alpha (serine), PKC beta (serine), PKC gamma (serine), and PKC mu (serine). Human MnSOD was also analyzed using Scansite, the following putative consensus sequences for phosphorylation sites were obtained: AMP kinase (serine), calmodulin dependent kinase 2 (serine), casein kinase 1 (serine), Erk1 kinase (serine), Cdk2 kinase (serine), Cdk5 kinase (serine) PKC alpha (serine, threonine), PKC beta (serine, threonine), PKC gamma (serine, threonine), PKC delta (serine, threonine), PKC epsilon (threonine), and PKC zeta (serine). In all cases, except for casein kinase 1 for CuZnSOD, the obtained sequences for *R. sylvatica* encompassed the predicted putative phosphorylation sites for both CuZnSOD and MnSOD in humans.

4.7. *Structural stability of CuZnSOD and MnSOD*

The structural stability of CuZnSOD and MnSOD was evaluated by testing enzyme sensitivity to urea denaturation. This was evaluated by incubating muscle extracts from both control and frozen frogs for 24 h at ~4°C in the presence of 0–7 M urea. Urea-denatured protein was then catabolized by 30 min proteolysis with thermolysis and then the remaining amount of native protein was assessed by Western blotting. The calculated C_m value (concentration of urea that results in a 50% loss of folded enzyme) was 0.72 M for CuZnSOD from muscle of frozen frogs, which was not significantly different than the value of 0.62 M for CuZnSOD in control muscle extracts (Table 2.3; Figure 2.7). The calculated C_m value was 2.53 M for MnSOD from frozen frog muscle extracts, which was not significantly different than the value of 2.92 M for MnSOD in control muscle extracts (Table 2.3; Figure 2.8).

5. Discussion

SOD is ubiquitous among living organisms, from prokaryotes to eukaryotes, and protects organisms from superoxide radicals that can oxidize and damage cellular macromolecules (Fridovich, 1995; McCord, 1995; Iqbal et al., 1989). Diverse human disease states are associated with abnormalities in the structure, function or levels of SOD including: several forms of anemia, thalassemia, molecular dystrophy and cystic fibrosis; dengue fever; lateral sclerosis; rheumatoid arthritis; ischemic injury; Parkinson's disease; Alzheimer's disease and cancer (Panchenko et al., 1979; Mavelli et al., 1984; Mizuno, 1984; Ratovski et al., 1996; Concetti et al., 1976; Ray et al., 1984; Gonzales et al., 1999; Stieber et al., 2000; Famulari et al., 1990; Zemlan et al., 2000; Rhodin et al., 2000). Given the importance of SOD function for the survival and quality of life in humans, as well as other organisms, there is great interest in characterizing unique forms SOD, as well as the elucidation of SOD regulation under conditions that mimic disease-like states. Many of the diseases mentioned above have in common fluctuations in oxygen levels or a reduction in blood flow. To identify the importance of SOD action in both normal and disease states, various experimental approaches have been used such as knockout experiments (Scott et al., 2010; Li et al., 1995; Kokoszka et al., 2001). These have had varying success; for example, MnSOD knockout experiments with animals are uniformly lethal, making it difficult to assess the actual role of MnSOD in the disease state (Melov et al., 1999; Kokoszka et al., 2000). Another way to better understand the role of SOD in disease, specifically the role of MnSOD, is to characterize the behavior and properties of CuZnSOD and MnSOD from animals that can withstand extreme conditions such as oxygen limitation, reduced blood flow, dehydration and ischemia. Studies examining the

characteristics of SOD regulation in different situations of environmental stress have included: the red eared slider (*Trachemys scripta elegans*) which shows decrease in SOD activity in the liver and brain during anoxia (Willmore and Storey, 1997), the leopard frog (*Rana pipiens*) which shows a reduction in SOD activity in muscle tissue during severe dehydration (Hermes-Lima and Storey, 1998), the ground squirrel (*Spermophilus citellus*) which show a reduction in CuZnSOD expression in white adipose tissue during hibernation (Vucetic et al., 2013), the African clawed frog (*X. laevis*) which shows MnSOD protein and transcript levels rose in the liver during estivation, the common carp (*Cyprinus carpio*) which shows increases in SOD activity in kidney and skeletal muscle during hypoxia (Lushchak et al., 2005), the garter snake (*Thamnophis sirtalis parietalis*) which shows an increase in SOD activity during freezing (Hermes-Lima and Storey, 1993), the spadefoot toad (*Scaphiopus couchii*) where SOD activity decreased upon exit from estivation (Grundy and Storey, 1998), the marine periwinkle (*Littorina littorea*) where SOD activity decreased in the hepatopancreas as well as foot muscle during anoxia (Pannunzio and Storey, 1998), and the land snail (*Otala lactea*) where SOD activity rose in hepatopancreas and foot muscle during estivation (Hermes-Lima and Storey, 1995). In the case of freeze-tolerance, ischemic stress is imposed on the cells of *R. sylvatica* (Storey and Storey, 1986). The link between free radical production in disease-induced and naturally occurring ischemic events has been widely characterized, from oxidative stress experienced during the ischemic episode, to the burst production of ROS upon re-oxygenation during reperfusion (Ferrari et al., 1991; Simpson and Lucchesi, 1987; Zweier and Talukder, 2006). It is clear from these studies that SOD plays a unique role in animal ability to overcome stressful conditions, and the data presented in this chapter not

only agree with this, but suggest another level of complexity in the mitigation of free radical damage sustained by overwintering frogs.

5.1. *Kinetic changes of CuZnSOD and MnSOD during freezing*

The kinetic properties of wood frog skeletal muscle SODs changed significantly between control and frozen conditions, indicating possible regulation during freezing. Past studies have shown a potential decrease in total SOD activity in muscle, kidney and heart of *R. sylvatica* during freezing, but those experiments used the method of Paoletti *et al.* (1986) which measures SOD activity using the reduction of NADH (Joanisse and Storey, 1996). That method of measuring total SOD activity has been shown to elicit different activity profiles than the NBT reduction method used in this experiment (Paoletti *et al.*, 1986). The use of NADH as a reporter molecule could well be associated with a high degree of background activity due to the presence of many NADH utilizing enzymes found endogenously in tissues. In the present study, CuZn and Mn SODs were explored independently and in a purified state, CuZnSOD exhibited a 1.52 fold higher maximal activity in the muscle of frozen frogs in comparison to control frogs, whereas MnSOD showed no change in maximal activity during freezing. An increased CuZnSOD activity could allow for increased cytoplasmic superoxide scavenging potential during freezing, as well as during reperfusion.

The apparent K_m for $O_2^{\cdot -}$ of CuZnSOD did not change significantly between control and frozen states (Table 2.3). However, in the case of MnSOD, the apparent K_m decreased from 0.201 mM under control conditions to 0.133 mM during frozen

conditions. This reduction in the apparent K_m of $O_2^{\cdot-}$ would suggest that MnSOD has a higher affinity for $O_2^{\cdot-}$ during freezing. An increase in affinity for superoxide would increase the scavenging ability of MnSOD, and increase the protection against the formation of superoxide at a major site of production, the mitochondria. Since superoxide is a charged molecule, it is not thought to be membrane permeable (Florian et al., 2004; Mao and Poznansky, 1992). Therefore, an increase in mitochondrial production of superoxide could lead to a compromised mitochondrial membrane, and ultimately the destruction of the cell due to oxidative damage. Mitochondrial superoxide production has been shown to trigger apoptosis, while cytosolic production of $O_2^{\cdot-}$ has been linked to many cell signaling events (Thannickal and Fanburg, 2000). This would suggest that CuZnSOD is more likely to have a role in regulating cell signaling events, whereas MnSOD is more likely to have a role in keeping $O_2^{\cdot-}$ levels in the mitochondria low, staving off apoptotic cell death. This is of special interest to wood frogs since the muscle tissue thaws later than internal organs such as the heart or liver (Rubinsky et al., 1994). Since muscle thaws last in *R. sylvatica*, during the spring thaw, rapid and successful disproportionation of $O_2^{\cdot-}$ would aid a rapid recovery of muscle functionality. Improved affinity for $O_2^{\cdot-}$ by MnSOD in the frozen frog may aid the peripheral tissue during thawing, allowing for reintroduction of re-oxygenated blood into the tissue without sustaining a buildup of oxidative stress.

5.2. *Regulation of CuZnSOD and MnSOD transcript and protein levels during freezing*

The CuZnSOD sequence discovered in this study showed 62% amino acid identity when compared to human MnSOD (accession number NP_000445) at 90% coverage whereas wood frog MnSOD showed 84% amino acid identity as compared to human (accession number NP_000627) at 99% coverage. Frog transcript and protein levels did not change significantly between control and 24 h frozen frog muscle for either CuZnSOD or MnSOD. This is important as it highlights the probable importance of kinetic modification of both forms of SOD during freezing as the means of adjusting SOD functionality. Since protein amounts do not change, the $V_{\max}$ difference for CuZnSOD from control versus frozen frogs cannot be attributed to an enhanced amount of CuZnSOD in the frozen state, and therefore, is likely due to a true functional difference in enzyme activity. Since MnSOD maximal activity did not change significantly during freezing, this result simply highlights the importance of the decreased K_m for $O_2^{\cdot -}$. During periods of stress, like freezing, it is too energetically costly to increase the amounts of enzymes present and it is more energetically favourable to modify the existing enzyme to achieve a more active state (Storey and Storey, 1984; Storey and Storey, 2004; Storey, 2004). Since there were no significant changes in the transcript levels for either CuZnSOD or MnSOD, it would appear that an increase in production of new SOD molecules either during freezing, or immediately following freezing is unlikely.

The fact that there was no decrease in mRNA or protein levels of SOD could be indicative of the importance of SOD for freezing survival. Furthermore, SOD activity

levels have already been shown to be more than three times greater in *R. sylvatica* muscle when compared to *R. pipiens*, a freeze intolerant frog (Joannis and Storey, 1996). This may be sufficient to overcome $O_2^{\cdot -}$ production produced by freezing and subsequent thawing in conjunction with the increase in $O_2^{\cdot -}$ affinity outlined in this study.

5.3. Phosphorylation of CuZnSOD and MnSOD

Investigation into the relative phosphorylation state of CuZnSOD did not give any evidence for a phosphorylated form of CuZnSOD. This suggests that the V_{max} difference discovered for CuZnSOD during freezing is not controlled by reversible phosphorylation. V_{max} changes can arise for many reasons, one of which may be a result of allosteric regulation. Although no mechanism for control is proposed in this chapter, allosteric regulation of CuZnSOD has been proposed in disease states such ALS, and lateral sclerosis have been reported to change the activity of CuZnSOD (Schuyler et al., 2011; Das and Plotkin, 2013). It is unclear at this time what caused the change in V_{max} for CuZnSOD during freezing, although the changes remain an important point of investigation for future studies.

Investigation into the relative phosphorylation state of MnSOD identified a significant difference in global phosphorylation levels (2.87 fold) between control and frozen states, as well as significant differences in the relative phosphorylation of serine (2.37 fold) and tyrosine (1.27 fold) residues (Figure 2.5). This suggests that the phosphorylation of MnSOD is a strong candidate for eliciting the increase in affinity for superoxide. Although it is impossible to discern the exact cause of the change in

phosphorylation, it is clear that the more phosphorylated form of MnSOD is more active. Increased affinity for $O_2^{\cdot -}$ would likely enable MnSOD from the frozen frog to prevent freeze-induced free radical damage, as well as prepare for the eventual burst production of free radicals during reperfusion.

Previous studies have shown that levels of IP3, a second messenger of PKC, increase as freezing progresses (Holden and Storey, 1996), adding credence to the possibility that SOD may be controlled by phosphorylation, and that it may be phosphorylated by PKC. This would suggest that PKC may become more active as freezing progresses, suggesting that phosphorylation of MnSOD by PKC would benefit the frog as freezing intensifies, or as a preparatory mechanism to deal with reperfusion. Investigation into the putative phosphorylation sites of MnSOD, based on the predicted protein sequence (Figure 2.6C) yielded several PKC phosphorylation sites, supporting PKC as a candidate to phosphorylate MnSOD during freezing. Interestingly, human MnSOD also demonstrated a larger number of potential PKC phosphorylation sites, suggesting the possibility for PKC mediated phosphorylation in humans. In all cases, except for casein kinase 1 for CuZnSOD, the obtained sequences for *R. sylvatica* encompassed the predicted putative phosphorylation sites for both CuZnSOD and MnSOD in humans.

5.4. *Stability of CuZnSOD and MnSOD*

Stability analysis of CuZnSOD did not show any significant differences between the enzymes from control and 24 h frozen muscle (Figure 2.7). When comparing

CuZnSOD and MnSOD, it is clear that MnSOD is far more resistant to urea denaturation in comparison to CuZnSOD. Upon closer observation of the tertiary structure of both CuZnSOD and MnSOD from *X. laevis*, it can be seen that MnSOD has more structured regions than CuZnSOD which may lead to its apparent greater stability (Carugo et al., 1996; Purello et al., 2005). Greater stability may aid MnSOD as it is found at a major site of superoxide production, the mitochondria. Furthermore, MnSOD did show a significantly different C_m between control and frozen muscle, suggesting a more stable form of MnSOD in the frozen state (Figure 2.8). Covalent modifications of proteins can result in either a more rigid or a more fluid structure. There is evidence of a correlation between phosphorylation events and a subsequent stabilization of protein, as has previously been seen for the enzymes hexokinase, creatine kinase and glucose-6-phosphate dehydrogenase in *R. sylvatica* (Dieni and Storey, 2009, 2010, 2011). Covalent modification via phosphorylation may also increase accessibility of the active site, which can affect kinetic parameters. The observed phosphorylation event may then be affecting stability as well as increasing accessibility to the active site, thereby increasing the affinity for $O_2^{\cdot -}$ in response to freezing.

5.5. Conclusion

The regulation of SOD in the freeze tolerant frog, *R. sylvatica* can provide useful insights into the effects of ischemia on free radical mediation. CuZnSOD may have increased capacity to deal with free radical production, although the exact mechanism of control is not understood. MnSOD from frozen frogs has a lower K_m , is more stable, and

exhibits increased phosphorylation at serine and tyrosine residues as compared with controls. The evidence presented in this chapter suggests that phosphorylation may stabilize and activate MnSOD during freezing. Augmented MnSOD activity may increase the ability of *R. sylvatica* to overcome oxidative stress associated with ischemia or reperfusion. This study can also provide valuable insight into disease pathology by offering a unique observation of the role of MnSOD during ischemia. MnSOD, along with many mitochondrial proteins, has a high degree of similarity across species. In this experiment, MnSOD from *R. sylvatica* was found to have 84% amino acid identity when compared to human MnSOD, as well as many predicted phosphorylation sites for PKC phosphorylation. This striking similarity suggests that researchers should explore possible phosphorylation events in human disease states as a method for reactivating MnSOD mutants, or in the case of ischemic tumour growth, exploring the possibility of deactivating MnSOD via de-phosphorylation as a method of triggering apoptosis.

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Table 2.1. Typical purification and yield of *Rana sylvatica* muscle CuZnSOD

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Fold Purification	Yield (%)
Crude extract	185.6	1230	6.63	-	100
DEAE ⁺	9.0	837	93.08	14.0	68.1
Copper Chelate	0.096	133	1385.4	209.0	10.8

Table 2.2. Typical purification and yield of *Rana sylvatica* muscle MnSOD

Purification Step	Total Protein (mg)	Total Activity (U)	Specific Activity (U/mg)	Fold Purification	Yield (%)
Crude extract	185.6	1230	6.63	-	100
DEAE ⁺	9.0	837	93.08	14.0	68.1
Copper Chelate	0.159	247	1550.69	234.0	20.1

Table 2.3. CuZnSOD and MnSOD kinetic parameters from purified control and frozen *R. sylvatica* muscle. * - indicates a significant difference from the corresponding control value, $p < 0.05$.

Enzyme parameters	Control	24h Frozen
<i>CuZnSOD</i>		
K_m superoxide (mM)	0.17 ± 0.019	0.14 ± 0.011
V_{max} (U/mg)	1390 ± 10	$2100 \pm 17^*$
C_m urea (M)	0.62 ± 0.03	0.72 ± 0.10
<i>MnSOD</i>		
K_m superoxide (mM)	0.20 ± 0.014	$0.13 \pm 0.019^*$
V_{max} (U/mg)	1550 ± 85	1570 ± 79
C_m urea (M)	2.53 ± 0.34	$2.92 \pm 0.22^*$

Figure 2.1. (A) Typical elution profile for control SOD on a DEAE+ column. (B)

Typical elution profile for control SOD on a copper chelate column.

Figure 2.2. Copper chelate column elution profile for SOD superimposed with western blot analysis showing the cross-reactivity of anti-CuZnSOD (dotted line) and anti-MnSOD (dashed line) with individual fractions.

Figure 2.3. Immunoblot analysis of (A) purified CuZnSOD from the muscle of control *R. sylvatica* and (B) purified MnSOD from the muscle of control *R. sylvatica*.

Figure 2.4. (A) RT-PCR analysis of *sod* transcript levels in frog muscle. (B) Western blotting analysis of the amount of SOD protein in frog muscle. No significant differences were observed using the Student's t-test ($n=4$, $P<0.05$).

Figure 2.5. Relative phosphorylation levels of MnSOD from muscle of control and 24 h frozen frogs using Pro-Q diamond phospho-protein staining to analyze relative total phosphorylation of the enzyme, and dot-blot analysis of residue-specific phosphorylation.

* - Significantly different from the corresponding control value, $p < 0.05$.

A CuZnSOD

KAGCVMKG**S**SEVTGVVRFEQEEDGPV**T**VTGQITGLTDGKHGFHIHTYGDN
 TNGCVSAGPHFNPQGKTHGGPDDEVVRHVVDLGNITSAAGVADINIKDKLI
 CLTGEHSIVGRTAVVHEKEDDLGKGGDNES

B

S – PKC delta
T – Erk1 Kinase

C MnSOD

AEEKYAEALAKGDVTAQVSLQPALKFNNGGGHVNHSIFWTNL**S**PSGGGEPQ
 GELLKAI**T**RDFG**S**FEKFKDKL**TT**MSVAVQGS**S**GWGWLGYNKESNRLQVIAC
 ANQDPLQGTTGLIPLLIDVWEHAYYLQYK

D

S(1) – Erk1 kinase
S(2) – PKC alpha/beta/gamma/zeta, Calmodulin dependent kinase 2
S(3) – PKC epsilon/delta/mu, Casein kinase 1
T(1) – PKC mu
T(2) – PKC epsilon/delta
T(3) – Calmodulin dependent kinase 2, Akt kinase

Figure 2.6. Predicted phosphorylation sites for CuZnSOD and MnSOD. (A) Partial amino sequence for CuZnSOD, highlighting predicted phosphorylation sites (bold, underlined). (B) Kinases predicted to phosphorylate CuZnSOD at the amino acid sites highlighted in A. (C) Partial amino sequence for MnSOD, highlighting predicted phosphorylation sites (bold, underlined). (D) Kinases predicted to phosphorylate MnSOD at the amino acid sites highlighted in C.

Figure 2.7. Stability of CuZnSOD from crude extracts of frog muscle. Samples were incubated for 24 h at 4°C with varying concentrations of urea then treated with pulse proteolysis to digest denatured CuZnSOD. Samples were then analyzed by western blotting and band densities of urea-treated samples are plotted relative to the initial band densities of untreated enzyme samples.

Figure 2.8. Stability of MnSOD from crude extracts of frog muscle. Samples were incubated for 24 h at 4°C with varying concentrations of urea then treated with pulse proteolysis to digest denatured MnSOD. Frozen MnSOD showed a significant difference when compared to MnSOD from control frogs as observed using student's t-test ($n=4$, $P<0.05$).

Chapter 3:

**Passive regeneration of glutathione: Purification
and properties of glutathione reductase
regulation from the freeze-tolerant North
American wood frog, *Rana sylvatica*.**

1. Introduction

As the frog freezes, the overall reducing environment of cells increases as oxygen is depleted as the final electron acceptor during aerobic metabolism (Storey, 1996). Since oxygen is exhausted in the frozen state, the electron transport chain is fully reduced, essentially priming the cell for reactive oxygen species (ROS) production. In order to combat the increasingly reduced state of the cell, as well as the burst production of free radicals during reperfusion, *R. sylvatica* has markedly higher levels of reduced glutathione (GSH) (219 ± 17 nmol/g wet weight) in skeletal muscle than its non-freezing cousin, *R. pipiens* (19.9 ± 1.8 nmol/g wet weight) (Joannis and Storey, 1996). The levels of GSH in the muscle of *R. sylvatica* were also observed to increase significantly during freezing in *R. sylvatica* to 308 ± 21 nmol/g wet weight (Joannis and Storey, 1996). An increase in total GSH levels could counteract an increase in production of ROS during freeze-induced ischemia or reperfusion during thawing.

GSH is the most abundant non-protein thiol in cells and plays a primary role as an antioxidant in mammalian cells (Meister, 1995). The role of GSH as an antioxidant can be divided into two main functions: direct interaction with ROS or as an electron donor to antioxidant enzymes such as glutathione peroxidase (GPX), glutathione-S-transferase (GST) and glutaredoxin (Meyer *et al.*, 2009). Glutathione exists in two forms, the reduced form (GSH) or the oxidized form (GSSG), the latter arising from the disulfide linkage of two GSH molecules. Synthesis of the tripeptide GSH is catalyzed by two enzymes, γ -glutamylcysteine synthetase (γ -GCS) which synthesizes γ -glutamylcysteine from glutamate and cysteine, and GSH synthetase (GS) which forms GSH from glycine and γ -glutamylcysteine (Forman *et al.*, 2008).

Glutathione reductase (GR)(E.C. 1.8.1.7) (also known as glutathione disulfide reductase or GSR) is a secondary antioxidant enzyme involved in the reduction of glutathione disulfide (GSSG) into two glutathione molecules (GSH). Specifically, GR catalyzes the following reaction:

Primary antioxidant enzymes use GSH to detoxify ROS species and glutathione reductase is the primary enzyme tasked with actively recycling oxidized GSSG back to GSH. In vertebrates, GR typically has only one gene, *gsr*, as in *Xenopus* and humans. GR from *X. tropicalis* or *X. laevis* has a molecular weight of 51.8 kDa as reported in the UniProtKB database (B1WBJ3_XENTR, Q58E89_XENLA) (Klein *et al.*, 2002). The active site of GR has two key tyrosine residues that stabilize NADPH and GSSG during catalysis (Krauth-Siegel *et al.*, 1988).

GR plays a major role in maintaining the GSH/GSSG ratio in the cell. Maintenance of this ratio is crucial, since GSH deficiencies cause severe oxidative shock to cells and can lead to apoptotic or necrotic cell death (Galluzzi *et al.*, 2007; Circu and Aw, 2008). In fact, keeping GSH levels high has been shown to be beneficial in apoptotic resistant models (Friesen *et al.*, 2004; Armstrong *et al.*, 2002). Although GSH is found in high amounts (mM levels) in the cytoplasm, GSH also exists in many organelles in the cell including the mitochondria, peroxisomes, nuclear matrix, and the ER (Forman *et al.*, 2008). Mitochondria are incapable of de novo synthesis of GSH, which suggests that the importance of GSH is amplified in the mitochondria, the organelle with perhaps the highest continuous generation of ROS in association with running the electron transport system (Galluzzi *et al.*, 2007; Circu and Aw, 2008). This emphasizes the crucial role of

mitochondrial GR in recycling GSH from GSSG. It has been proposed that both GR and GSH must be actively transported into the mitochondrial matrix (Franco and Cidlowski, 2009). The roles of mitochondrial and cytosolic levels of GSH have been explored independently, and oxidative stress has been linked to both in a similar fashion, suggesting that their roles may in fact not be independent of one another (Lash, 2006; Garcia-Ruiz and Fernandez-Checa, 2006).

Cytosolic and mitochondrial GSH pools seem to be critically important to counteract the production of ROS during reoxygenation of cells, and reactivation of the ETS in the mitochondria following ischemic stress (Muyderman *et al.*, 2007). This may cause certain sites of burst ROS production, such as the mitochondria, to overwhelm the GSH pools at the site of insult during or proceeding ischemic stress. One of the main ROS produced in the mitochondria is superoxide (O_2^-). O_2^- cannot cross the mitochondrial membrane, and either reacts with GSH or other antioxidants or is converted into H_2O_2 by MnSOD. GR plays a pivotal role in maintaining the redox state of the cell, specifically the GSSG/GSH ratio, and maintenance of the mitochondrial ratio of GSSG/GSH has also been linked to the suppression of apoptosis (Marchetti *et al.*, 1997; Muydermann *et al.*, 2007).

GR has been widely studied and characterized from humans and traditional animal models, however, less is known about its role in disease states, or its role in aiding survival in the freezing frog. This chapter presents the first investigation of the potential method of regulation of GR in the leg muscle of *R. sylvatica*, comparing control and frozen states, and provides evidence of possible regulation by glucose levels as well as a

general characterization of the enzyme, including the propensity to become phosphorylated *in vitro*.

2. Materials and Methods

2.1. Chemicals

All biochemicals were from BioShop (Burlington, ON, Canada) with a few exceptions; oxidized glutathione (GSSG) was from Sigma (St. Louis, MO), Cibacron blue column was from Affiland (Ans, Belgium), hydroxyapatite Bio-Gel[®] HTP Gel column was from Bio-Rad (Hercules, CA) and potassium phosphate, monobasic was from J.T. Baker Chemical Company (London, UK).

2.2. Animals

Male wood frogs were cared for and sampled as in Chapter 2 section 2.2.

2.3. Preparation of muscle tissue lysates for protein purification

For protein purification, samples of frozen muscle tissue were homogenized 1:5 w:v in ice-cold homogenizing buffer A [50 mM potassium phosphate (KPi) buffer, pH 7.2, containing 30 mM β -glycerophosphate, 1 mM EGTA, 1 mM EDTA, 15 mM β -mercaptoethanol, 10% glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were then centrifuged at $13,500 \times g$ at 4 °C and the supernatant was collected for use in protein purification.

2.4. *Purification of GR*

A 5 cm column of Sephadex G-50 in a syringe barrel was equilibrated in buffer A and centrifuged at 500 g in a bench-top centrifuge for 2 min to remove excess buffer. An aliquot of 2 mL of frog muscle tissue supernatant was applied to the column and centrifuged again. The resulting eluant was collected. A hydroxyapatite column (1.5 cm × 2 cm) was then washed with 20 mL of buffer A to equilibrate the column. The eluant collected from the Sephadex G50 column was applied to the hydroxyapatite column and washed with 20 mL buffer A to remove unbound proteins. GR was eluted from the hydroxyapatite column with a linear gradient of 0-3.5 M KCl in buffer A. The 4 fractions (with highest activity) of 900 µL were collected and 10 µL from each fraction was assayed to detect GR activity (see kinetic assays section below for methodology). The fractions of peak GR activity were pooled and diluted 10-fold with buffer A. The diluted fractions were applied to a Cibacron blue column (1.5 cm × 10 cm) pre-washed with 50 mL of buffer A. The column was washed with a further 50 mL of buffer A to remove unbound protein and then bound protein was eluted with a linear gradient of 0-2 M KCl in homogenization buffer A. The top 4 fractions (with highest activity) of 450 mL were collected and 20 µL from each fraction was assayed to detect GR activity. To assess enzyme purity, aliquots of GR from different stages of the purification procedure were first assayed for protein concentration as described in chapter 2 section 2.7, then combined with an equal volume of 2× SDS loading buffer (100 mM Tris buffer, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercapotethanol), boiled for 5 min, and then 20 µL samples were run of on SDS-PAGE as described in chapter 2 section 2.7.

2.5. *Kinetic assays*

GR was assayed using a modified version of the method of Di Ilio (1983). Assay conditions were: 50 mM KPi buffer (pH 7.2), 2 mM GSSG, 0.25 mM NADPH, and 10 μ L tissue extract. One unit of enzyme activity is the amount that oxidizes 1 μ mol of NADPH per minute at 25°C. The amount of NADPH was measured at 340 nm in a Thermo Labsystems Multiskan spectrophotometer (Thermo Scientific, Waltham, MA, USA). Enzyme affinity for GSSG was determined in the presence versus absence of 75 mM urea or 300 mM glucose. Activation of GR by adenosine triphosphate (ATP), adenosine diphosphate (ADP), adenosine monophosphate (AMP), magnesium (Mg^{2+}), manganese (Mn^{2+}), potassium (K^+), calcium (Ca^{2+}), or zinc (Zn^{2+}) was also tested. Data were analyzed using the Kinetics v.3.5.1 program (Brooks 1992).

2.6. *ProQ Diamond Phosphoprotein Staining*

Muscle GR from control and frozen frogs was purified as described in section 2.4. The top four fractions based on activity were pooled, and protein levels in the pooled fractions were quantified using the Coomassie blue dye-binding method. Aliquots of the pooled fractions were then mixed 1:1 v:v with SDS loading buffer (100 mM Tris buffer, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercapotethanol), subsequently boiled for 5 minutes and stored at -20°C until used.

Equal volumes of each sample were loaded on a 10% SDS-PAGE gel. The gel was run at 180 V for 55 min in running buffer (0.5 M Tris, 5 M glycine, 0.5% w/v SDS). The gel was removed and washed in fixing solution (50% v/v methanol, 10% v/v acetic

acid) twice for 10 min, then left in fixing solution overnight at 4°C followed by 3 washes with ddH₂O for 10 min. The gel was then stained with ProQ Diamond Phosphoprotein stain (Invitrogen, Eugene, OR) for 90 min and washed 3 times with ddH₂O for 10 min. The gel was covered during staining (and for the remainder of the protocol) with aluminum foil to prevent the photosensitive stain from interacting with light. To minimize non-specific background, the gel was washed in ProQ Diamond destaining solution (20% v/v acetonitrile, 50 mM sodium acetate, pH 4) for 45 minutes, and then washed 3 times in ddH₂O for 10 minutes. The bands on the gel were visualized using the ChemiGenius Bioimaging System (Syngene, Frederick, MD) to assess the relative intensities of the fluorescent bands. The fluorescence of the bands was quantified using the accompanying GeneTools software.

2.7. *In vitro* Incubation to Stimulate Protein Kinases

Frog muscle tissue extracts, prepared as previously described in section 2.2, were filtered through a G50 spun column equilibrated in incubation buffer (50 mM KPi, 10% v:v glycerol, 30 mM β -mercaptoethanol, pH 7.2). Aliquots of the filtered supernatants were incubated for 12 hours at 4°C with specific inhibitors and stimulators of protein kinases, as described in Macdonald and Storey (1999). Each aliquot was mixed 2:1 v:v with the appropriate solutions to stimulate protein kinases. Each solution was prepared in incubation buffer and the following incubation conditions were used:

(a) STOP conditions: 2.5 mM EGTA, 2.5 mM EDTA and 30 mM β -glycerophosphate (designed to inhibit both kinases and phosphatases).

(b) Stimulation of endogenous kinases: 5 mM Mg-ATP, 30 mM β -glycerophosphate, 1 mM cAMP (to stimulate PKA), 1 mM cGMP (for PKG), 1.3 mM CaCl_2 + 7 $\mu\text{g/mL}$ PMA (for PKC), 1 mM AMP (for AMPK), and 1 U of calf intestine calmodulin + 1.3 mM CaCl_2 (for CaMK).

GR was then purified as previously described in section 2.4 and samples were analyzed using Pro-Q diamond phosphoprotein stain as described in section 2.6. Figure 3.2 shows the elution profile for control and frozen preparations from a DEAE⁺ column. There was no significant difference in the elution profiles. Western blotting was carried out as described in chapter 1 section 2.7, with the following primary antibodies: (1) rabbit anti-phosphoserine (Cat #618100, Invitrogen, Carlsbad, CA, USA); (2) rabbit anti-phosphothreonine (Cat. # 718200, Invitrogen, Carlsbad, CA, USA); or (3) rabbit anti-phosphotyrosine (Cat # 615800, Invitrogen, Carlsbad, CA, USA).

2.8. *Arrhenius Plots*

Maximal GR activity was determined at 5°C increments starting from 5°C and ending at 45°C. The reaction temperature was altered by placing the Thermo Labsystems Multiskan spectrophotometer into a VWR International BOD 2020 Incubator (Sheldon Manufacturing Inc., Oregon USA) set to the desired temperature. Microplates filled with assay mixture (but without enzyme) were equilibrated in the same incubator for several minutes until the desired temperature was reached (as measured by a telethermometer). Plates were then placed into the spectrophotometer and reactions were initiated by the

addition of enzyme. Arrhenius plots were constructed from these experiments and the activation energy (E_a) was calculated.

2.9. *Determination of protein stability*

Differential scanning fluorimetry (DSF) is a high throughput method that monitors the thermal unfolding of proteins in the presence of a fluorescent dye (Niesen *et al.*, 2007). Purified control and 24 h frozen GR were aliquoted to a concentration of approximately 0.1 $\mu\text{g}/\mu\text{L}/\text{well}$ into the wells of a 96-well, thin-walled PCR plate along with the dye SYPRO Orange (40X final concentration, Invitrogen) to a total volume of 20 μL . PCR plates were then sealed with sealing tape and placed into a BioRad iCycler5 PCR instrument. SYPRO Orange fluorescence was monitored as described by Biggar *et al.* (2012). Briefly, SYPRO Orange was excited through the transmission of light through the FAM filter ($485 \pm 30 \text{ nm}$), with the subsequent emission of light through the ROX filter ($625 \pm 30 \text{ nm}$). Measurements were taken every 30 s at 1°C increments from 25°C to 97°C . Subsequent analysis of the fluorescent data using OriginPro 8.5 and the Boltzmann distribution curve yielded the midpoint temperature of the protein unfolding transition, known as the protein melting temperature (T_m), for control and frozen frog muscle GR.

2.10. *Statistical analysis*

Comparison of enzyme kinetics, relative protein phosphorylation and protein stability were performed using a Student's t test, two-tailed, assuming unequal variances. A probability of $P < 0.05$ was considered significant.

3. **Results**

3.1. *Purification of GR from the muscle of control and frozen frogs*

The purification scheme used for wood frog muscle GR is shown in Table 3.1. The procedure used ion exchange chromatography on a Bio-Gel[®] HTP Gel hydroxyapatite column (Figure 3.1A), and Cibacron blue chromatography (Figure 3.1B). GR eluted from the Cibacron blue column at approximately 1 M KCl. Frog muscle GR was purified 134.9 fold with an overall yield of activity of 34.2% (Table 3.1). The final specific activity of GR was 124.73 mU/mg of protein (Table 3.1). The success of the purification process was assessed using SDS-PAGE with Coomassie blue staining of the gel (Figure 3.3). This showed that GR was purified to near homogeneity as there was one band corresponding to correct molecular weight of ~50 kDa for GR (Figure 3.3).

3.2. *Kinetic characterization of GR*

Kinetic parameters of purified muscle GR were assessed to search for differences between control and frozen animals. The K_m for GSSG and the V_{max} of GR from the muscle of *R. sylvatica* did not change significantly between control and frozen animals

($p > 0.05$) (Table 3.2). However, when assayed in the presence of different additives, the K_m value for GSSG changed. In the presence of 75 mM urea, the K_m for GSSG increased significantly (1.57 fold, $p < 0.05$) in comparison to the K_m of GSSG in untreated samples (Table 3.2). Oppositely, in the presence of 300 mM glucose, the K_m for GSSG decreased significantly to 50% of the measured K_m of GSSG in untreated samples. In the presence of both 75 mM urea and 300 mM glucose, K_m for GSSG was significantly different from the K_m of GSSG in both urea treated and untreated samples but not different from the K_m in the presence of glucose.

The control form of GR was activated when assayed in the presence of ATP (1.42 fold), ADP (1.37 fold) and AMP (1.35 fold) (Table 3.2). K_a values for ATP or ADP K_a were similar at 0.076 and 0.071 mM, respectively. AMP had a K_a (0.16 mM) of more than 2 fold greater than that of either ATP or ADP (Table 3.2).

Mn^{2+} inhibited GR activity, showing an I_{50} value (inhibitor concentration that reduced activity by 50%) of 4.44 mM (Table 3.3). Ca^{2+} inhibited GR activity, with an I_{50} of 5.12 mM (Table 3.2). Mg^{2+} and K^+ did not inhibit GR activity up to 10 mM.

3.3. *Post-translational modification of GR*

To test whether GR was susceptible to reversible phosphorylation the purified GR from muscle of both control and frozen frogs was run on an SDS-PAGE gel and stained with ProQ Diamond phosphoprotein stain. GR did not react with the ProQ Diamond phosphoprotein stain, resulting in no apparent banding (Figure 3.3).

Crude extracts of skeletal muscle from control and frozen frogs were incubated to stimulate the activity of endogenously present kinases and GR was then purified as described in section 2.7. The enzyme showed a clear band at ~50 kDa when stained for total phosphorylation (Figure 3.5). However, there was no significant difference in band intensities between GR purified from control and frozen incubated samples.

Immunoblotting was used to assess residue-specific phosphorylation of muscle GR. Purified GR reacted with antibodies testing for phosphorylation on serine residues, threonine residues and tyrosine residues (Figure 3.6). However, relative phosphorylation levels of serine, threonine or tyrosine residues did not change between control and frozen states ($P > 0.05$).

3.4. Scansite Prediction of Phosphosites

The Scansite application from the Massachusetts Institute of Technology (<http://scansite.mit.edu/>) was used to analyze GR sequences from two other frogs, *Xenopus laevis* and *X. tropicalis* to search the proteins for putative phosphorylation sites (Figure 3.7). GR protein sequences showed 94.75% identity between *X. tropicalis* and *X. laevis*. Multiple putative phosphorylation sites were predicted to occur on GR based on the presence of consensus sequence motifs for different protein kinases. Only those kinases predicted to phosphorylate GR from both *X. laevis* and *X. tropicalis* are reported. Putative consensus sequences for phosphorylation sites of Akt kinase (threonine), AMP kinase (serine, threonine), calmodulin dependent kinase 2 (serine, threonine), casein kinase 1 (serine, threonine), casein kinase 2 (serine, threonine), EGFR kinase (tyrosine), Fgr

kinase (tyrosine), PKA (serine, threonine), PKC alpha (serine, threonine), PKC beta (serine, threonine), PKC delta (serine), PKC epsilon (serine), PKC gamma (serine, threonine), and PKC mu (serine) were predicted for GR.

Human GR shows 81.75% identity when compared to *X. laevis* and *X. tropicalis*. Human GR was analyzed for comparison using Scansite, resulting in the following putative consensus sequences for phosphorylation sites: Akt kinase (serine, threonine), AMP kinase (serine, threonine), calmodulin dependent kinase 2 (serine), casein kinase 1 (threonine), casein kinase 2 (threonine), EGFR Kinase (tyrosine), Erk1 Kinase (serine, threonine), Fgr Kinase (tyrosine), GSK3 kinase (serine, threonine), PKA (threonine), PKC alpha (serine, threonine), PKC beta (serine, threonine), PKC delta (serine, threonine), PKC epsilon (threonine), PKC gamma (serine, threonine), PKC mu (serine, threonine) and PKC zeta (serine) were predicted for GR (Figure 3.7).

3.5. Arrhenius Plots

Maximum GR activity was measured at 5°C increments starting from 5°C and ending at 45°C using purified GR from the leg muscle of *R. sylvatica*. Purified GR demonstrated a positive correlation between temperature and activity (Figure 3.8).

Arrhenius plots were created by plotting $\ln(V_{\max})$ versus $1/\text{temperature in Kelvin}$. The E_a was then calculated as 40.7 ± 0.9 kJ/mol (Table 3.2; Figure 3.8).

3.6. Stability of GR

The structural stability of GR was evaluated by testing enzyme sensitivity to thermal denaturation using differential scanning fluorimetry as described in section 2.8. There was no significant difference in the pattern of thermal denaturation of purified GR between control and frozen frogs (Figure 3.9). GR purified from the muscle of *R. sylvatica* showed a peak at 68°C for both control and frozen frogs. The calculated T_m value (temperature that results in a 50% loss of folded enzyme) was 64.7°C for GR from control frog muscle extracts, which was not significantly different than the value of 63.8°C for GR from frozen muscle extracts (Table 3.2; Figure 3.9).

4. Discussion

Abnormalities in GR activity have been linked to disease states including Parkinson's disease and Alzheimer's disease, in which an increase in GSSG concentration was linked with greater apoptotic cell death (Diaz-Hernandez *et al.*, 2005; Merad-Boudia *et al.*, 1998; Aksenov and Markesbery, 2001). An increase in the activity of GR has been observed in rheumatoid arthritis (Mulherin *et al.*, 1996) and in cancer, changes in the expression level and activity of GR have also been found (Mullineaux and Creissen, 1997). Although the exact role of GR in disease is still being explored, knockout and knockdown experiments with GR have proven to be lethal (Pretsch, 1999; Rogers *et al.*, 2004; Rogers *et al.*, 2006). Specifically, elevated levels of GSSG due to oxidative stress have been directly linked to apoptotic signaling events in these experiments. Since, the function of GR seems paramount for the continued survival and

quality of life in humans, exploration of GR function in organisms than can endure long periods of time under conditions that mimic disease-like states is of great interest to understanding the role of GR in disease. A common theme observed for the diseases mentioned above is an increase in oxidative stress imposed on both the whole cell and at the mitochondrial level due to fluctuations in blood flow and oxygen deprivation.

Organisms experiencing changes in GR regulation under oxygen deprived states include: the red eared slider turtle (*Trachemys scripta elegans*) which shows fluctuations in GR activity as well as decreased GSH levels in the liver, heart and muscle during anoxia (Willmore and Storey, 1997), the leopard frog (*Rana pipiens*) which shows a reduction in GR activity in muscle tissue during severe dehydration (Hermes-Lima and Storey, 1998), the common carp (*Cyprinus carpio*) which shows increases in GR activity in the brain during hypoxia (Lushchak *et al.*, 2005), the spadefoot toad (*Scaphiopus couchii*) where GR activity in the liver, heart and kidney was significantly lower during estivation (Grundy and Storey, 1998), and the marine periwinkle (*Littorina littorea*) where GR activity decreased in the hepatopancreas during anoxia as well as foot muscle upon exit from anoxia (Pannunzio and Storey, 1998).

In line with the animals discussed above, the freeze-tolerant frog also experiences low oxygen, specifically in the form of ischemia, which has been repeatedly demonstrated to include oxidative stress and a burst production of ROS in the reperfusion recovery episode (Ferrari *et al.*, 1991; Simpson and Lucchesi, 1987; Zweier and Talukder, 2006). Studies involving the overexpression or increased activity of GR in the mitochondria have shown increased resistance to oxidative stress (Foyer 1995; Mockett 1999). Therefore, through exploration of the role of GR in maintaining GSH pools during

freezing in *R. sylvatica*, with particular importance in the mitochondria, the regulation of GR in the frozen frog presented in this chapter can provide important information for both the survival of the animal and insight into disease states by staving off signaling of apoptosis upon reoxygenation.

4.1. *Kinetic changes of GR in the face of increasing glucose*

The kinetic properties of wood frog skeletal muscle GR did not change significantly when comparing GR purified from control and frozen animals (Table 3.2). Since GR is an abundant enzyme in muscle cells, and found in several subcellular locations, it may be less energetically costly to modify the entire GR pool in order to deal with the stress imposed by freezing. However, covalent modification of proteins is only one of many methods to change enzyme activity or function. *R. sylvatica* increases the levels of select osmolytes to extreme levels during freezing such as glucose (>200 mM levels) which seem to affect the enzymatic function of GR (Storey and Storey, 2004).

GR purified from the muscle of *R. sylvatica* demonstrated significant changes in affinity for GSSG when tested in the presence of the physiological concentrations of glucose or urea that are encountered during freezing (Table 3.2). GR showed a significant decrease in affinity for GSSG when subjected to physiological levels of urea (75 mM) that can be present during freezing (Table 3.2). This phenomenon was overwhelmed by the effects of high glucose, since purified GR showed a significant increase in affinity for GSSG when subjected to physiological levels of glucose alone or glucose + urea (Table 3.2). As previously mentioned, *R. sylvatica* elevates glucose levels in order to prevent the

osmotic loss of water from cells due to freezing of extracellular water. The frog may be benefitting from a preexisting hyperglycemic response to augment the function of GR.

Ischemia stress must be removed in order to return glucose levels to normal, therefore, the positive effects of glucose on GR activity would likely remain throughout the burst production of ROS during reperfusion. Once the initial oxidative stress imposed on the cells due to prolonged ischemia has been surmounted, freezing, and subsequently the glucose levels will gradually return to normal levels, and the glucose-induced increase in GR affinity for GSSG will also return to normal. This is of note, since the GSSG/GSH ratios are involved in many signaling pathways, and could be disrupted by maintaining elevated GR affinity beyond the necessary time to overcome reperfusion induced ROS production.

In humans, high glucose has been suggested to increase the production of free radicals, and ultimately cell death and reduced proliferation, in diabetic and glucose treated human cell lines (Curcio and Ceriello, 1992; Durante *et al.*, 1988; Ceriello *et al.*, 1993; Tesfamaia and Cohen, 1992). However, the effect of elevated or added GSH has demonstrated protective effects on glucose-treated human cell lines (Marfella *et al.*, 1995). Previous studies on the effects of elevated glucose levels on antioxidant enzymes have demonstrated up-regulation in the presence of high glucose, suggesting that glucose can stimulate the activity of antioxidant enzymes (Ceriello *et al.*, 1996). It is clear from these studies, as well as the evidence of glucose-activated GR in this chapter, that the relationship between glucose levels, free radicals and antioxidant enzyme activity is linked.

4.2. Kinetic changes of GR due to common metabolites

The effect of Ca^{2+} on GR is likely linked to damage of the mitochondrial membrane, which would lead to an increase in the cytoplasmic Ca^{2+} concentration (Halliwell, 1992). Leakage of Ca^{2+} has been widely characterized in the mitochondrial signaling of apoptosis (Pinton *et al.*, 2008). It has also been suggested that freeze-thaw cycles can disrupt Ca^{2+} transport in muscle, suggesting that as Ca^{2+} levels return to normal, GR activity could be influenced by the change in Ca^{2+} levels (Storey and Storey 2004; Halliwell, 1992).

Cellular Mn^{2+} exists mainly in complexes with proteins and a rise in free metals is usually due to damage of proteins, and subsequent release of the bound metals from the protein structure (Valko *et al.*, 2005). It would appear that the inhibition of GR could be linked to cellular damage and subsequent release of Mn^{2+} when proteins are damaged; however, it is unclear at this time what the role of Mn^{2+} is in inhibiting GR activity in *R. sylvatica*.

R. sylvatica ATP levels have been shown to drop during freezing (Storey and Storey, 1985). ATP, ADP and AMP were observed to activate GR similarly (Table 3.2) suggesting that any activation of GR by changes in adenylate ratios during freezing, is unlikely.

4.3. Phosphorylation of GR during freezing and induced phosphorylation of GR by endogenous kinases

Reversible protein phosphorylation has been demonstrated as an important method of modifying key enzymes involved in the success of *R. sylvatica* during freezing (Dieni and Storey, 2008; Dieni and Storey, 2009; Dieni and Storey, 2010; Dieni and Storey, 2011; Abboud and Storey, 2013; Chapter 2 and 4). The potential phosphorylation of GR during freezing was therefore explored in order to determine if this same method of regulation was used to aid GSSG/GSH homeostasis during freezing. There was, however, no evidence of changes in the phosphorylation state of GR between control or frozen states as assessed by ProQ diamond staining (Figure 3.4). This lack of phosphorylation suggested that any differences in GR activity are not due to a freeze-induced phosphorylation event.

The phosphorylation state of GR was nonetheless explored as a potential method of further regulating the protein and to discover if different phosphorylation profiles are possible between the control and frozen states. The activities of endogenous kinases were stimulated in frog muscle homogenates as described in section 2.7, resulting in the phosphorylation of frog muscle GR (Figure 3.5). Figure 3.2 shows the elution profile for control and frozen preparations from a DEAE⁺ column. There was no significant difference in the elution profiles, seemingly suggesting that no frozen state-specific phosphorylation site exists. Further analysis of the induced phosphorylation of GR suggested that phosphorylation was achieved on serine, threonine and tyrosine residues (Figure 3.6). This is the first study to show phosphorylation of GR *in vitro*.

However, phosphorylation of GR does not seem to play a role in freeze tolerance of *R. sylvatica*, but, it highlights the propensity of GR for phosphorylation, which may play other roles in the regulation of GR in the frog. Investigation into the putative phosphorylation sites on GR, based on sequences from *X. laevis* and *X. tropicalis*, showed the presence of putative sites for serine, threonine and tyrosine kinases supporting the notion that endogenous kinases in *R. sylvatica* muscle can phosphorylate GR (Figure 3.7). Several of the kinases stimulated in the endogenous incubation study were among those predicted to phosphorylate GR, including AMPK, CamK, PKA and PKC. This suggests that although altered phosphorylation of GR is not part of freeze-tolerance in *R. sylvatica*, it could play a role in other cellular functions. Interestingly, human GR also shows potential serine, threonine and tyrosine predicted phosphorylation sites, suggesting the possibility for GR phosphorylation in humans. Although GR shows the propensity for phosphorylation, more work must be conducted to elucidate the physiological role of phosphorylation of this enzyme. It is clear, however, that the phosphorylation of GR during freezing in *R. sylvatica* is unlikely to have a role in maintenance of the GSH/GSSG levels.

4.4. *Thermal stability and temperature dependent activity of GR*

GR purified from both control and frozen frogs was explored via DSF, and showed no significant differences in enzyme structural stability between states (Figure 3.9). This provides further proof that GR from control and frozen animals is not functionally different. Past studies of GR have demonstrated the thermodynamic stability

of GR activity, and have similarly shown that GR remains active across a broad range of temperatures (Ohtsuka *et al.*, 1994; Lopez-Barea and Chi-Yu, 1979; Rescigno and Perham 1994). The T_m determined for frog GR shows that denaturation takes place at a relatively high temperature of approximately 64°C, suggesting the GR is a very stable enzyme (Table 3.2, Figure 3.9). The calculated E_a was 40.7 kJ/mol which suggests that the reaction catalyzed by GR is endothermic in nature, and that the reaction may be hindered at low temperatures.

4.5. Conclusion

This study of the regulation of GR in the freeze tolerant frog, *R. sylvatica* provides useful insights into the maintenance of the cellular redox states as well as the GSSG/GSH ratios involved in apoptotic cell signaling. GR from the muscle of *R. sylvatica* does not seem to be covalently modified during freezing, although it does show the propensity to be phosphorylated through activation of endogenous kinases. The regulation of GR activity during freezing seems to be related to the changing cellular environment, specifically increases in glucose concentrations. GR from *R. sylvatica* seems to have coupled an increase in affinity for GSSG with a natural increase in the concentration of glucose during freezing. Although cytosolic concentrations of GSH are significantly higher in *R. sylvatica* in comparison to other closely related frog species, an increase in GR activity may be vital to maintaining GSH/GSSG levels, specifically in the mitochondria. The mitochondrial production of ROS has been linked to apoptotic signaling, and GR activity may be augmented in order to combat the mitochondrial

production of ROS during thaw-induced reperfusion. GR, along with many antioxidant-related proteins, has a high degree of similarity across species. The similarity observed in mitochondrial proteins could allow for researchers to explore possible roles for GR in a controlled ischemic state, such as freezing in *R. sylvatica*. It is clear from this study and others, that the role of GSH and GR maintenance of the mitochondrial redox environment during freezing in *R. sylvatica* should be explored in greater detail, as it could provide valuable insight into disease pathology by offering a unique observation of the role of GR during ischemia.

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Table 3.1. Typical purification and yield of *Rana sylvatica* muscle GR

Purification Step	Total Protein (mg)	Total Activity (mU)	Specific Activity (mU/mg)	Fold Purification	Yield (%)
Crude extract	73.6	69.3	0.94	-	100
Sephadex G50	63.6	63.0	0.99	1.1	91.0
Hydroxylapatite	0.55	25.8	46.91	49.9	37.2
Cibacron Blue	0.19	23.7	124.73	134.9	34.2

Table 3.2. Kinetic parameters of GR purified from muscle of control and frozen *R.**sylvatica*

Enzyme parameters	Control	24h Frozen
K _m GSSG (mM)	0.054 ± 0.006	0.048 ± 0.003
V _{max} (mU/gww)	125 ± 6.1	121 ± 5.3
T _m	63.8 ± 0.4	64.7 ± 0.4
K _m GSSG+Urea (mM)	0.085 ± 0.009*	
K _m GSSG+Glucose (mM)	0.027 ± 0.002*	
K _m GSSG+Glucose+Urea (mM)	0.031 ± 0.002*	
K _a ATP (mM)	0.076 ± 0.02	
Fold activation ATP	1.42	
K _a ADP (mM)	0.071 ± 0.01	
Fold activation ADP	1.37	
K _a AMP (mM)	0.16 ± 0.03	
Fold activation AMP	1.35	
I ₅₀ Mn ²⁺ (mM)	4.44 ± 0.33	
I ₅₀ Mg ²⁺ (mM)	No inhibition ≤10 mM	
I ₅₀ K ⁺ (mM)	No inhibition ≤10 mM	
I ₅₀ Ca ²⁺ (mM)	5.12 ± 0.99	
E _a (kJ/mol)	40.7 ± 0.9	

To test the effects of additives on K_m, assays were run with the addition of 75 mM urea or 300 mM glucose. K_a and I₅₀ values were determined at K_m values for GSSG. Means ± SEM, n= 8. * - Significantly different from control value without additives.

Figure 3.1. (A) Typical elution profile for GR on a hydroxyapatite column. (B) Typical elution profile for GR on a Cibacron blue column.

Figure 3.2. DEAE⁺ elution profiles for purified GR from control and 24 h frozen muscle after incubation to stimulate protein kinases.

Figure 3.3. Purified GR from the muscle of control *R. sylvatica*. (A) crude muscle homogenate, (B) GR-containing eluant after Sephadex G50 chromatography, (C) GR-containing eluant after hydroxylapatite chromatography, (D) purified GR from Cibacron blue chromatography, and (E) molecule weight standards. GR protein is the prominent band at ~50 kDa indicated by the arrow.

Figure 3.4. Absence of bands indicates no apparent phosphorylation of muscle GR from either control or 24 h frozen frogs as assessed by Pro-Q diamond phosphoprotein staining.

Figure 3.5. Relative phosphorylation levels of GR from control and 24 h frozen frog muscle after total kinase incubations as assessed by Pro-Q diamond phosphoprotein staining. Bands on the left are for GR from control muscle; on the right are 24 h frozen.

Figure 3.6. Relative phosphorylation levels of wood frog muscle GR after incubations to stimulate total kinases; control and 24 h frozen conditions are compared using Western-blot analysis of site-specific phosphorylation. Data are means $\pm$ SEM, $n=4$. There were no significant differences between control and frozen values, $p<0.05$.

<i>X.tropicalis</i>	MHKPASDSPS	GNGHLPRYYD	<u>Y</u> LVVGGGSGG	LASARRAAEL	GARTAVVE <u>S</u> <u>S</u>	KLGGTCVNVG
<i>X.laevis</i>	MHKVPDSDSHS	SDGHLPRYFD	<u>Y</u> LVVGGGSGG	LASARRAAEL	GARTAVVE <u>S</u> <u>S</u>	KLGGTCVNVG
<i>X.tropicalis</i>	CVPKKIMWNA	AMHSEYIHDH	ADYGFEIPDV	KFTWKVIEKEK	RDAYVSRLND	IYQNNLQKAQ
<i>X.laevis</i>	CVPKKIMWNA	AIHSEYIHDH	EDYGFETSAI	KFTWKVIEKEK	RDAYVSRLND	IYQNNLQKAQ
<i>X.tropicalis</i>	IEIIRGNANF	<u>T</u> <u>S</u> DPEPTVEV	NGQKYSAPHI	LIATGGKPSM	PSDAELPG <u>S</u>	LGITSDGFFE
<i>X.laevis</i>	IEIIRGQANF	<u>T</u> <u>S</u> DSEPTVEV	NGQKYIAPHI	LIATGGKPSM	PSDAEVP <u>S</u>	LGITSDGFFQ
<i>X.tropicalis</i>	LTDLPRR <u>S</u> IV	VGAGYIAVEI	AGILSALGSK	<u>A</u> <u>S</u> LLIRQDKV	LR <u>T</u> FDSIISS	NC <u>T</u> EELENAG
<i>X.laevis</i>	LTDLPRR <u>S</u> VV	VGAGYIAIEI	AGILSALGSK	<u>A</u> <u>S</u> LLIRQDKV	LR <u>T</u> FDSMISS	NC <u>T</u> EELENAG
<i>X.tropicalis</i>	VEVWKYAQVK	<u>S</u> VKKST <u>T</u> GLE	INVQCSMPGR	KP <u>T</u> VRTIQDV	DCLLWAIGRD	PNTEYLGLEN
<i>X.laevis</i>	VEVWKYAQVK	<u>S</u> VKKS <u>A</u> <u>T</u> GLE	INVQCSMPGR	KP <u>T</u> VRTIQDV	DCLLWAIGRD	PNTEDLGLEN
<i>X.tropicalis</i>	LGLELDEKGH	IVVDEFQNTS	RKGVYAVGDV	CGRALLTPVA	IAAGRKL <u>S</u> HR	LFEGQEDSKL
<i>X.laevis</i>	LGLELDEKGH	IVVDEFQNTS	RKGVYAVGDV	CGRALLTPVA	IAAGRKL <u>S</u> HR	LFEGQEDSKL
<i>X.tropicalis</i>	DYNNIPTVVF	SHPPIGTVGL	<u>T</u> EEEAV <u>T</u> AKG	RENVKVYTTTS	FSPMYHVVTR	RK <u>T</u> KCVMKLV
<i>X.laevis</i>	DYDNIPTVVF	SHPPIGTVGL	<u>T</u> EEEAV <u>T</u> AKG	RENVKVYTTTS	FSPMYHAVTR	RK <u>T</u> KCVMKLV
<i>X.tropicalis</i>	CVGKEEKVVG	LHMQGLGCDE	MLQGFAVAIK	MGATKKDFDN	TVAIHP <u>T</u> SSE	ELVTLR
<i>X.laevis</i>	CVGKEEKVVG	LHMQGLGCDE	MLQGFSAIK	MGATKKDFDN	TVAIHP <u>T</u> SSE	ELVTLR

Figure 3.7. A comparison of the predicted serine (S), threonine (T) and tyrosine (Y) phosphorylation sites on GR that are shared between *X. tropicalis* and *X. laevis*. Predicted phosphorylation sites are shown in bold and underlined.

Figure 3.8. Arrhenius plot for purified GR from the muscle of control *R. sylvatica*. Data are means $\pm$ SEM, $n=4$.

Figure 3.9. Differential scanning fluorimetry analysis of the thermal stability of purified GR from control and 24 h frozen frog muscle. Frozen GR showed no significant difference when compared to GR from control frogs, n=8 independent trials.

Chapter 4:

A hydrogen peroxide safety valve: The reversible phosphorylation of catalase from the freeze tolerant North American wood frog, *Rana sylvatica*.

1. Introduction

Formation of H_2O_2 in animal tissues can arise directly from enzymatic sources, specifically a number of different oxidases including xanthine oxidase, monoamine oxidase, and amino acid oxidase (Sies, 1974). However, H_2O_2 formation due to superoxide dismutase activity is considered to be the major cellular source (Boveris et al., 1972). Although O_2^- can spontaneously dismutate, superoxide dismutase catalyzes this reaction at much higher rates. Superoxide radicals have been linked to the mitochondrial formation of H_2O_2 via the respiratory chain (Loschen et al., 1971; Loschen et al., 1974). The mitochondria are a major site of H_2O_2 formation, due in large part to the superoxide formed via complexes I and III of the electron transport system (Drose and Brandt, 2008). Specifically, complex III releases O_2^- into the intermembrane space, while complex I releases O_2^- into the inner mitochondrial matrix space where MnSOD produces H_2O_2 (Boveris et al., 1972; Loschen et al., 1971; Loschen et al., 1974). *R. sylvatica* muscle superoxide dismutase activity seems to be boosted in the mitochondria (chapter 2), which could lead to the increased formation of H_2O_2 .

H_2O_2 is generated in a site specific manner and the local concentration of H_2O_2 is modulated by its generation and subsequent removal. The diffusion of H_2O_2 away from the site of production and across membranes creates a H_2O_2 gradient (Antunes and Cadenas, 2000). Furthermore, the sites of H_2O_2 removal, via catalase in the peroxisomes, glutathione peroxidase and peroxiredoxins in the cytosol and other organelles further drives this diffusion gradient from the site of production to the site of removal (Antunes and Cadenas, 2000; Mueller et al., 2009; Sobatta et al., 2013). Under normal cellular

concentrations, peroxidases seem to play a much larger role in catalyzing the reduction of H_2O_2 in comparison to other enzymatically driven reduction reactions. This is in large part due to the significantly higher affinity for H_2O_2 by the peroxiredoxins (Kim et al., 1988). Peroxiredoxins are estimated to be responsible for the reduction of greater than 90% of the mitochondrial and cytosolic H_2O_2 under normal cellular conditions (Cox et al., 2010; Winterbourn, 2008). However, peroxiredoxins, like most peroxidase driven reactions, have limited flux and are inactivated via H_2O_2 reaction with cysteine thiolate in peroxiredoxins in the face of elevated levels of H_2O_2 (Flohe, 2010; Winterbourn, 2013; Hall et al., 2011). This means that under conditions such as ischemia/reperfusion, peroxiredoxins would likely be inactive.

Catalase (CAT) (E.C. 1.11.1.6) is a primary antioxidant enzyme responsible for catalyzing the decomposition of hydrogen peroxide into water and oxygen. There are three known types of CAT studied to date, however, only the mammalian model will be discussed herein. Mammalian CAT is a tetrameric protein containing four identical heme-containing subunits (Young and Woodside, 2001). The exact mechanism by which CAT decomposes H_2O_2 is very complex; however, it can be simplified as follows:

In this proposed mechanism, CAT reacts with H_2O_2 to form an intermediate called compound I which reacts with a second H_2O_2 molecule to return CAT to the native state (Andersson et al., 1991). It has been suggested that a key histidine residue in the active site is involved in the proton transfer (Andersson et al., 1991).

Interestingly, CAT is a major NADPH binding enzyme, showing significantly high specificity for NADPH over NADH or the corresponding oxidized forms (Fita and Rossman, 1985; Kirkman et al., 1984). The function of NADPH is not fully understood, however, several proposed roles have recently emerged. The first role proposed for NADPH has been a protective one, in which NADPH may hinder the inactivation of CAT by H_2O_2 by oxidizing bound NADPH (Kirkman et al., 1999). The second is as an alternative reduction pathway for CAT at low H_2O_2 concentrations (Olson and Bruice, 1995; Ivancich et al., 1996; Hoffschir et al., 1998; Putnam et al., 2000). The last, and most interesting with respect to this thesis, is as a regulatory protein, releasing NADPH when the cell is under peroxidative stress (Kirkman et al., 1984). The release of NADPH from CAT could augment the activities of glutathione reductase in conjunction with glutathione peroxidase to help remove excess H_2O_2 (Kirkman et al., 1984).

Although there are several primary antioxidant enzymes involved in the detoxification of ROS species, CAT is the primary enzyme tasked with actively decomposing high levels of H_2O_2 and some other cytotoxic oxygen derivatives (Gaetani et al., 1996; Mueller et al., 1997). CAT is encoded by a similar gene, *cat*, in both frogs (*Xenopus tropicalis*, *X. laevis*) and humans. CAT from *X. tropicalis* and *X. laevis* has a molecular weight of 60 kDa as reported in the UniProtKB database (F6PYM8_XENTR, Q4FZM6_XENLA) (Klein et al., 2002). CAT has a significantly lower affinity for H_2O_2 , than the peroxidase family of enzymes, and is often thought to carry out the reduction of H_2O_2 under extreme or toxic conditions (Kim et al., 1988).

Increases in mitochondrial H_2O_2 concentrations and loss of the enzymatic ability to reduce H_2O_2 , has been shown to play a role in signaling during hypoxia, inflammation,

and apoptosis (Finkel, 2012). Elevated H_2O_2 during ischemia / reperfusion has been demonstrated to overwhelm delicate balances in cellular H_2O_2 distribution, maintained by compartmentalized peroxidases, often triggering apoptosis under oxidative stress (Clement et al., 1998). Although it is unclear how H_2O_2 stimulates apoptosis, it is clear that CAT is important in humans and traditional animal models, however, less is known about its role in disease states, or its role aiding survival in the freezing frog. This chapter presents the first investigation of the potential method of regulation of CAT in the leg muscle of *R. sylvatica*, comparing control and frozen states, and provides evidence of CAT regulation by reversible protein phosphorylation during freezing, including the possibility of multiple phosphorylation states *in vitro*.

2. Materials and Methods

2.1. Chemicals

All biochemicals were from BioShop (Burlington, ON, Canada) with a few exceptions; hydrogen peroxide (H_2O_2) was from Caledon Labs (Ontario, Canada), the Cibacron blue column was from Affiland (Ans, Belgium), hydroxyapatite Bio-Gel[®] HTP Gel column was from Bio-Rad (Hercules, CA) and potassium phosphate, monobasic was from J.T. Baker Chemical Company (London, UK).

2.2. Animals

Wood frogs were cared for and sampled as described in chapter 2 section 2.2.

2.3. *Preparation of muscle tissue lysates for protein purification*

For protein purification, samples of frozen muscle tissue were homogenized 1:5 w:v in ice-cold homogenizing buffer A [20 mM potassium phosphate (KPi) buffer, pH 7.2, containing 15 mM β -glycerophosphate, 1 mM EGTA, 1 mM EDTA, 10 mM β -mercaptoethanol, 5% v/v glycerol and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. Homogenates were then centrifuged at $13,500 \times g$ at 4 °C and the supernatant collected for use in protein purification.

2.4. *Purification of CAT*

A hydroxyapatite column was equilibrated in homogenization buffer A. A 3 mL aliquot of frog muscle extract was applied to the column (1.5 cm $\times$ 2 cm) and washed with 20 mL of buffer A to remove unbound proteins. CAT was eluted from the hydroxyapatite column with a linear gradient of 0-3.5 M KCl in buffer A. Fractions of 900 μ L were collected and 10 μ L from each fraction was assayed to detect CAT activity (see kinetic assays section for methodology). The top 7 fractions of peak CAT activity were pooled and diluted 10-fold with buffer A. The diluted fractions were applied to a Cibacron blue column (1.5 cm $\times$ 10 cm) pre-washed with 30 mL of buffer A. The Cibacron blue column was then washed with 30 mL of buffer A to remove unbound protein. Bound proteins were eluted with a linear gradient of 0-2 M KCl in buffer A. Fractions of 450 μ L were collected and 10 μ L from each fraction was assayed to detect CAT activity. The top 14 fractions of peak CAT activity were pooled and diluted 10-fold with buffer A. The diluted fractions were applied to a DEAE⁺ column (1.5 cm $\times$ 20 cm)

pre-washed with 50 mL of buffer A. The DEAE⁺ column was then washed with 50 mL of buffer A to remove unbound protein. Bound proteins were eluted with a linear gradient of 0-1 M KCl in buffer A. Fractions of 900 mL were collected and 10 μ L from each fraction was assayed to detect CAT activity, the top 12 fractions were pooled for control CAT, while the top 9 fractions were pooled for frozen CAT. The purity of CAT was determined by combining aliquots of samples 2:1 v:v with 2 $\times$ SDS loading buffer (100 mM Tris buffer, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercapotethanol), boiling for 5 min, and then running 30 μ L samples purified from each successive purification on SDS-PAGE, as described in section 2.6.

2.5. *Kinetic assays*

CAT was assayed using a modified version of the method of Aebi (1984). Assay conditions were: 50 mM KPi buffer (pH 7.2), 40 mM H₂O₂, and 10 μ L enzyme preparation. One unit of enzyme activity is the amount that reduces 1 μ mol of H₂O₂ per minute at 25°C. The amount of H₂O₂ was measured at 240 nm in a Thermo Labsystems Multiskan spectrophotometer (Thermo Scientific, Waltham, MA, USA). Data were analyzed using the Kinetics v.3.5.1 program, developed by Brooks (1992).

2.6. *Gel electrophoresis*

CAT was purified as described in section 2.4. Protein separation was achieved by running 30 μ L of CAT samples on 10% SDS-PAGE gels. Sample aliquots were loaded onto polyacrylamide gels together with PageRuler[®] pre-stained molecular weight

standards (Thermo Scientific; Cat# 26616) and separated using a discontinuous buffer system. Electrophoresis was carried out at 180 V for 65 min using the BioRad Mini-Protean 3 system with 1x Tris-glycine running buffer (0.05 M Tris, 0.5 M glycine, 0.05% w/v SDS). Protein banding was visualized with Coomassie blue.

2.7. *ProQ Diamond phosphoprotein staining*

Enzyme extracts from muscle of both control and frozen frogs were purified as described in section 2.4. The top fractions based on activity were pooled, and protein levels in the pooled fractions were quantified using the Coomassie blue dye-binding method. Aliquots of the pooled fractions were then mixed 2:1 v:v with SDS loading buffer (100 mM Tris buffer, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercapotethanol) and subsequently boiled for 5 minutes and stored at -20°C until used. The gel was then run as in section 2.6 above and then subjected to ProQ diamond staining as described in chapter 3 section 2.7.

2.8. *Western blotting analysis of post-translational modifications of CAT*

Electrophoresis was carried out as described in section 2.6. Proteins on the gel were then electroblotted onto polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA) using a BioRad mini Trans-Blot cell. The transfer was carried out at 160 mA for 1.5 h. Following the transfer, membranes were washed in TBST (10 mM Tris, pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) for 3×5 min. The membranes were blocked using 0.1% polyvinyl alcohol in TBST for 30 min. After blocking, the membranes were

probed with primary antibody diluted 1:500 v:v in TBST for 3 h at room temperature (RT) with the following primary antibodies: (1) rabbit anti-phosphoserine (Cat #618100, Invitrogen, Carlsbad, CA, USA); (2) rabbit anti-phosphothreonine (Cat. # 718200, Invitrogen, Carlsbad, CA, USA); or (3) rabbit anti-phosphotyrosine (Cat # 615800, Invitrogen, Carlsbad, CA, USA). The membranes were washed 3×5 min with TBST at RT and probed with goat anti-rabbit–peroxidase secondary antibody for 20 min. Membranes were washed again 3×5 min in TBST at RT. Blots were developed with enhanced chemiluminescence reagents. Images were captured using a ChemiGenius Bio-Imaging system with GeneSnap software and densitometry analysis performed using GeneTools software (Syngene, Frederick, MD). The intensity of the immunoreactive bands were standardized against corresponding Coomassie blue stained bands. The intensity of the immunoreactive bands from control muscle tissue was set to a reference value of 1 whereas the intensity of standardized immunoreactive bands from frozen muscle tissue was expressed as a fold change.

2.9. *In vitro* incubation to stimulate protein kinases and phosphatases

Frog muscle extracts from control animals, prepared as described in section 2.2, were filtered through a G50 spun column equilibrated in incubation buffer (50 mM KPi, 10% v/v glycerol, 30 mM β -mercaptoethanol, pH 7.2). Aliquots of the filtered supernatants were incubated for 12 hours at 4°C with specific inhibitors and stimulators of protein kinases, as described in Macdonald and Storey (1999). Each aliquot was mixed

2:1 v:v with the appropriate solutions to stimulate protein kinases. Each solution was prepared in incubation buffer and the following incubation conditions were used:

- (a) STOP conditions: 2.5 mM EGTA, 2.5 mM EDTA and 30 mM β -glycerophosphate.
- (b) Stimulation of total endogenous kinases: 30 mM β -glycerophosphate, 2 mM AMP, 2 mM cAMP, 10 mM ATP, 2 mM cGMP, 2.6 mM CaCl_2 , 14 $\mu\text{g/mL}$ phorbol myristate acetate, 20 mM MgCl_2 and 10 mM Na_3VO_4 .
- (c) Stimulation of total endogenous kinases: 10 mM MgCl_2 and 10 mM CaCl_2 .
- (d) Stimulation of endogenous AMP-activated kinase (AMPK): 1 mM AMP, 5 mM ATP, 10 mM MgCl_2 , 30 mM β -glycerophosphate, 5 mM Na_3VO_4 .
- (e) Stimulation of endogenous calcium-calmodulin dependent kinase (CaMK): 1 U of calf intestine calmodulin + 1.3 mM CaCl_2 , 5 mM ATP, 10 mM MgCl_2 , 30 mM β -glycerophosphate, 5 mM Na_3VO_4 .
- (f) Stimulation of endogenous cyclic-AMP dependent protein kinase (PKA): 1 mM cAMP, 5 mM ATP, 10 mM MgCl_2 , 30 mM β -glycerophosphate, 5 mM Na_3VO_4 .
- (g) Stimulation of endogenous cyclic-GMP dependent protein kinase G (PKG): 1 mM cGMP, 5 mM ATP, 10 mM MgCl_2 , 30 mM β -glycerophosphate, 5 mM Na_3VO_4 .
- (h) Stimulation of endogenous protein phosphatases 1 + 2A (PP1+PP2A): 2 mM EDTA, 2 mM EGTA, 30 mM Na_3VO_4 .
- (i) Stimulation of endogenous protein phosphatase 2B (PP2B): 2 mM EDTA, 1 μM okadaic acid, 5 mM Na_3VO_4 .
- (j) Stimulation of endogenous protein phosphatase 2C (PP2C): 2 mM EGTA, 1 nM cypermethrin, 1 μM okadaic acid, 5 mM Na_3VO_4 .

CAT from control and frozen muscle was then purified as previously described in section 2.4. Samples were then analyzed as described in section 2.5 and the K_m for H_2O_2 was compared for each condition.

2.10. Determination of protein stability

Stability parameters of purified CAT from control and 24 h frozen frogs were analyzed using differential scanning fluorometry as described in chapter 3 section 2.10 and the T_m for each was recorded.

2.11. Statistical analysis

Comparison of enzyme kinetics, relative protein phosphorylation and protein stability were performed using a Student's t test, two-tailed, assuming unequal variances. A probability of $P < 0.05$ was considered significant. Comparison of the K_m for H_2O_2 after incubations stimulating endogenous kinases and phosphatases were performed using analysis of variance followed by the Tukey post hoc test, assuming unequal variances. A probability of $p < 0.05$ was considered significant.

3. Results

3.1. Purification of CAT from the muscle of control and frozen frogs

The purification scheme used for wood frog muscle CAT is shown in Table 4.1. The procedure used chromatography on a Bio-Gel[®] HTP Gel hydroxyapatite column

(Figure 4.1A), Cibacron blue chromatography (Figure 4.1B), and DEAE⁺ ion exchange column (Figure 4.1C, 4.1D). Control CAT eluted from the DEAE⁺ ion exchange column yielding a first peak at approximately 200 mM KCl and a second peak at approximately 300 mM KCl (Figure 4.1C). Frozen CAT eluted from the DEAE⁺ ion exchange column in one peak at 300 mM KCl (Figure 4.1D). Frog muscle CAT was purified 105.4 fold with an overall yield of activity of 11.4% (Table 4.1). The final specific activity of CAT was 2308 mU/mg of protein. The success of the purification process was assessed using SDS-PAGE with Coomassie blue staining of the gel (Figure 4.2). This showed that CAT was purified to near homogeneity as there was one strong band corresponding to the correct molecular weight of ~60 kDa for CAT (Figure 4.2).

3.2. Kinetic characterization of CAT

Kinetic parameters of purified muscle CAT were assessed to determine the differences between the enzyme isolated from control and frozen frogs. The K_m for H₂O₂ and the V_{max} of CAT from the muscle of *R. sylvatica* were both demonstrated to change between control and frozen animals ($p < 0.05$) (Table 4.2). The K_m for H₂O₂ decreased significantly (by 36.4%) in frozen frogs in comparison to the K_m of H₂O₂ in control animals (Table 4.2). The V_{max} of CAT from frozen frogs increased significantly (1.48 fold) over the measured V_{max} of CAT in control animals (Table 4.2). To properly assess frog muscle enzyme kinetics, all recoverable peaks of pure enzyme were combined for each condition prior to kinetic analysis.

3.3. *Post-translational modification of endogenous CAT*

To test whether or not purified frog muscle CAT was modified by reversible phosphorylation, the peak fractions (based on activity) from the purification profiles for control and frozen frogs were combined and run on SDS-PAGE gels, and stained with ProQ Diamond phosphoprotein stain. When band densities were quantified, they showed a 1.73 fold higher band intensity ($p < 0.05$) for CAT from muscle of frozen frogs as compared to the enzyme from control frogs (Figure 4.3).

Immunoblotting was then used to further assess residue-specific phosphorylation of muscle CAT. Purified CAT reacted with antibodies specific for phosphorylation at serine residues, threonine residues or tyrosine residues (Figure 4.3). The relative phosphorylation levels of serine and tyrosine residues increased significantly for frozen CAT as compared with control ($p < 0.05$, Figure 4.3).

CAT from muscle of control frogs was purified as described in section 2.4 and the two resulting peaks from the final DEAE chromatography step were assessed independently. Both produced clear band at ~60 kDa but showed differential levels of total phosphorylation when subjected to ProQ diamond phosphoprotein staining. When band densities were quantified, Peak II showed a 3.33 fold higher band intensity ($p < 0.05$) as compared to Peak I from control frogs (Figure 4.4).

3.4. *In vitro posttranslational modification of CAT*

To test whether or not reversible phosphorylation of CAT could account for the observed changes in K_m for H_2O_2 that were seen in vivo between control and frozen

states, incubations were set up in which crude extracts of muscle were stimulated by endogenous kinases and phosphatases. CAT was purified as previously described in section 2.4., and subsequent effects on CAT K_m H_2O_2 were measured. Incubations which stimulated endogenous phosphatases yielded multiple peaks of CAT activity. The first major peak was observed at ~200 mM KCl with an obvious cleft at ~325 mM KCl, followed by the second major peak at ~400 mM KCl (Figure 4.5A). Incubations which stimulated total endogenous kinases resulted in a single sharp peak on DEAE+ at ~400 mM KCl (Figure 4.5B).

The K_m of H_2O_2 was measured after incubation with the specific endogenous kinases and phosphates as described in section 2.9 (Figure 4.6). Stimulation of AMPK resulted in a significant decrease in the K_m for H_2O_2 for CAT from control frogs, but not frozen frogs ($p < 0.05$). Stimulation of CAMK had no significant effect on the K_m for H_2O_2 for CAT from either control or frozen frogs ($p < 0.05$). Stimulation of PKA resulted in a significant decrease in the K_m for H_2O_2 for CAT in control frogs, but not for the enzyme from frozen frogs ($p < 0.05$). Stimulation of PKG had no significant effect on the K_m for H_2O_2 for CAT in control or frozen frogs ($p < 0.05$). Stimulation of PP1 resulted in no significant changes in the K_m for H_2O_2 for CAT from either control or frozen frogs ($p < 0.05$). However, stimulation of PP2B resulted in a significant increase in the K_m for H_2O_2 for CAT in frozen frogs, but not in control frogs ($p < 0.05$). Stimulation of PP2C resulted in a significant increases in the K_m for H_2O_2 for CAT from both control and frozen frogs ($p < 0.05$).

3.5. Scansite Prediction of Phosphosites

The Scansite application from the Massachusetts Institute of Technology (<http://scansite.mit.edu/>) was used to analyze the CAT sequences from two frog species, *X. laevis* and *X. tropicalis*, in a search for putative phosphorylation sites. These two CAT protein sequences showed 93.18% identity. Only those kinases predicted to phosphorylate CAT from both *X. laevis* and *X. tropicalis* are reported. Putative consensus sequences for phosphorylation sites of Akt kinase (serine, threonine), AMP-activated kinase (serine, threonine), calcium-calmodulin dependent kinase 2 (threonine), Cdc2 kinase (serine, threonine), Cdk5 kinase (serine, threonine), EGFR Kinase (tyrosine), Erk1 (threonine), GSK3 (serine), Lck kinase (tyrosine), PDGFR kinase (tyrosine), PKA (serine, threonine), PKC alpha (serine), PKC beta (serine), PKC delta (serine), PKC epsilon (serine), PKC gamma (serine), PKC mu (serine, threonine), PKC zeta (serine), and Src kinase (tyrosine) were predicted for CAT (Figure 4.7).

Human CAT shows 89.96% identity when compared to *X. laevis* and *X. tropicalis*. Human CAT was analyzed for comparison using Scansite, resulting in the following putative consensus sequences for phosphorylation sites: Akt kinase (serine, threonine), AMP-activated kinase (threonine), calmodulin dependent kinase 2 (serine), casein kinase 1 (threonine), casein kinase 2 (serine), Cdc2 kinase (serine, threonine), Cdk5 kinase (serine, threonine), EGFR Kinase (tyrosine), Erk1 Kinase (threonine), Lck kinase (tyrosine), PDGFR kinase (tyrosine), PKA (serine, threonine), PKC alpha (serine), PKC beta (serine), PKC delta (serine), PKC epsilon (serine), PKC gamma (serine), PKC mu (serine, threonine) and PKC zeta (serine), and Src kinase (tyrosine) were predicted for CAT.

3.6. Stability of CAT

The structural stability of CAT was evaluated by testing enzyme sensitivity to thermal denaturation measured using differential scanning fluorimetry as described in section 2.10. There was no significant difference in the thermal denaturation properties of purified CAT between control and frozen frogs (Figure 4.8). CAT purified from the muscle of *R. sylvatica* showed a peak at 60°C for control frogs and 61°C for frozen frogs. The calculated T_m value (temperature that results in a 50% loss of folded structure) was 56.25°C for purified CAT from muscle of control frogs, which was not significantly different from the value of 56.67°C for purified CAT from muscle of frozen frogs (Table 4.2; Figure 4.8).

4. Discussion

The role of CAT in disease is unclear. Knockout and knockdown experiments of CAT have proven to be non-lethal, however, deleterious effects have been reported including increases incidence of diabetes (Sies, 1986; Mo et al., 1995; Hamilton et al., 2012). Disruption in CAT activity and expression has been observed in many disease states including diabetes, in which decreases in catalase expression contribute to disease development (Goth, 2000; Goth and Eaton, 2000). A complete loss of catalase is the phenotype expressed in Acatlasia or Takahara's disease (Ogata, 1991). Reduced CAT activity has been reported in patients suffering from Parkinson's disease, schizophrenia as well as atherosclerosis (Goth and Vitai, 1996). CAT gene mutations, leading to a dysfunctional enzyme, have been observed in diabetes, hypertension and vitiligo (Goth et

al., 2004). Functional, endogenous CAT seems to be important for the quality of life, and exploration of CAT function in the wood frog, which can endure long periods of time under conditions that mimic disease-like states, could yield a greater understanding the role of CAT in disease.

Many of the aforementioned disease states include disruption in blood flow or oxygen deprivation, often accompanied by an increase in the oxidative stress imposed on the organism and individual cells. Previous studies exploring organisms experiencing changes in CAT regulation under oxygen deprived states or reduced blood flow include: the ground squirrel (*Spermophilus citellus*) which shows increased CAT expression during hibernation (Vucetic et al., 2013), the African clawed frog (*Xenopus laevis*) which exhibits increased CAT expression in both the muscle and liver in response to whole body dehydration (Malik and Storey, 2011), the common carp (*Cyprinus carpio*) which shows increased CAT activity in the brain and kidneys during hypoxia (Lushchak *et al.*, 2005), the land snail (*Otala lactea*) in which CAT activity decreases in foot muscle during estivation (Hermes-Lima et al., 1998), the leopard frog (*Rana pipiens*) which shows an increase in CAT activity in muscle tissue during severe dehydration and anoxic conditions (Hermes-Lima and Storey, 1998), the spadefoot toad (*Scaphiopus couchii*) where CAT activity in the liver and heart were significantly higher, while being lower in the kidney during estivation (Grundy and Storey, 1998), and the marine periwinkle (*Littorina littorea*) where CAT activity increased in the hepatopancreas during anoxia as well as in hepatopancreas and foot muscle upon exit from anoxia (Pannunzio and Storey, 1998), and the red eared slider turtle (*Trachemys scripta elegans*) which shows decreased CAT activity in the heart kidney and brain during anoxia (Willmore and Storey, 1997).

In addition to oxygen deprivation, the freeze tolerant frog also experiences ischemia which has been repeatedly demonstrated to imposed oxidative stress on the animal during the ischemic period, and a burst production of ROS following the ischemic episode (Ferrari *et al.*, 1991; Simpson and Lucchesi, 1987; Zweier and Talukder, 2006). The wood frog likely experiences similar increases in H_2O_2 , attributed to the ischemic nature of freezing. Interestingly, introduction of exogenous CAT has been shown to attenuate apoptosis (Sancho et al., 2003). Therefore, through exploration of the role of CAT as a key enzyme involved in the reduction of H_2O_2 at high levels, the regulation of CAT in the frozen frog presented in this chapter can provide important information for both the survival of the animal and insight into disease states by staving off signaling of apoptosis upon reoxygenation.

4.1. *Kinetic changes of CAT during freezing*

The apparent K_m for H_2O_2 of CAT significantly decreased in the frozen state compared to the control state (Table 4.2). This reduction in the apparent K_m of H_2O_2 would suggest that CAT has a higher affinity for H_2O_2 during freezing. An increase in affinity for H_2O_2 would increase the reducing ability of CAT, and increase protection against the formation of very high levels of H_2O_2 . As previously mentioned, CAT has a significantly lower affinity for H_2O_2 than peroxidases, suggesting that the role of CAT may be a panic switch of sorts (Kim et al., 1988). Although CAT is absent in the organelle that is the major source of H_2O_2 production, the mitochondria, H_2O_2 is membrane permeable and in large concentrations will follow a concentration gradient

from sources of production to sources of reduction such as the peroxisome (Sies, 1974). This is of special importance due to the fact that peroxidases are known to be inhibited at high concentrations of H_2O_2 , possibly shifting the role of the main H_2O_2 reducer from the peroxidases to CAT (Flohe, 2010; Winterbourn, 2013; Hall et al., 2011; Wood et al., 2003). Therefore, an increase in production of H_2O_2 could lead to a disruption of H_2O_2 mediated apoptosis signaling, typically held in check by peroxidase activities, and ultimately lead to the destruction of the cell due to oxidative stress induced signaling of apoptosis. CAT has been proposed to take a more central role in H_2O_2 reduction under such circumstances (Wood et al., 2003).

4.2. *Phosphorylation of CAT*

Investigation into the relative phosphorylation state of CAT identified a significant difference in global phosphorylation levels (a 1.73 fold increase) in the frozen state as compared with the control, as well as significant increases in the relative phosphorylation of serine (1.60 fold) and tyrosine (1.27 fold) residues (Figure 4.3). Interestingly, control CAT demonstrated multiple peaks of CAT activity during elution off a DEAE⁺ column (Figure 4.1C). Previously, shifts to the right in elution profiles for proteins bound to a positively charged ion exchange column have been attributed to phosphorylation events, since the addition of phosphate groups adds a large negative charge to a protein (Dawson and Storey, 2012; Lant and Storey, 2011; Dawson and Storey, 2011; Holden and Storey, 2011; Bell and Storey, 2010; Dieni and Storey, 2009; Abnous and Storey, 2008). Hence, it is probable that the CAT peaks eluting at lower

versus higher salt concentrations represent low versus high phosphorylation states of the enzyme. To investigate this experimentally, the phosphorylation state of each peak was directly analyzed for CAT from control frogs. A significantly higher level of phosphorylation (3.33 fold higher) was observed for CAT in the second peak, as determined by ProQ diamond phospho-protein staining (Figure 4.4). This difference in phospho-staining, in conjunction with the two peak elution profile, provides evidence for multiple phosphorylation forms of CAT from the muscle of *R. sylvatica*.

To further analyze CAT posttranslational modification, enzyme preparations were incubated under conditions that stimulated endogenous protein phosphatase or protein kinase activities. After subsequent purification, the protein phosphatase treated enzyme showed the same two peak elution profile as was observed in control frogs (Figure 4.5A) whereas after stimulation of endogenous kinases and subsequent purification, only one sharp peak was discovered in the elution profile, similar to the situation in frozen frogs (Figure 4.5B). This adds strong support for the proposal that the two peaks of CAT activity found in control frogs represent low versus high phosphate forms of the enzyme and that freezing stimulates the conversion of CAT into the high phosphate form.

The phosphorylation phenomenon observed for CAT from *R. sylvatica* was explored further in an attempt to elucidate which kinases and phosphatases are responsible for the reversible phosphorylation of CAT during freezing, and to explore possible functional consequences of phosphorylation. After incubation under conditions which stimulated AMPK and PKA action, CAT from control frogs showed a decrease in the K_m for H_2O_2 to values similar to (for AMPK) or about 50% lower than (for PKA) that seen for CAT purified from frozen frogs (Figure 4.6). Conversely, under conditions

which stimulated PP2B or PP2C, CAT from frozen frogs showed an increase in the K_m of H_2O_2 to values similar to CAT purified from control frogs (Figure 4.6). This suggests that the phosphorylation of CAT is a strong candidate for eliciting the apparent increase in affinity for H_2O_2 observed during freezing. These data would suggest that the more phosphorylated form of CAT (with the lower K_m value) is the more active form. Increased affinity for H_2O_2 would likely enhance the ability of CAT in the muscle of frozen frogs to minimize/prevent freeze-induced oxidative stress, as well as prepare for the eventual burst production of free radicals during thawing reperfusion.

Previous studies have shown that AMPK activity increases (4.5 fold) during freezing and the percentage of free catalytic subunit of PKA (PKAc) in the muscle of frozen frogs is elevated (3.62 fold) within 2 minutes after freezing begins (Rider and Storey, 2006; Holden and Storey, 1996). The level of PKAc remains significantly high through the duration of freezing, and 24 h after thawing, and the activity of AMPK remains elevated after thawing 24 h. Hence, this provides further support for the idea that CAT is modified by protein phosphorylation during freezing, and that phosphorylation may be mediated by either AMPK or PKA in frozen frogs. This would suggest that CAT may become more active as freezing begins and remain active throughout the freeze-thaw cycle, suggesting that phosphorylation of CAT by AMPK or PKA would benefit the frog both during freezing and as a preparatory mechanism to deal with reperfusion during thawing.

To further explore the viability of phosphorylation as a mechanism of CAT control in the freezing frog, an investigation into the putative phosphorylation sites present on CAT was undertaken by analyzing the sequences of two genome-sequenced

frog species (Figure 4.7). This analysis showed the presence of putative AMPK and PKA phosphorylation sites on frog CAT, supporting the idea that AMPK and/or PKA are candidates to phosphorylate CAT during freezing. Interestingly, human CAT also showed the predicted AMPK and PKA phosphorylation sites, suggesting the possibility for AMPK or PKA mediated phosphorylation regulation of the enzyme in humans.

4.3. *Thermal stability of CAT*

CAT purified from both control and frozen frogs was explored via DSF to determine whether the enzymes showed differences in thermal stability which would be indicative of substantive conformational changes in the enzyme between the two states. However, no significant differences in CAT thermal stability were found between the two states (Figure 4.8). This does not exclude to possibility of improved stabilization of CAT in the face of H₂O₂ insult. The stability of CAT has been extensively studied, demonstrating the thermodynamic stability of CAT activity, and has shown that CAT remains active across a broad range of temperatures, pH, and H₂O₂ concentrations (Nadler et al., 1986; Yumoto et al., 1990; Brown-Peterson and Salin, 1993; Maricinkeviciene et al., 1995; Fraaije et al., 1996). The T_m values found in the present study showed that denaturation of frog muscle CAT takes place at a relatively high temperature of approximately 56°C, suggesting that CAT is a very stable enzyme and unlikely to be denatured under environmentally encountered temperatures (Table 4.2, Figure 4.8).

4.4. Conclusion

The regulation of CAT from muscle of the freeze tolerant frog, *R. sylvatica*, can provide useful insights into the effects of ischemia on free radical mediation. CAT from frozen frogs showed significant modifications compared with the control enzyme including a lower K_m for H_2O_2 , a higher V_{max} , and increased phosphorylation at serine and tyrosine residues. Specifically, phosphorylation of CAT by AMPK and PKAc was suggested. The evidence presented in this chapter suggests that phosphorylation may activate CAT during freezing. Augmented CAT activity may increase the ability of *R. sylvatica* to overcome oxidative stress associated with ischemia or reperfusion. This study can also provide valuable insight into disease pathology by offering a unique observation of the role of CAT during ischemia. CAT has a high degree of similarity across species. The discovery of phosphorylation events for CAT is of interest for human application since CAT from frogs was found to have 90% amino acid identity when compared to human CAT, as well as similar predicted phosphorylation sites. This striking similarity suggests that researchers should explore possible phosphorylation and subsequent activation of CAT in human disease states since increased CAT activity has been previously attributed to amelioration of disease symptoms.

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Table 4.1. Typical purification and yield of *Rana sylvatica* muscle CAT.

Purification Step	Total Protein (mg)	Total Activity (mU)	Specific Activity (mU/mg)	Fold Purification	Yield (%)
Crude extract	110.4	2418	21.9	-	100
Hydroxylapatite	1.10	1666	1513	69.1	68.9
Cibacron Blue	0.85	1599	1876	85.7	66.2
DEAE ⁺	0.12	276	2308	105.4	11.4

Table 4.2. Kinetic parameters of CAT purified from muscle of control and frozen *R. sylvatica*. * - Significantly different between control and frozen values, $p < 0.05$. $n = 8$.

Enzyme parameters	Control	24h Frozen
$K_m \text{ H}_2\text{O}_2 \text{ (mM)}$	20.7 ± 0.87	$13.2 \pm 0.50^*$
$V_{\max} \text{ (U/mg)}$	2308 ± 18.5	$3412 \pm 55.8^*$
$T_m \text{ (}^\circ\text{C)}$	56.3 ± 0.10	56.7 ± 0.17

Figure 4.1. (A) Typical elution profile for CAT on a hydroxyapatite column. (B) Typical elution profile for CAT on a Cibacron blue column. (C) Typical elution profile for CAT from control muscle on a DEAE⁺ column. (D) Typical elution profile for CAT from frozen muscle on a DEAE⁺ column. Solid line indicates [KCl].

Figure 4.2. Stepwise purification of CAT samples from the muscle of control *R. sylvatica*. (A) CAT-containing eluant after hydroxylapatite chromatography, (B) CAT-containing eluant after Cibacron blue chromatography, (C) molecule weight standards, and (D) purified CAT from DEAE⁺ chromatography. CAT protein is the prominent band at ~60 kDa indicated by the arrow.

Figure 4.3. Relative phosphorylation levels of CAT as assessed from incubations of purified frog muscle extracts from control and 24 h frozen frogs with detection using ProQ Diamond phospho-protein stain or Western blotting for site-specific phosphorylation. * - Significantly different from the corresponding control value, $p < 0.05$, $n = 6$.

Figure 4.4. Relative phosphorylation levels of control muscle CAT from Peak I and Peak II as assessed by Pro-Q diamond phospho-protein staining. * - Significantly different from the peak 1 value, $p < 0.05$, $n = 5$.

Figure 4.5. DEAE⁺ elution profiles for muscle CAT from control frogs and incubated under conditions that stimulated protein phosphatases (A) or protein kinases (B). Solid line indicates [KCl].

Figure 4.6. Effects of in vitro incubations of purified CAT of muscle under conditions which stimulated the activities of endogenous protein kinases or phosphatases on the resulting K_m values for H_2O_2 of CAT from control and frozen *R. sylvatica*. * - Significantly different from corresponding STOP condition using the Dunnett's test, $p > 0.05$, $n = 8$.

<i>X.tropicalis</i>	MAGNKEKAPT	QMNQWKEARG	SQKPSVLTTG	AGHPVGDKLN	LLTAGPRGPL	LVQDVVFTDE
<i>X.laevis</i>	MANNKEKADT	QMNQWKETRG	SQKPNVLTG	AGHPVGDKLN	LLTVGPRGPL	LVQDVVFTDE
<i>X.tropicalis</i>	MAHFDREIRP	ERVVHAKGAG	AFGYFEVTHD	ITQYCKANVF	GKVGKR <u>TP</u> VA	ARFS <u>TV</u> VAGEA
<i>X.laevis</i>	MAHFDREIRP	ERVVHAKGAG	AFGYFEVTHD	ITQYCKAKVF	EKVGKR <u>TP</u> VA	ARFS <u>TV</u> VAGEA
<i>X.tropicalis</i>	G <u>SP</u> DTIRDPR	GFAVKMYTEE	GNWDLT <u>GNN</u> T	PIFFIRDAIL	FPSFVHSQKR	NPQTHMKDPD
<i>X.laevis</i>	G <u>SP</u> DTIRDPR	GFAVKMYTED	GNWDLT <u>GNN</u> T	PIFFIRDAIL	FPSFVHSQER	NPQTHMKDPN
<i>X.tropicalis</i>	MVWDFWSLRP	ESLHQV <u>S</u> FLF	SDRGIPDGHR	HMNGYG <u>S</u> HTF	KLVNCKDEAV	<u>Y</u> CKFHFKTDQ
<i>X.laevis</i>	MVWDFWTLRP	ESLHQV <u>S</u> FLF	SDRGIPDGHR	HMNGYG <u>S</u> HTF	KLVSCKDEAV	<u>Y</u> CKFHFKTDQ
<i>X.tropicalis</i>	GIRNL <u>T</u> LEKA	EQLAASDPDY	GIRDLY <u>E</u> EAIA	AGNYPSWTFY	IQIMTFEQAE	KFPFNPFDDVT
<i>X.laevis</i>	GIRNL <u>T</u> VERA	EQLAASDPDY	GIRDLY <u>E</u> EAIA	AGNYPSWTLY	IQTMTFQQA	KFPFNPFDDVT
<i>X.tropicalis</i>	KVWPHGDYPL	IPVGKMVLSR	NPTN <u>Y</u> FAEVE	QLAFDPSNMP	PGIEP <u>SP</u> DKM	LQGRLF <u>S</u> YPD
<i>X.laevis</i>	KVWPHGDYPL	IPVGKMVLSQ	NPKN <u>Y</u> FAEVE	QLAFDPSNMP	PGIEP <u>SP</u> DKM	LQGRLF <u>S</u> YPD
<i>X.tropicalis</i>	THRHRLGANY	LQLPVNCPYK	ARVANYQRDG	PMCFTDNQGG	APNYYPNSFS	APEQQPQFRE
<i>X.laevis</i>	THRHRLGANY	LQLPVNCPYK	VRVANYQRDG	PMCFTDNQGG	APNYYPNSFS	APEQQPQFRE
<i>X.tropicalis</i>	HRFRVSADVE	RYNSANDDNV	TQVREFYLKV	LNEEERQRLC	ENIVGHLKEC	QLFIQKRTVK
<i>X.laevis</i>	HKFRVSADVD	RYNSAVDDNV	TQVRDFYLVN	LNEEERQRLC	ENLAGHLKEC	QLFIQKRAVK
<i>X.tropicalis</i>	NFSDVHPDYG	SRIQALLDKH	NAKCPKKEV	GPNTQHSSYL	TSKEKSNL	
<i>X.laevis</i>	NFSDVHLDYG	SRIQALLDKH	NAKCTKKECA	RPNTQHSSYL	TSKEKSNL	

Figure 4.7. A comparison of the predicted phosphorylation sites for CAT from *X.*

tropicalis and *X. laevis* which are shared between the two frog species. Predicted phosphorylation sites are shown in bold and underlined.

Figure 4.8. Differential scanning fluorimetry analysis of the thermal stability of purified CAT from control and 24 h frozen frog muscle. CAT from frozen frogs showed no significant difference when compared to CAT from control frogs, $n=8$.

Chapter 5:

General Discussion

1. General Discussion

Oxygen radicals are continuously produced by all oxygen-consuming organisms and changes in their expression have been linked to many pathological disease states. All known oxygen-consuming organisms have built-in defenses to protect against attack by oxygen radicals. Protection against damage by ROS can be classified as three distinct groups: i) the prevention of free radical generation, ii) termination of free radical propagation, and iii) repair of the damage incurred. Termination is accomplished through the activities of enzymes tasked with detoxification of ROS and through low molecular weight metabolites that act as scavengers of toxic oxygen species. Although production of ROS is encountered at all times, certain physiological conditions are associated with increased oxidative stress including: hyperoxia (Barga de Quiroga et al., 1988), hypoxia (Bhat et al., 1992), ingestion of toxic substances (Lee and Berenbaum, 1993), exposures to extremes of cold and heat (Barga de Quiroga, 1992), or ultraviolet radiation (Shick et al., 1981). In some cases ROS production actually occurs following physiological stresses, such as the oxidative stress that arises during recovery from ischemia. Endurance of freezing and entry into hibernation or estivation are all accompanied by depression of metabolic rate and oxygen consumption (Storey and Storey, 1990). In these cases, the organism may endure prolonged ischemia or hypoxia. During freezing, animals experience a cutoff of the blood supply to tissues, while hibernation and estivation subjects animals to wide variations in oxygen levels. Ischemia experienced during free-thaw episodes is a more stressful state since plasma-borne delivery of oxygen and other substances to tissues is cut off and CO₂ and other wastes cannot be removed. Following

ischemia, reoxygenation of blood, rapidly increased metabolic rate and a burst of ROS production is coupled with increased levels of iron, copper (Chevion et al., 1993) and hypoxanthine (Rubin et al., 1992). Animals that endure freezing have adapted to circumvent the injurious consequences of oxidative stress upon recovery. They do this by continually maintaining higher levels of antioxidants than are present in comparable species that are intolerant of the stress, through stress-mediated alterations of antioxidant enzyme activity, or by up-regulation of antioxidant enzyme gene expression during the initial stress, in anticipation of increased generation of ROS upon recovery. Examples of these strategies can be found in tissues of the wood frog, *R. sylvatica*, and the spadefoot toad, *S. couchii*, which maintain high levels of antioxidant enzymes (and alter antioxidant enzyme function) while frozen or estivating, and the estivating land snail, *O. lactea*, which synthesizes antioxidant enzymes as a defence against stress that occurs when oxygen consumption is rapidly increased upon arousal (Joanisse and Storey, 1996; Grundy and Storey, 1998; Hermes-Lima and Storey, 1998). In all cases these animals experience suppressed metabolic states that are reversed during thawing or arousal with a rapid increase in oxygen consumption. The freeze-thaw transition is functionally analogous to an ischemia/reperfusion event (Joanisse and Storey, 1996), which in mammalian systems is well known to cause substantial free radical damage (Campos et al., 1993; Downey, 1990; Hossain et al., 1995).

Previous studies were conducted on crude tissue extracts in order to test which mechanisms of oxidative damage avoidance were used in wood frogs (Joanisse and Storey, 1996). These studies quantified activities of antioxidant enzymes and the amount of soluble glutathione in wood frogs during freezing (Joanisse and Storey 1996). These

results provided evidence that frogs seem to enter freezing with thawing in mind; that is, they employ an anticipatory strategy that sets them up during freezing with biochemical adaptations to deal with ROS stress and damage upon thawing reperfusion. The results clearly showed that the maximal activities of select antioxidant enzymes were augmented, and glutathione levels were higher in frozen animals. Thus, it would seem that maintenance and induction of the frog's antioxidant complement of enzymes and soluble glutathione is essential for survival during freezing. In addition, the activity of XO, which generates ROS, was not significantly higher in frozen frogs in comparison to control frogs, and the ratio of XO to XDH remained unchanged (Joanisse and Storey, 1996). The conversion of XDH to XO and the subsequent increase in the ratio of XO/XDH are predictive criteria for oxidative stress during ischemia and reperfusion (Hearse et al., 1986), and it would seem that frogs recovering from the frozen state are able to avoid the expected ROS damage upon reperfusion (Joanisse and Storey, 1996). In addition to XO/XDH ratios, tests used to measure oxidative damage reported that oxidative damage was noticeably absent in frozen and thawed frogs, as assessed using the TBARS test and the xylanol orange test (Joanisse and Storey, 1996). These data suggest that the frogs are able to prevent the formation of oxygen damaged molecules rather than deal with damaged tissues, which is energetically favorable for the animal. Since the frog has been shown to exit the frozen state in an energy-limited state (Storey and Storey, 1986; Storey, 1987), prevention of the formation of ROS-damaged molecules may be critical since the frog may simply lack the appropriate energy reserves to repair or resynthesize oxidatively damaged molecules in a timely manner upon thawing.

It is unlikely that increased numbers of oxygen radicals were formed during freezing since oxygen consumption is essentially zero in this state in comparison to the tissues of control frogs. The rate of generation of free radicals is directly proportional to metabolic rate (i.e. oxygen consumption) and should be lower in frozen frogs. However, recovery from freezing involves the rapid increase in metabolic rate which should stimulate the burst production of oxygen radicals. Indeed, hallmarks of oxidative damage including lipid peroxidation and GSSG/GSH ratios increased following arousal from estivation in both land snails, *O. lactea* (Hermes-Lima and Storey, 1998) and toads, *S. couchii* (Grundy and Storey, 1998). XO/XDH ratios were also significantly higher upon exit from estivation in toads, *S. couchii* (Grundy and Storey, 1998). In the case of *O. lactea*, increased activities of antioxidant enzymes were observed. However, little was known of their kinetic parameters, and in the case of the estivating toad, antioxidant enzymes activities of some of the enzymes studied in this thesis actually dropped over the estivation period. In both cases, changes in the antioxidant enzymes activity alone were not sufficient to disrupt the formation of oxidative damage due to production of ROS. In stark contrast to the general reduction of antioxidant enzymes in the estivating toad, GST activities were observed to increase (Grundy and Storey, 1998). Increased GST activities were suggested to play a pivotal role in reversing the effects of oxidative damage upon arousal, and it has been suggested that the estivating toad as well as the estivating land snail are more likely to detoxify ROS damage after exit from a metabolically depressed state, rather than prevent ROS formation (Grundy and Storey, 1998; Hermes-Lima and Storey 1998).

R. sylvatica has demonstrated none of the hallmarks of oxidative stress, and it has been suggested that ROS formation is prevented through constitutively higher antioxidant enzymes and glutathione levels throughout freezing (Joanisse and Storey, 1996). However, increased antioxidant enzymes was also observed in *O. lactea*, yet lipid peroxides and changes in the GSSG/GSH ratios were observed upon exit from metabolic rate depression (Hermes-Lima and Storey, 1998). The studies designed in this thesis explored the possibility of preventative measures employed by *R. sylvatica* to detoxify oxygen radicals before oxidative damage can occur.

1.1. Purification of Antioxidant Enzymes

A common problem for protein biochemists studying the function of native proteins is the phenomena of competing activity profiles. Multiple enzymes can sometimes act upon the same substrates or products which skews data collected when competing enzymes are present in the protein preparation. This is of particular importance for studies of antioxidant enzymes since many of these enzymes have similar functions, or even a second function that mimics another antioxidant enzyme. This can be clearly seen in the case of CAT, which can exhibit GPx activity (Singh *et al.*, 2007), and GST and GPx which have been demonstrated to play similar roles in detoxifying xenobiotics and hydroperoxide adducts (Salinas and Wong, 1999). Another cause for concern is the reactivity of the substrates and products produced in antioxidant enzyme-catalyzed reactions. Superoxide, glutathione and hydrogen peroxide (the three main molecules studied in this thesis) have all been linked to a wide range of biochemical

reactions and pathways in mammalian cells. Therefore, antioxidant enzymes were purified to homogeneity or near-homogeneity in order to ensure that the measured differences between the enzymes from control and frozen frogs were the result of the enzyme of interest, and not linked to competing enzymes. This phenomenon will also be explored when comparing previously collected data of *R. sylvatica* antioxidant enzymes obtained without the aid of protein purification.

1.2. *Superoxide Dismutase*

SOD, an essential antioxidant enzyme, was purified from frog muscle with a final specific activity between 1390-2100 U/mg protein for CuZnSOD and 1550-1570 U/mg protein for MnSOD for the enzyme from both control and frozen animals, with yields of ~11% and ~20% respectively. Both the specific activities and yields obtained for each isoform of SOD was in line with values obtained from other animals (Rigo *et al.*, 1975). Superoxide is a very reactive molecule, particularly in the face of elevated amounts of metal or metal-containing proteins. In previous studies of *R. sylvatica*, total SOD activity was measured as rising from 14 U/mg protein to nearly 40 U/mg protein over the course of a freeze-thaw cycle (Joanisse and Storey, 1996). This is likely due to an increase in endogenously present molecules, and enzymes such as xanthine oxidase, that can react with superoxide. The assay used in previous studies has also been demonstrated to exhibit greater error and NAD(P)H-utilizing methods are prone to errors in biological samples (Berridge *et al.*, 2005). Therefore, it was critical to purify SOD. The purified form of CuZnSOD from frozen frogs exhibited a higher maximal activity in comparison to the

control form, which is in line with the increases seen with nearly all other SODs studied. CuZnSOD is a more abundant eukaryotic protein than MnSOD. The observed stress-responsive changes in activity for CuZnSOD (but not MnSOD) seen in chapter 2 probably account for the changes in total SOD that are observed in wood frogs and other animals when fluctuations in total SOD activity have been discovered.

The successful separation of isoforms allowed for the independent characterization of MnSOD. Proper characterization of MnSOD has proven elusive, since flash-frozen tissues are prone to mitochondrial destabilization and studies of mitochondria-specific enzymes have been left largely unexplored. Chapter 2 presents one of the first studies of the mitochondrial form of SOD in an animal that undergoes hypometabolism. MnSOD activity remained uniform when comparing the control and frozen states, which would have otherwise been masked by changes in CuZnSOD activity. However, MnSOD demonstrated unique modification and enzyme function in the frozen state, highlighting its possible importance to surviving repeat freeze-thaw cycles in *R. sylvatica*. Augmented MnSOD activity would increase the protection against superoxide production at the major site where it is formed, the mitochondria. Superoxide production during thawing and the attendant reoxygenation of tissues would likely result mostly from superoxide production from the mitochondria, since the ETC would be primed for radical production in its fully reduced state. In line with this rationale for SOD function, mitochondrial superoxide production has been shown to trigger apoptosis, whereas cytosolic production of superoxide has been linked to many cell signaling events (Thannickal and Fanburg, 2000). This would suggest that CuZnSOD is more likely to have a role in regulating cell signaling events during freeze/thaw, whereas MnSOD is

more likely to have a role in keeping $O_2^{\cdot-}$ levels in the mitochondria low to stave off apoptotic cell death. This is of special interest to wood frogs since the muscle tissue thaws later than internal organs such as the heart or liver (Rubinsky et al., 1994). Later thawing could mean that the muscle is under prolonged ischemic stress until circulation is restored which could be one reason for the delay in recovery of skeletal muscle functionality as compared with the reinstatement of heart beat and breathing (Layne and First, 1991; Kling *et al.*, 1994). Furthermore, improved affinity for $O_2^{\cdot-}$ by MnSOD in the frozen frog may aid the peripheral tissue during thawing, allowing for reintroduction of re-oxygenated blood into the tissue without sustaining a buildup of oxidative stress.

1.3. Catalase

It is critical to think of the consequences of changes in SOD function and how these will impact the rest of the cell. In this thesis, up-regulation of SOD activity and function was documented in chapter 2, which leads to the inevitable conclusion that H_2O_2 levels would increase accordingly. It is for this reason that CAT was explored in detail, since CAT plays a pivotal role in the breakdown of H_2O_2 when H_2O_2 levels are much higher than normal (Kim *et al.*, 1988). CAT was purified from frog muscle with a final specific activity between 2308-3412 U/mg protein for the enzyme from both control and frozen animals, with yields of ~11%. Previous studies of CAT from *R. sylvatica* showed no significant changes in CAT activity in whole tissue extracts; however, changes in glutathione peroxidase activities were observed (Joanisse and Storey, 1996). CAT was assayed by directly measuring the decomposition of H_2O_2 in solution in the previously

mentioned study, as well as in chapter 4 of this thesis. Under normal cellular conditions peroxiredoxins, peroxidases, GSTs, as well as CAT all contribute to catalyzing the reduction of H_2O_2 (Rabilloud et al., 2002; Wagner et al., 2002; Koo et al., 2002). GSTs are largely ignored when discussing H_2O_2 reduction, as only select isoforms exhibit this potential, and those that do show very poor H_2O_2 reducing ability. Whole cell extract or whole tissues extract assays are unable to distinguish the source of H_2O_2 breakdown, and classical studies are likely measuring the total capacity of these and other H_2O_2 reducing enzymes. In chapter 4, CAT was purified to homogeneity, and it was discovered that CAT from the muscle of frozen frogs was modified and its activity and affinity for H_2O_2 were augmented. Increased CAT activity is likely required to deal with increases in H_2O_2 produced from elevated SOD activity previously outlined in chapter 3. In this sense, CAT and SOD work in concert to detoxify the main oxygen radicals produced by multiple ischemic episodes encountered during freezing.

Through exploration of the differences in affinity of the different H_2O_2 reducing enzymes, it can be concluded that their substrate affinities in a normal mammalian system are as follows: peroxiredoxins>peroxidases>CAT (Cox et al., 2010; Winterbourn, 2008; Flohe, 2010; Winterbourn, 2013; Hall et al., 2011). Peroxiredoxins are likely to work best at low H_2O_2 concentrations and are inactivated in high $[\text{H}_2\text{O}_2]$, and peroxidases are known to have limited flux. Therefore, when exploring the relative rate of H_2O_2 reduction, the order is believed to be reversed as follows: CAT>peroxidases>peroxiredoxins (Flohe, 2010; Winterbourn, 2013; Hall et al., 2011). This poses an interesting potential point of further study since the affinity of frog muscle peroxidases/peroxiredoxins is not known in either control or frozen frogs. However,

human GPx has a K_m value for H_2O_2 that ranges from 9-19 mM (Zheng *et al.*, 2008), which is below the measured K_m for H_2O_2 of CAT from control frogs, yet encompasses the measured value for CAT from frozen frogs, as reported in chapter 4. The diffusion of H_2O_2 away from the site of production and across membranes using H_2O_2 gradients has been discussed (Antunes and Cadenas, 2000; Mueller *et al.*, 2009; Sobatta *et al.*, 2013). Although this is merely speculative, it could very well be that CAT from frozen frogs is activated as a protective ‘vacuum’ in the face of elevated H_2O_2 , drawing H_2O_2 away from the peroxidases (with lower, or similar affinity) to the peroxisome where CAT resides. This could potentially help to detoxify H_2O_2 that is produced in the greatly dehydrated cells of frozen tissue. Indeed, if 65% of total body water is frozen as extracellular ice, the intracellular concentrations of all solutes in the remaining cell water can be automatically elevated by 3-fold apart from any additional effect of stress-induced increased synthesis of H_2O_2 or other products. Hence, H_2O_2 concentrations could rise substantially during prolonged freezing due to freeze concentration of the cell and/or during thawing reperfusion due to a burst of ROS production during reoxygenation. Furthermore, CAT might become the favored route for H_2O_2 detoxification in frozen tissues because peroxidases or peroxiredoxins might be compromised if the supply of the reduced cofactors needed for their reaction, glutathione or thioredoxin, runs short during long term freezing.

1.4. *Glutathione Reductase*

Glutathione reductase is not a traditional antioxidant enzyme; it is considered

more of a secondary antioxidant enzyme because it recycles an antioxidant molecule, converting GSSH back to GSH. However, GR does seem to play an important role in the antioxidant defense system of the frozen frog. As previously reported, GSH levels are much higher in *R. sylvatica* than in non-tolerant species (Joanisse and Storey, 1996), and as such, a more active GR was expected in frozen frogs in order to keep GSSG/GSH ratios intact. Initial experiments performed in chapter 3 suggested that GR was, in fact, not regulated at all in frozen animals. However, when explored in the face of elevated levels of common metabolites, GR exhibited changes in kinetic parameters. This provides a complex picture of regulation in the frozen animal. Many of the metabolites explored in chapter 4 are known to, or thought to, change throughout the course of freeze-thaw cycles. Specifically, glucose and urea are known to vary greatly in *R. sylvatica* between the control and frozen states (Storey and Storey, 2004). It is interesting that high-glucose shows an effect on GR, as constitutively high antioxidant enzymes have recently been reported for hypoglycemic states (Dey and Lakshmanan, 2013). Glutathione has been shown to play a protective role in hypoglycemia, demonstrating positive effects on oxidative stress and hypoglycemia mediated damage in diabetes (Rochette et al, 2014; Dey and Lakshmanan, 2013). Clearly the interaction between glutathione and glutathione-using enzymes with respect to variations in glucose levels is an area that requires further attention. Chapter 3 provides yet another interesting link between glucose and oxidative stress, and it appears that GR from frog muscle exists in a glucose sensitive form, with an amelioration of function in the face of elevated glucose.

1.5. Concluding Remarks

In conclusion, this thesis provides an in-depth analysis of the front-line antioxidant defense systems in the muscle of the freeze tolerant frog, *R. sylvatica*. Each successive chapter explores the mediation of ROS production from the primary source, the mitochondria, and the byproducts as they migrate away from the source of production. Through phosphorylation-mediated increases in the function of the mitochondrial superoxide scavenger, MnSOD, the frog is likely able to eliminate superoxide before it can cause serious harm to the muscle tissue. However, increased SOD activity results in an increase in H₂O₂ production, potentially to a point that is lethal to most animals (particularly in freeze dehydrated cells). This means that under conditions such as ischemia/reperfusion, primary H₂O₂ reducing peroxiredoxins would likely be inactivated in the face of greatly increased H₂O₂ levels. H₂O₂ is generated in a site specific manner and the local concentration of H₂O₂ is modulated by its generation and subsequent removal. The diffusion of H₂O₂ away from the site of production and across membranes creates a H₂O₂ gradient (Antunes and Cadenas, 2000). Furthermore, the sites of H₂O₂ removal, via catalase in the peroxisomes, glutathione peroxidase and peroxiredoxins in the cytosol and other organelles further drives this diffusion gradient from the site of production to the site of removal (Antunes and Cadenas, 2000; Mueller et al., 2009; Sobotta et al., 2013). Increases in the affinity for H₂O₂ and activity of CAT, seemingly as a result of protein phosphorylation of CAT, is likely to aid the breakdown of elevated H₂O₂ levels encountered as a result of multiple freeze-thaw episodes or of long-term sustained freezing events. Finally, increased glutathione turnover is achieved via positive effects of glucose and other effectors on the secondary antioxidant enzyme

GR. In summary, then, *R. sylvatica* has shown a unique method of dealing with ROS production, employing a preventative measure of defense in lieu of the more widespread post-ROS damage repair mechanisms seen in many other vertebrates experiencing variations in oxygen availability.

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Appendix A:

List of Publications

- Childers CL, Dawson NJ, Storey KB.** (2015) Purification and properties of glutamate dehydrogenase from the African clawed frog, *Xenopus laevis*. (Completed).
- Dawson NJ, Childers CL, Storey KB.** (2014) Using native denaturation and differential scanning fluorimetry to assess protein stability; an exploration into subtle differences in protein isoforms. (Submission in progress).
- Dawson NJ, Katzenback BA, Storey KB.** (2014) Free-radical first responders: The characterization of MnSOD and CuZnSOD regulation during freezing of the freeze-tolerant North American wood frog, *Rana sylvatica* (syn *Lithobates sylvaticus*). (Submission in progress, *Biochim Biophys Acta Gen Subj*).
- Dawson NJ, Katzenback BA, Storey KB.** (2013-2014) Purification and characterization of a urea sensitive lactate dehydrogenase from the liver of the African clawed frog, *Xenopus laevis*. *J Comp Physiol B*. (DOI: 10.1007/s00360-014-0824-1).
- Shahriari A, Dawson NJ, Bell RAV, Storey KB.** (2013) Stable suppression of lactate dehydrogenase activity in the foot muscle of *Littorina littorea* and the potential role of acetylation as a novel regulatory mechanism. *Enz Res* Article ID 461374.
- Dawson NJ, Biggar K, Storey KB.** (2013) Characterization of fructose-1,6-bisphosphate aldolase during anoxia in the tolerant turtle, *Trachemys scripta elegans*: An assessment of enzyme activity, expression and structure. *PLOS ONE* 8(7): e68830.
- Dawson NJ, Bell RAV, Storey KB.** (2013) Purification and properties of white muscle lactate dehydrogenase from the anoxia-tolerant turtle, the red eared slider, *Trachemys scripta elegans* *Enz Res* Article ID 784973.
- Dawson NJ, Biggar K, Storey KB.** (2012) Real-time protein unfolding: a simple method for determining the kinetics of native protein denaturation (K_{ND}) using a quantitative real-time thermocycler. *BioTechniques* 53:231-238.
- Dawson NJ, Storey KB.** (2012) Regulation of tail muscle hexokinase in the anoxia-tolerant freshwater crayfish, *Orconectes virilis*. *HOAJ Biol.* 1(1).
- Bell R, Dawson NJ, Storey KB.** (2012) Insights into the in vivo regulation of glutamate dehydrogenase from the foot muscle of an estivating land snail. *Enz Res.* Article ID 317314.
- Dawson NJ, Storey KB.** (2011) An enzymatic bridge between carbohydrate and amino acid metabolism: regulation of glutamate dehydrogenase by reversible phosphorylation in a severe hypoxia-tolerant crayfish. *J Comp Physiol B.* 182(3): 331-40.

Dawson NJ, Storey KB. (2011) Regulation of tail muscle arginine kinase by reversible phosphorylation in an anoxia-tolerant crayfish. *J Comp Physiol B*. 181(7): 851-9.

Appendix B:

Communications at scientific meetings

- Dawson NJ, Katzenback BA, Storey KB.** (2014) Free-radical first responders: The characterization of MnSOD and CuZnSOD regulation during freezing of the freeze-tolerant North American wood frog, *Rana sylvatica*. Canadian Oxidative Stress Consortium, June 11-13, 2014, Carleton University, Ottawa, Ontario. Poster Presentation.
- Dawson NJ, and Storey KB.** (2013) Threonine phosphorylation of catalase from the freeze tolerant frog, *Rana sylvatica*; a preparatory mechanism. CRYO2013, 50th Annual Meeting, Society for Cryobiology, Bethesda, Maryland, July 28-31, 2013. Poster Presentation.
- Dawson NJ, Biggar KK, and Storey KB.** (2012) Real-time protein unfolding: A method for determining the kinetics of native protein denaturation using a quantitative real-time thermocycler. 2nd China Canada Systems Biology Symposium and 19th Methods in Protein Structure Analysis Conference June 25-28, 2012, Ottawa Convention Centre, Ottawa, Ontario. Poster Presentation.
- Dawson NJ, and Storey KB.** (2012) Threonine phosphorylation of catalase from the freeze tolerant frog, *Rana sylvatica*; A preparatory mechanism. 2nd China Canada Systems Biology Symposium and 19th Methods in Protein Structure Analysis Conference June 25-28, 2012, Ottawa Convention Centre, Ottawa, Ontario. Poster Presentation.
- Dawson NJ, and Storey KB.** (2011) An anoxic energy reserve: Purification and properties of argine kinase from the tail muscle of *Oronectes virilis*. 26th Annual meeting, Federação de Sociedades de Biologia Experimental (FeSBE), Rio de Janeiro, Brazil, August 24-27. Poster Presentation.
- Dawson NJ, and Storey KB.** (2011) An enzymatic bridge between carbohydrate metabolism and amino acid metabolism: Regulation of glutamate dehydrogenase in the tail muscle of *Orconectes virilis*. 26th Annual meeting, Federação de Sociedades de Biologia Experimental (FeSBE), Rio de Janeiro, Brazil, August 24-27. Poster Presentation.
- Dawson NJ, and Storey KB.** (2011) Oxidative damage; the allosteric regulation of glutathione reductase in the freezing frog, *Rana sylvatica*. Canadian Society of Zoology Annual Meeting. Ottawa, ON. Oral Presentation.
- Dawson NJ, and Storey KB.** (2010) An anoxic energy reserve: Purification and properties of argine kinase from the tail muscle of *Oronectes virilis*. 7th Annual Ottawa Carleton Institute of Biochemistry Symposium. Ottawa, ON. Poster Presentation.

- Dawson NJ, and Storey KB.** (2009) An enzymatic bridge between carbohydrate metabolism and amino acid metabolism: Regulation of glutamate dehydrogenase in the tail muscle of *Orconectes virilis*. Canadian Society of Zoology Annual Meeting. Toronto, ON. Poster Presentation.
- Dawson NJ, and Storey KB.** (2009) Purification and properties of arginine kinase from the freshwater crayfish, *Orconectes virilis*; regulation of muscle energetic during anoxia by reversible phosphorylation. 12th Annual Chemistry and Biochemistry Graduate Research Conference, Concordia University, Montreal, Que. Oral Presentation.
- Dawson NJ, and Storey KB.** (2008) An enzymatic bridge between carbohydrate metabolism and amino acid metabolism: Regulation of glutamate dehydrogenase in the tail muscle of *Orconectes virilis*. 5th Annual Ottawa Carleton Institute of Biochemistry Symposium. Ottawa, ON. Poster Presentation.
- Dawson NJ, and Storey KB.** (2007) GDH regulation by reversible phosphorylation during estivation of the land snail, *Otala lacteal*.
- Dawson NJ, and Koschinsky M.** (2006) A Study of the Folding Kinetics of Various Apo(a) Isoform Sizes. Queen's University Undergraduate Research Conference. Oral Presentation.

Appendix C:

Contributions to Chapters

Chapter 1 General Introduction

- Chapter 1 was completed in its entirety by Neal Dawson.

Chapter 2 Free-radical first responders: The characterization of CuZnSOD and MnSOD regulation during freezing of the freeze tolerant North American wood frog, *Rana sylvatica*.

- Chapter 2 was completed by Neal Dawson and Barbara Katzenback. Barbara Katzenback contributed the transcript and protein data for MnSOD. All other sections were completed by Neal Dawson.

Chapter 3 Passive regeneration of glutathione: Purification and properties of glutathione reductase regulation from the freeze tolerant North American wood frog, *Rana sylvatica*.

- Chapter 3 was completed in its entirety by Neal Dawson.

Chapter 4 A hydrogen peroxide safety valve: The reversible phosphorylation of catalase from the freeze tolerant North American wood frog, *Rana sylvatica*.

- Chapter 4 was completed in its entirety by Neal Dawson.

Chapter 5 General Discussion

- Chapter 5 was completed in its entirety by Neal Dawson.