Regulation of skeletal muscle glycolysis during dehydration in the aestivating African clawed frog, *Xenopus laevis*.

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Abstract

Seasonally arid conditions can trigger African clawed frogs (*Xenopus laevis*) to enter aestivation. This process includes whole body dehydration that at high levels can create hypoxic conditions due to impaired blood circulation and increase the need for glycolytic energy production. This thesis examines hexokinase (HK) and lactate dehydrogenase (LDH) purified from skeletal muscle of control versus dehydrated (~30% body water lost) frogs. Studies analyzed substrate affinities, urea effects, thermal stability and protein posttranslational modifications (PTM) to understand how enzyme properties are modified under dehydration stress. Muscle HK and LDH showed regulation by reversible protein phosphorylation and nitrosylation. These PTM's correlated with reduced affinities for glucose by HK and lactate by LDH, overall lower V_{max} for LDH in both directions, and altered thermal stabilities. The two enzymes responded to the same PTMs, which suggests that coordinated controls over these first and last enzymes of anaerobic glycolysis contribute to dehydration responsive pathway regulation.

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List of Abbreviations

Acetyl-CoA – Acetyl coenzyme A

AMP, ADP, ATP- Adenosine mono,-di,-or triphosphate

DEAE- Diethylaminoethyl

EDTA- Ethylenediamine tetraacetate

EGTA- Ethyleneglycol bis tetracacetate

G6P- Glucose-6-phosphate

G6PDH- Glucose-6-phosphate dehydrogenase

HK- Hexokinase

$\mathbf{I_{50}}$- Inhibitor concentration reducing enzyme velocity by 50%

$\mathbf{K_m}$- Substrate concentration producing half maximal enzyme activity

LDH- Lactate dehydrogenase

NAD, NADH- Oxidized and reduced nicotinamide adenine dinucleotide

NaF- Sodium fluoride

PAGE – Polyacrylamide gel electrophoresis

PKA- cAMP-dependent protein kinase

PKG- Protein kinase G

PMSF- phenylmethysulfonyl fluoride

PP2A- Protein phosphatase type-2A

$\mathbf{P_i}$ – Inorganic phosphate

PTM- Posttranslational modification

PVDF- Polyvinylidene difluoride

SDS- Sodium dodecyl sulfate

$\mathbf{T_m}$ **–** Melting temperature

TCA- Tricarboxylic acid cycle (citric acid cycle)

TRIS – tris(hydroxymethyl)aminomethane

$\mathbf{V_{max}}$ – Maximal enzyme velocity

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Chapter 1:

General Introduction

1.1. Dehydration stress and metabolic rate depression

Seasonally hot weather imposes the challenge of dehydration stress on many organisms. Those without proper dehydration survival adaptations (i.e. dehydration intolerant species) can face multiple forms of lethal damage. When experiencing dehydration stress, several types of pressures and damage can occur: (a) water loss; (b) reduced access to food; (c) increased concentration of damaging osmolytes in the blood and tissues; (d) reduced blood flow, due to increased viscosity, leading to ischemia (Secor and Lignot 2010; Hillman 1980). Generally, death caused by dehydration is due to an interruption of normal cellular processes resulting from a disruption of energy metabolism caused by these consequences of dehydration.

In order to survive exposure to dehydration over the hot dry months of the year, many organisms have developed a variety of behavioral, physiological and metabolic adaptations. Aestivation is a summer or dry season period of inactivity triggered by restricted water and food availability. The primary aim for aestivators is to conserve energy, retain body water, ration the use of stored fuel reserves, deal with nitrogenous end products, and stabilize organs, cells, and macromolecules of over many weeks or months of inactivity (Storey and Storey 2012). For instance the spadefoot toad, *Scaphiopus couchii,* is found in Arizona and California deserts and is a well-documented terrestrial aestivator surviving 9-10 months each year beneath the drying desert soil. During this time the toads can lose 50% of their total body water and build up about 300 mM urea in their cells as an osmolyte to slow water loss and store nitrogen in a less toxic form (Cowan and Storey 2002). The spadefoot toad has the physiological adaptations of a large water reserve in its bladder, which

helps delay dehydration of tissues since these amphibians are able to reabsorb water from the bladder. The toads also have the behavioral adaptation of burrowing under the soil where they assume a crouched position with limbs drawn in close to the body, which helps them to both resist water loss from the body and to absorb moisture from the surrounding soil over the first months of inactivity when the soil is still moist. However, these adaptations only function to slow dehydration, which inevitably does occur. The toads are able to survive the lengthy duration of inactivity and the eventual severe water loss to the surrounding soil by dropping their metabolic rate to just 20-30% of the rate of non-aestivating animals and switching to a reliance on stored lipid reserves rather than ingested food for energy (Cowan et al., 2000).

Metabolic rate depression refers to an animal's ability to lower its energetic needs and is the key metabolic adjustment that allows many animals to survive in unfavorable environmental conditions. Metabolic rate depression has two main functions. First, it reduces an animal's energy needs so the energy stores they have amassed during their active season are sufficient to last the months of inactivity without feeding. Second, metabolic rate depression reduces an animal's oxygen requirements so when dehydration induced hypoxia sets in, and oxygen is less available to tissues, the cells are prepared to handle the stress (Storey and Storey 1990). Other species such as African lungfish, *Protopterus* sp., have evolved to withstand dehydration by regulating their moisture loss through the formation of a cocoon during aestivation; this is also true of a variety of species of water-holding frogs (see below). This behavioral adaptation functions similar to the burrowing of

the spadefoot toad as it serves to delay dehydration. The lungfish also uses metabolic rate depression to lower rates of oxygen consumption during aestivation by as much as 50 to 85% (Fishman et al., 1986; Storey 1988). Both the toad and lungfish employ aestivation as a survival strategy to withstand periods of drought and low food availability, lowering their metabolic rates to conserve energy and oxygen use (Secor and Lignot 2010).

Cocoon forming frogs such as *Neobatrachus* and *Cyclorana* present an interesting example of metabolic rate control as they become a closed system once they form their cocoon out of epidermal layers that are shed during aestivation (Cartledge et al., 2007). Survival of aestivation in this case is dependent on large bladders, dehydration tolerance and metabolic rate depression (Reynolds et al., 2011). When the soil surrounding them in their burrows dries to a point where the influx of water across their skin is negative, these frogs form cocoons from multiple layers of shed skin, which reduces their evaporative water loss by 0.8% to 38% compared to non-cocooned rates (Cartledge et al., 2006). Once formed however, they must be able to survive on their internal energy reserves and manage waste accumulation without the ability to excrete potentially harmful accumulations (Cartledge et al., 2007).

1.2. The African clawed frog and dehydration stress

The African clawed frog (*Xenopus laevis*) is an aquatic frog that exhibits a significant natural dehydration tolerance that includes both physiological and biochemical adaptations to enable its survival during seasonal dry conditions

(Romspert 1976). The native range of *X. laevis* is throughout southern Africa, which experiences a dry season during which the ponds where these frogs live frequently dry up. Behaviorally, clawed frogs react to drying conditions in one of two ways. They either migrate to nearby larger bodies of water (typically moving at night) or they burrow into the mud of their drying pond and aestivate for 2–3 months (Balinsky et al., 1967). Aquatic frog species typically have small bladder capacities of just 2-8% of total body mass. This compares with the water-holding cocoon-forming frogs that can have bladder reserves with a holding capacity of 50% of their body mass at the start of aestivation and that are slowly reduced over time. By utilizing the water reserve in the bladder, cocooned frogs are able to keep plasma osmolality low during aestivation (Cartledge et al., 2007). Similarly, the spadefoot toads, which can hold 30% of their body mass as bladder water, or other terrestrial anurans such as *Bufo cognatus* that can store up to 45% of their body mass in their bladder, use this water to help maintain osmotic balance under seasonally dry conditions. By contrast, the bladder holding capacity of the aquatic *X. laevis is* only 1% of their body mass and these frogs also do not form a barrier (e.g. cocoon) to help retard evaporative water loss across the skin (Ruibal 1962 as in Jorgensen 1997; McClanahan Jr. 1967). Hence, their chief strategy to retard water loss under dry conditions is through chemical means, by increasing the osmolality of their body fluids through the synthesis of urea (Cartledge et al., 2007; Storey 2000). Urea has been demonstrated to function as both an osmoprotectant and cryoprotectant in *Rana sylvatica* and Malik and Storey (2009) reported that *Xenopus* that had been dehydrated close to the lethal limit showed an increase in plasma urea to 55 mM as

compared to 1.66 mM in controls (Costanzo and Lee 2005). Therefore, clawed frogs present an example of mainly cellular adaptation to dehydration, as they cannot store water to delay dehydration once the surrounding soil has a negative water balance to their skin but they can alter their metabolic defenses. *X. laevis* aestivation is characterized by a dehydration lethal limit of about 32-42% of their total body water, which is lower than the tolerance of many of the other anuran aestivators but much greater in comparison to mammals (Romspert 1976; Storey and Storey 2012)(see also Chapter 2 Methods).

1.2.1. Overview of dehydration tolerance by clawed frogs

In order to counteract the gradual loss of body water as the mud of their ponds dries, *X. laevis* undergo some classic adaptations known to other aestivators: 1) they build up osmolytes to reduce water loss across their skin into the drying mud where they are buried; and 2) they enter a state of hypometabolism to reduce the energy cost of their cellular functions during their aestivation. Studies focused on long-term aestivation in *X. laevis* show a reduction in oxygen consumption and suppression of the activities of various metabolic enzymes (Merkle 1989; Merkle and Hanke 1988; Onishi et al., 2005). However, an adverse effect of dehydration, often causing death in *Xenopus,* is an increase in blood viscosity and a decrease in blood volume that impairs proper oxygen delivery to tissues and causes hypoxia and ischemia (reduced delivery of fuels to cells and removal of wastes), stress on the heart to pump thickened blood, and ultimately circulatory failure. Hillman (1978) showed a strong decrease in maximal oxygen consumption rates and circulatory

oxygen delivery capabilities during dehydration in *X. laevis* leading to hypoxia/anoxia and ultimately dehydration-related death. Likewise, Malik and Storey (2009) found that the percent blood volume of packed red blood cells increased from 40% to 50% when under elevated dehydration stress. Before death, however, the gradual decrease in perfusion of peripheral tissues requires the recruitment of glycolysis to help sustain the ATP demands of tissues. It has been demonstrated previously in leopard frogs, *Rana pipiens,* that compromised circulatory oxygen delivery to organs, due to dehydration-related hypoxic stress, results in a decline in whole body oxygen consumption and a rise in lactate levels, which is the main product of anaerobic glycolysis (Churchill and Storey 1995). This cardiovascular deficit as a result of dehydration also hinders the capacity of animals to increase aerobic metabolism during induced activity. For example, when *X. laevis* has lost approximately 30% of its initial body mass by dehydration, it loses its ability to right itself (Hillman 1980). In exercising *X. laevis*, a physiological situation that mimics the oxygen-compromised situation during aestivation, anaerobic glycolysis is thought to account for more than 70% of the total energy produced (Miller 1983).

1.2.2. Glycolysis

When *X. laevis* are highly dehydrated (~28% body water lost), blood circulation is slowed creating hypoxic conditions and shifts metabolic ATP production from primarily oxidative phosphorylation to a greater dependence on glycolysis (Malik and Storey 2009; Hillman 1978; Hillman et al., 2009; Belkin 1968).

Anaerobic glycolysis helps support the ATP requirements of the aestivating frog but it has two major limitations. First, glycolysis usually results in a significant acidification of cells both from ATP hydrolysis and lactic acid build up and, second, it results in a significantly lower ATP yield per glucose molecule in comparison to aerobic oxidative phosphorylation (Hochachka and Mommsen 1983). Many animals can overcome the reduced rate of ATP production by lowering and matching their metabolic needs with the amount of ATP they can produce. This metabolic rate depression also helps to minimize cellular acidification by reducing the harmful buildup of byproducts of metabolism (Hand and Hardewig 1996).

Glycolysis begins with the formation of glucose-6-phosphate (G6P) either by catabolism of endogenous cellular glycogen or the uptake of glucose from the blood. Glucose is phosphorylated by hexokinase (HK) to produce G6P, which is subsequently converted to pyruvate by a 10-enzyme pathway. The pyruvate produced has two main fates – conversion to acetyl-CoA and aerobic catabolism by mitochondria when oxygen is plentiful, or conversion to lactate by lactate dehydrogenase (LDH) when oxygen is low or absent. In terms of ATP production, glycolysis is much less efficient than oxidative phosphorylation, but it can be self-sustaining because the LDH reaction regenerates the NAD+ needed by the glyceraldehyde-3-phosphate dehydrogenase (G3PDH) step and thereby maintains redox balance (Berg et al. 2002). Studies focused on long-term aestivation in *X. laevis* have previously shown a reduction in oxygen consumption and suppression of the activities of various metabolic enzymes (Merkle 1989; Merkle and Hanke 1988; Onishi et al., 2005). Metabolic adaptations supporting hypoxia (or anoxia) tolerance

can include substantial reserves of fermentable substrate (glycogen), changes in the activities of glycolytic enzymes in organs, elevated buffering capacity to deal with acidosis, and metabolic rate depression (Storey and Storey 1990). Interestingly, lactate levels in other dehydration tolerant frogs, such as *Rana pipiens* and *Pseudacris crucifer,* have been shown to increase in various tissues indicating that the switch to fermentative ATP production is a solution to poor circulation (Churchill and Storey 1994, 1995). *P. crucifer* is freeze tolerant as well as dehydration tolerant and it is thought that high dehydration tolerance is an evolutionary precursor to freeze tolerance because frozen frogs must survive total anoxia as well as extensive cellular dehydration when circulation is halted by the conversion of as much as 65% of total body water into ice (Voituron et al., 2009).

1.3 Protein post-translational modifications

Reversible protein phosphorylation plays a regulatory role in almost every aspect of life (Ciesla et al., 2011). Phosphorylation is regulated by the opposing actions of protein kinases and protein phosphatases, often providing essentially on-off control to critical cellular processes (Deribe et al., 2010). The main concern for long-term stress survival in a hypometabolic state is energy conservation, which includes reprioritizing energy expenditures and suppressing overall energy consumption by cellular processes. Utilizing strategies to enhance energy efficiency in regulating protein or enzyme function is a crucial step in achieving this. With limited energy production possible in the dehydrated state, major changes in transcription and translation cannot be accomplished. Therefore, alternatives that are both energy efficient, functionally effective, and readily reversible are applied.

Posttranslational control of protein/enzyme function via reversible protein phosphorylation is one major strategy that can be used. For example, pyruvate kinase from the tissues of *Otala lactea*, the desert snail, undergoes reversible phosphorylation in response to aestivation or anoxic stress. The removal of phosphate groups turns pyruvate kinase into the active form, a process that is accomplished in under 10 minutes when arousal is initiated (Whitwam et al., 1990). Phosphorylation is a mechanism for regulation that is quickly reversible upon the return of favorable conditions.

In recent years, it has become known that a variety of other posttranslational modifications of proteins can be just as influential as phosphorylation. For example, nitrosylation occurs at cysteine thiols and metal centers of a broad spectrum of proteins. Since the nitrosylated residues are typically acid-base or hydrophobic structure motifs, they are thought to be redox based signals. Therefore, nitrosylation is thought to be a principal effector mechanism in redox-based regulation of cellular function (Tennenbaum and White 2006; Hess et al., 2005; Stamler et al., 2001). Furthermore, protein acetylation has emerged as a prevalent modification in enzymes that mediate metabolism. Zhao et al. (2010) found that most enzymes in glycolysis, gluconeogenesis, the tricarboxylic acid (TCA) cycle, the urea cycle, fatty acid metabolism and glycogen metabolism in human liver tissue can be found to be acetylated and that the concentration of the metabolic fuels are what determine the acetylation status (Zhao et al., 2010). In fact, recently it has been demonstrated that lysine acetylation regulates components of cellular autophagy. The main substrate for this reaction is acetyl-CoA, a major substrate of aerobic metabolism, which led

Webster et al. (2014) to suggest that there is a direct link between fuel metabolites and enzyme regulation. Ubiquitination is a posttranslational modification (PTM) thought to regulate apoptosis. The ubiquitination of proteins involved in apoptosis is thought to destabilize them and target them for proteasome degradation (Vucic et al., 2011). Hence, one method of stabilizing cellular proteins during hypometabolism would be to block the ubiquitination of proteins. This can be done by using other PTMs such as acetylation to modify and thereby block the amino acid sites that could otherwise be ubiquitinated, helping to slow down protein turnover, extend the life of proteins and reducing energy expensive processes like protein synthesis (Vucic et al., 2011; Storey and Wu 2013). It has been demonstrated that acetylation, methylation, and nitrosylation can all affect LDH from *R. sylvatica* with significant changes in acetylation and ubiquitination of the enzyme occurring in response to dehydration (Abboud et al., 2013). Similarly, LDH from the liver of the anoxia tolerant fresh water turtle, *Trachemys scripta elegans,* demonstrated an altered level of acetylation, increasing 70% along with the increased phosphorylation after exposure to 20 h of anoxic submergence (Xiong et al., 2012).

1.4 Objectives and hypotheses

The African clawed frog can survive ~30% loss of total body water and the related hypoxia in its natural aestivation. A coordinated suppression of energy expensive processes is required to respond to the demands placed on the organism during this period of stress. Furthermore, specific regulatory controls over key enzymes may be needed to either enhance anaerobic ATP production or suppress

ATP use for nonessential functions to achieve strong metabolic rate depression. Based on this, I propose the following hypotheses about the regulation of selected enzymes that are involved in energy metabolism in the skeletal muscle of the African clawed frog, *X. laevis.*

1) **Hypothesis**: Aestivation survival depends on carbohydrate fuels to produce ATP via fermentative glycolysis. Strict control over the catabolism of glucose will therefore be required. **Prediction**: Hexokinase will be differentially regulated in hydrated versus dehydrated states to manage the entry of glucose into glycolysis.
2) **Hypothesis**: During dehydration-induced hypoxia of the peripheral limbs, anaerobic production of ATP will be enhanced to compensate for a reduced capacity for oxygen-dependent ATP production in the mitochondria. **Prediction**: Lactate dehydrogenase, the final enzyme in anaerobic glycolysis will be regulated to promote the conversion of pyruvate to lactate under hypometabolic, oxygen-limited conditions.
3) **Hypothesis**: In order to achieve the regulations predicted above the posttranslational modifications made to the enzymes must alter their structural and functional properties. These PTMs will help to control the use of cellular resources in the dehydrated state. **Prediction**: Reversible posttranslational modifications will play a part in enzyme regulation during dehydration.

Two crucial enzymes representing the beginning and end of glycolysis are explored in this thesis.

1.5 Hexokinase

The first committed step of glycolysis is the conversion of glucose to G6P by HK as follows:

Glucose + ATP →G6P + ADP + H^+

HK is an important enzyme to study because its product, G6P, can enter several cellular pathways besides glycolysis, including glycogen synthesis for carbohydrate storage, the pentose phosphate pathway for the production of NADPH and sugar phosphates, and in some organs, dephosphorylation of G6P in order to export glucose. Therefore, chapter 2 explores the kinetic and regulatory properties of HK from skeletal muscle of dehydrated frogs.

1.6 Lactate dehydrogenase

Glycolysis to the level of pyruvate results in the net synthesis of 2 ATP molecules for every glucose molecule catabolized. However, this process does not maintain redox balance. Under aerobic conditions, the NADH generated in glycolysis is reoxidized by the actions of shuttles that move reducing equivalents into the mitochondria whereas under anaerobic conditions, redox must be rebalanced without the involvement of oxygen-dependent mitochondrial metabolism. In the cytosol, LDH plays this role by converting pyruvate to lactate while oxidizing NADH back to NAD^+, which is in limited supply in the cell. The LDH reaction is as follows:

Pyruvate + NADH + H^+ → lactate + NAD^+

Overall conversion of glucose to lactate is as follows:

$$\text{Glucose} + 2\ P_i + 2\ \text{ADP} \rightarrow 2\ \text{lactate} + 2\ \text{ATP} + 2\ H_2O$$

LDH is therefore crucial for maintaining redox balance in cells under hypoxic/anoxic conditions and hapter 3 explores the kinetic and regulatory properties of LDH from skeletal muscle of clawed frogs under dehydration stress.

Chapter 2:

Regulation of hexokinase by reversible posttranslational modifications in response to dehydration in the leg muscle from *Xenopus laevis*

2.1. Introduction

To survive in seasonally arid environments where both water and food availability is highly restricted, many animals enter prolonged periods of hypometabolism where ATP demand and metabolic fuel use are greatly reduced. By doing so, they extend the time that body fuel reserves can support survival in the face of environmental stress, and hopefully endure until environmental conditions are again conducive to active life. A major stress presented during aestivation is dehydration as body water is lost to the surrounding environment. As dehydration rises to high levels this leads to reduced blood volume and elevated blood viscosity which causes oxygen deprivation to tissues due to poor blood circulation (Churchill and Storey 1995). In response to dehydration-induced hypoxia, aestivators increase their reliance on anaerobic pathways, particularly the pathway responsible for fuel catabolism and ATP generation, namely glycolysis. As a result, there is a requirement for strict regulatory control over glycolytic rate during aestivation as it is critical for the maintenance of energy homeostasis and cell survival.

HK is the first enzyme involved in the breakdown and use of glucose as a fuel for the cell. The regulation of HK is therefore vital in controlling carbohydrate metabolism during dehydration stress (Dieni and Storey 2011). HK catalyzes the conversion of glucose and ATP to G6P and ADP. Glucose is widely recognized as a central compound in metabolism and glucose homeostasis as a whole is highly regulated in physiology (Polakof et al., 2011). Glucose is delivered to all organs of the body by the blood. Once glucose is transported into cells, it is rapidly phosphorylated by HK to form G6P, effectively trapping it for use in the cell since

G6P cannot diffuse through the cell membrane (Berg et al., 2002; Dawson and Storey 2012). G6P can be directed into many different pathways such as; glycolysis to produce ATP, the pentose phosphate pathway to form NADPH and various sugar phosphates, or is converted to glycogen for fuel storage (Dawson and Storey 2012). Therefore, the regulation of HK could slow many different processes and contribute to metabolic rate depression during aestivation while also sustaining the needs of anaerobic glycolysis for ATP production if oxygen is limiting. However, other intermediates of glycolysis can be also acquired from lipid metabolism and amino acid metabolism therefore the regulation of HK mainly influences the use of carbohydrates as a fuel source. As previously mentioned, carbohydrate metabolism is not the main fuel source used during aestivation by the spadefoot toad (or by aestivators in general). The spadefoot toad mainly relies on lipids and protein catabolism for the duration of its aestivation. Hence, a differential regulation of HK in response to dehydration in aestivators could be indicative of the type of fuels that the clawed frog relies on under stress.

Previous studies in our lab have shown that one of the mechanisms of HK regulation in response to stress is reversible protein phosphorylation. HKI and II from the skeletal muscle of hibernating ground squirrels, HK from the skeletal muscle of freeze-tolerant frogs and from the tail muscle of freshwater crayfish were all shown to be regulated by reversible phosphorylation (Abnous and Storey 2008; Dawson and Storey 2012; Dieni and Storey 2011). In the ground squirrel, the removal of phosphate groups through the addition of protein phosphatases in vitro, lowered the activity of HK. Interestingly in crayfish, the removal of phosphates had

no effect on the activity of HK, but a large effect on the stability of the enzyme in the face of urea (Dawson and Storey 2012). Therefore, due to the role of HK in gating glucose entry into glycolysis and the evidence from previous studies of differential regulation of HK in response to stress, the current work investigates the regulation of HK during dehydration in the skeletal muscle of X*. laevis*.

2.2. Methods

2.2.1. Animals and chemicals

Adult male *X. laevis* were obtained from a colony at the University of Toronto (Toronto, ON); mean body mass was 38.1 ± 2.1 g. Frogs were housed in buckets containing dechlorinated water at room temperature (~20°C). Animals were acclimated for 3 weeks prior to the initiation of dehydration experiments and were provided with 3-4 pellets of *Xenopus* CU Adult Frog diet per frog every other day (PMI Nutrition International). Water was changed and animals were fed every 2-3 days, but feeding was halted during the experimental days. Control animals were sampled from these conditions. For dehydration exposure, frogs were placed in closed dry plastic buckets at room temperature. Animals were weighed at ~12 h intervals to determine weight loss due to evaporation and were sampled when total body water exceeded 30 %. Total body water lost was calculated from the change in mass of the frogs over time: % water lost = [(Mi - Md) / (Wi × BWCi)] × 100 where, Mi is the initial mass of the frog, Md is the mass at any given weighing during the experimental dehydration, and BWCi is the initial body water content of frogs before dehydration, which was experimentally determined to be 0.741 ± 0.019 g

H_2O per gram body mass (Malik and Storey 2009). The mean percent body water lost by the frogs was 38.0 ± 1.6 % and occurred over 1.5-2 days. All frogs were killed by pithing and tissues were rapidly excised, flash frozen in liquid nitrogen and then stored at -80 °C until use. Animal care, holding and experimental procedures were approved by the Carleton University Animal Care Committee in accordance with the guidelines of the Canadian Council on Animal Care.

2.2.2. Assay

HK activity was assayed with a Thermo Labsystems Multiskan spectrophotometer at an absorbance of 340 nm. HK activity was measured by coupling the HK reaction to the glucose-6-phosphate dehydrogenase (G6PDH) reaction and monitoring the production of NADPH. Optimum assay conditions for the forward (NADPH producing) reaction for muscle HK from both control and aestivating frogs were determined to be 1.5 mM NADP, 5 mM glucose, 5 mM ATP-Mg, 1 unit of G6PDH and 20 mM Tris-HCl pH 8.0 in a total volume of 200 µl with 50 µl of muscle extract used per assay. Activity was measured in mU/mg soluble protein and routine assays were run at room temperature (22°C). K_m and V_{max} values were determined at constant, saturating co-substrate concentrations, as above.

Soluble protein concentrations were determined using the Coomassie blue G-250 dye-binding method with the Bio-Rad Laboratories prepared reagent and bovine serum albumin as the standard.

2.2.3. Preparation of Tissue Extracts

Samples of frozen leg muscle were homogenized 1:5 w:v in ice-cold homogenization buffer A: 50 mM Tris buffer, pH 8.0 containing 10 mM NaF, 2.5 mM EGTA, 2.5 mM EDTA, 10% v:v glycerol, 5 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF). Initial tests of phosphatase inhibitors showed that β-glycerophosphate yielded worse HK activity compared with NaF and hence NaF was used in the study. In addition, good long-term stability of muscle HK over time was not affected by the presence/absence of additional protease inhibitors. Muscle homogenates were centrifuged at 13,500 × g at 4°C for 30 minutes and the supernatant was decanted; the supernatant was held on ice until use.

2.2.4. Purification of Hexokinase

Low molecular weight metabolites and ions were found to interfere with the purification of HK. These were removed by Sephadex G-25 gel filtration. 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. Then a 500 µL aliquot of skeletal muscle extract was applied to the column and centrifuged again. The resulting eluant was collected. Ion exchange chromatography was used to purify HK from both control and dehydrated muscle. An aliquot of desalted extract was then applied to a DEAE Sephadex G50 column (1.5 cm × 5 cm) equilibrated in homogenization buffer. The column was washed with this same buffer and then eluted with a linear KCl gradient (0-1 M) in homogenization buffer. Fractions were collected and assayed and those with the highest activities were

pooled. The pooled fractions from the DEAE Sephadex G50 column were then applied to a Cibacron Blue column (1.5 cm × 5 cm) and eluted with a linear KCl gradient (0-2M). The purity of HK was checked by running eluent fractions on SDS-PAGE (as described under Western blotting) and then staining with Coomassie blue. The fractions with the highest purity were pooled for differential scanning fluorimetry and the remaining fractions were pooled for kinetic analysis and western blotting.

2.2.5. Western Blotting

Purified skeletal muscle extracts were 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), boiled for 5 min and stored at -20°C until used. Lanes on 8% SDS-PAGE gels were loaded with ~20 µg of protein and electrophoresis was carried out at 180 V for 55 min in running buffer (0.05 M Tris, 0.5 M glycine, 0.05% w/v SDS). Proteins were transferred to PVDF membranes at 160 mA for 90 min. Membranes were then blocked with 2.5% non-fat dried milk in Tris-buffered saline containing Triton-X (TBST: 20 mM Tris base, pH 7.6, 140 mM NaCl, 0.05% v/v Tween-20) for 20 min and washed three times with TBST. Membranes were then incubated with antibody overnight at 4°C. All antibodies used in this study were made in rabbits or mice and diluted 1:1000 v:v in TBST before use: rabbit anti-phosphoserine (Cat # 618100, Invitrogen, Carlsbad, CA, USA); rabbit anti-phosphothreonine (Cat. # 718200, Invitrogen, Carlsbad, CA, USA); mouse anti-phosphotyrosine (Cat # 700286, Invitrogen, Carlsbad, CA, USA); anti-acetyl (Santa Cruz Biotechnology; Cat. # sc 8663-R,), anti-SUMO 1 and 2/3

(generous gift from Dr. JM Hallenbeck, NINDS, NIH, Bethesda, MD); and anti-nitrosyl (Abcam; Cat. # ab50185). Unbound primary antibody was removed with three 5 min washes with TBST and the membrane was incubated with HRP-conjugated anti-rabbit secondary antibody (BioShop, diluted 1:4000 v:v in TBST) for 40 min and then washed. Immunoreactive bands were visualized by enhanced chemiluminescence and visualized on the ChemiGenius Bioimaging System (Syngene, Frederick, MD). Band intensities were quantified using GeneTools software. HK band intensities were normalized against the corresponding Coomassie blue stained band to correct for any variations in sample loading.

2.2.6. Differential Scanning Fluorimetry

HK protein unfolding was measured using a Bio-Rad IQ PCR instrument. Purified HK that was concentrated with a centricon (Ultracel-10K regenerated cellulose 10,000 NMWL, REF: UFC801024) to greater than 0.02 g/L was added with DSF buffer (100 mM potassium phosphate, pH 7.0, 150 mM NaCl), 40x diluted SYPRO orange dye (Invitrogen) and any additional reagents to a 20 µL final volume in thin-walled PCR plates. The plates were sealed and placed in the IQ PCR where the orange fluorescence was measured (excitation filter: 490 ± 20nm emission filter: 625 ± 30nm) over a 15˚C to 93˚C temperature gradient (1˚C increments with 30 s reads). Fluorescence intensity was analyzed using OriginPro 8.5. The Boltzmann distribution curve was used to calculate the midpoint temperature of the protein-unfolding transition, known as T_m (Biggar et al., 2012).

2.2.7. Data and statistical analysis

A Microplate Analysis (MPA) Program was used to analyze enzyme rates and kinetic parameters were derived using a nonlinear least squares regression computer program, Kinetics 3.51 (Brooks, 1992).

2.3. Results

2.3.1. Optimization of experimental conditions

The various components of the homogenization buffers, and assay conditions were evaluated prior to assessing kinetic parameters of HK from frog muscle. It was determined that the inclusion of EGTA and EDTA to the homogenization buffer did not reduce the recoverable activity of HK in the skeletal muscle of the frog in either the control or dehydrated conditions. The inclusion of the protein phosphatase inhibitor, β-glycerophosphate, significantly decreased the recoverable activity in comparison to NaF, which showed negligible effects on activity. Therefore, NaF, EGTA and EDTA were included in the standard homogenizing buffer.

2.3.2. HK Partial Purification

The purification scheme for skeletal muscle HK from *Xenopus* is shown in Table 2.1. Two affinity columns, DEAE+ Sephadex and Cibacron Blue, were used. Skeletal muscle HK was partially purified 3.4 fold with an overall yield of 8.86 %. The final specific activity of enzyme was 112.4 U/mg. The success of each purification step was assessed by electrophoresis on an SDS-PAGE gel stained with Coomassie blue (Figure 2.1). The end result of the purification showed that the enzyme was purified to homogeneity in the two initial fractions as there is only one

band corresponding to HK at the correct molecular weight of ~100 kDa (Figure 2.1, lane 4 and lane 5). These two initial fractions were used for DSF studies while the remaining fractions were pooled and used for kinetics. The same purification protocol was used to purify HK from skeletal muscle of dehydrated *Xenopus*.

2.3.3. HK Kinetics

Kinetic parameters of skeletal muscle HK were assessed to determine if there were any differences in enzyme functional properties between the enzyme isolated from control versus dehydrated frogs. As compared to control conditions, the Michaelis-Menten constant (K_m) for glucose of HK from dehydrated frogs increased by 2.5 fold whereas the K_m for ATP did not change (Table 2.2). Also, there was no change in the HK resistance to urea denaturation since the I_{50} (denaturant concentration that reduced enzyme activity by 50%) remained the same between control and dehydrated conditions.

2.3.4. Western Blotting

The potential posttranslational modifications of *Xenopus* HK were assessed by western blotting using the remaining partially purified preparations of control and dehydrated HK. A comparison of control versus dehydrated HK showed a significant change between the control and dehydrated HK forms with respect to nitrosylation, and phosphorylation on threonine and serine sites (Figure 2.2). Nitrosylation decreased by 27% while phosphorylation on threonine sites decreased by 21%. Phosphorylation on serine sites increased by 30% between the control and dehydrated HK forms.

2.3.5. Structural Stability of Xenopus Skeletal Muscle HK

Differential scanning fluorimetry (DSF) is an efficient method that assesses the thermal unfolding of proteins (Niesen et al. 2007). Heat is a denaturant to all enzymes and causes their hydrophobic core to become exposed allowing a hydrophobic dye to bind within these regions and resulting in the emission of light (fluorescence) that is detected. DSF was used to test the stability of purified control and dehydrated HK against heat. The T_m value, the temperature that results in 50% of protein unfolding, for HK was measured and Table 2.2 shows that HK purified from dehydrated muscle had a Tm value that was 8°C lower than that of the control enzyme.

2.4. Discussion

During aestivation *X. laevis* must be able to adapt to the gradual loss of its body water to the surrounding soil. The frogs inability to stop the surrounding soil from leaching its body water eventually leads to dehydration related hypoxia as the circulatory system become taxed with thicker blood (Churchill and Storey 1995). This study examined the effect of high dehydration in *X. laevis,* as seen during their aestivation, on the regulation of skeletal muscle HK. The results presented show that a decrease in HK binding affinity for glucose is accompanied by a change in the posttranslational modifications to the enzyme during dehydration (Table 2.2 and Figure 2.2). Furthermore, HK from dehydrated frogs showed a significantly lower melting temperature (as determined by DSF). This correlated with the changes in PTMs, which suggests that the PTMs caused a perturbation in enzyme structure that

led to a large reduction in its stability (a 7.7°C decrease in Tm value) in the face of increasing temperature (Table 2.2). This reduction in stability and lowered affinity for glucose could reduce HK function in vivo, reducing its ability to convert glucose into G6P and effectively slowing all pathways that use the hexose phosphate. G6P is directed into many different pathways, which can be regulated by reversible phosphorylation. For example, in crayfish (*Orconectes virilis*) G6PDH demonstrated lowered K_m values in response to anoxia that corresponded with phosphorylation by cyclic AMP or cyclic GMP protein kinases (PKA or PKG) (Lant and Storey 2011). For many years, the production of G6P by HK was thought to be regulated primarily by allosteric inhibition by the G6P product. However, it is now known that HK is posttranslationally modified, which suggests that G6P production is regulated before the critical product inhibition concentration is reached. In the aestivating snail *Oreohelix,* G6P levels were decreased during the transition into aestivation, which suggested a decrease in glycolytic flux through the regulation of HK in the investigated tissues (Rees and Hand 1990). This agrees with previous work such as on HK in crayfish *O. virilis* (Dawson and Storey 2012) and *R. sylvatica*, the wood frog, (Dieni and Storey 2011). Both of these species showed significant changes in kinetic parameters between the control and oxygen stressed states. Most pertinent to this study, *R. sylvatica* HK demonstrated an increase in the K_m for glucose during freezing stress which is similar to dehydration stress since cell water is lost into extracellular ice (Dieni and Storey 2011). Similar to *R. sylvatica* HK, the enzyme form dehydrated *Xenopus* in this study presented a 2.5-fold increase (Table 2.2) in the Michaelis-Menten constant for glucose, demonstrating a significant reduction in the

catalytic efficiency of HK during dehydration. The effect of decreasing the affinity for glucose would be a slowing of the production of G6P from glucose taken up from the blood into muscle. In the case of *R. sylvatica* this allows the glucose to pool in the cells and act as a cryoprotectant, but for *Xenopus* it might be one of the mechanisms that reduces carbohydrate catabolism and facilitates the change-over to other fuels during aestivation. Indeed, lipids are known to be the main fuel during hypometabolism in other anurans and protein catabolism also becomes a source of fuel later in aestivation when osmotic stress increases and stimulates urea biosynthesis (Pinder et al., 1992; Storey and Storey 1990).

The kinetic alteration identified in this study (change in K_m glucose) suggests that muscle HK from dehydrated and control *X. laevis* exists in two distinct PTM states. While the exact nature of these posttranslational modifications are not explicitly known, it seems that post-translational modifications of muscle HK during dehydration result in an overall reduction in HK function. The physiological implications of changes in enzyme function are often hard to establish. The changing cellular environment in which an enzyme resides during the prolonged exposure of *X. laevis* to dehydration must be taken into consideration. Urea accumulation in the plasma, liver and muscle tissue is one strategy used by aestivating amphibians, including *X. laevis*, to minimize water loss during aestivation (Janssens 1964; Wray and Wilkie 1995; Romspert 1976; Balinsky et al., 1961; Malik and Storey 2009; Hillman 1980). It is interesting to note that *X. laevis* muscle HK was not differentially affected by urea, suggesting that the modifications to dehydrated HK potentially aid the enzyme in remaining stable in the face of the denaturant during dehydration. It

has been previously demonstrated that LDH from the liver of *X. laevis* maintains the control level of activity in vitro when urea is present. Katzenback at al. (2014) concluded that the PTM changes allowed the enzyme to function normally in the face of this protein denaturant. Further kinetic analysis is required in order to determine if the affinity for glucose remains lowered in the presence of urea or if the PTMs that are causing the affinity changes are stabilizing when urea is present.

The data indicates that HK from dehydrated frogs was less stable in comparison to the control enzyme when temperature was used to unfold the enzyme. It is unknown at this time if the chemical stability of the enzyme is related to the temperature instability. A possible explanation is that there are structural changes made to withstand the increased urea concentration in a dehydrated cell. Increased structural flexibility may allow for the reaction to occur in the face of the denaturant. Furthermore, HK is known to associate with the mitochondrial membrane and has been demonstrated to have bound and unbound forms. Dawson and Storey (2012) suggested that the phosphorylation of crayfish HK controls its sub-cellular localization. During anoxia the mitochondrial source of ATP is compromised forcing HK to depend on cytosolic glycolysis for ATP and therefore favoring an unbound cytosolic HK during oxygen stress. Similarly, since dehydration induced hypoxia would alter the ATP output of *Xenopus* mitochondria, a similar shift to HK dependent on cytosolic ATP could occur. In a study to analyze the colocalization between HK and the mitochondria in response to metabolic changes, Lynch et al., (1991) studied the change in associated HK when glucose metabolism was inhibited. After glucose inhibition there was a 35% decrease in mitochondrial

associated HK, which suggests that the association is dependent on a functioning metabolism (Lynch et al., 1991). Metabolic rate depression, an overall reduction in glucose metabolism may influence HK binding ability. The decreased thermal stability in the dehydrated state may be due to this change in membrane association, however the mechanism of destabilization is unknown. Regardless, the apparent posttranslational changes that occur during dehydration stress in correlation with changes in enzymatic function suggest that the regulated function of HK may play a role in glycolytic flux control during dehydration.

Previous studies have shown that HK is subject to reversible phosphorylation (Dieni and Storey 2011; Abnous and Storey 2008). The data presented in this study indicate that *X. laevis* muscle HK is also a phosphoprotein, phosphorylated at serine and threonine residues (Fig. 3). Phosphorylation of *X. laevis* muscle HK on threonine residues is similar to the phospho-control reported for crayfish and wood frog HK. These two animals regulate HK in response to anoxia and freezing (Dawson and Storey 2012; Dieni and Storey 2011). The reduction in the phosphorylation on threonine residues of HK during dehydration stress suggests that reversible phosphorylation of HK may adjust enzyme function in response to changing conditions. However, the phosphorylation on the serine residues was elevated during dehydration, an event that directly opposes the previous phosphorylation regulation hypothesis. It is therefore unclear whether this PTM is solely responsible for the altered affinity for glucose.

Nitrosylation involves covalently adding a NO-moiety to a reactive cysteine thiol and is known to have a variety of effects on enzyme function (Hess et al., 2005).

Nitrosylation has previously been demonstrated to activate glucokinase (the liver-specific isozyme of HK) in the insulin secretion pathway (Hess et al., 2005). Concomitantly, in this study the reduction in nitrosylation during dehydration is correlated with a reduction in HK affinity for glucose, suggesting a similar mechanism of regulation for *X. laevis* muscle HK. Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) is also thought to be a potential target for nitrosylation, suggesting that overall metabolic regulation by nitrosylation may be similar to that known of phosphorylation events (Broillet, 1999). In this case, since the phosphorylation events on Ser and Thr of *X. laevis* HK are opposing, there is the potential that other PTMs play a role in the kinetic outcomes. It has been previously shown that nitrosylation at active site cysteines can play a regulatory role on enzyme activity (Bauer et al., 2001). It is unclear at this time whether the nitrosylation event is the cause of the kinetic outcome.

In conclusion, the regulation of *X. laevis* skeletal muscle HK during dehydration seems to be correlated with a complex interaction of posttranslational modifications. The substrate affinity and protein stability of HK decreases during dehydration as the enzyme has undergone dephosphorylation on threonine residues and denitrosylation while simultaneously undergoing an increase in phosphorylation on serine residues. All of these changes could adjust HK for a regulatory role in optimizing glycolytic flux during dehydration-related hypoxia. Furthermore, the HK from dehydrated tissue remained stable in the face of physiologically increased urea, but is less structurally stable in the face of increased

temperatures suggesting a fine-tuned structural change to withstand conditions in dehydrated cells.

Table 2.1: Purification of HK from leg muscle of control *X. laevis*.

Step	Total Protein (mg)	Total activity (mU)	Yield (%)	Fold Purification	Specific Activity (U/mg)
Crude	8.9	293.5			32.8
DEAE Sephadex	0.859	77.0	26.2	2.45	80.3
Cibacron Blue	0.231	26.0	8.86	3.43	112.4

Table 2.2: Comparison of kinetic and structural parameters of purified HK from muscle of control and dehydrated frogs assayed at 23°C. K_m and I_{50} data are means ± SEM, n=5. *Tm* values are means ±SEM, *n* = 6 independent determinations on purified enzyme. * - Significantly different from the corresponding control value using the Students t-test, $p<0.05$.

	Control	Dehydrated
Glucose K_m (mM)	0.74 ± 0.06	1.83 ± 0.37*
ATP K_m (mM)	4.14 ± 0.42	4.21 ± 0.31
Urea I_{50} (mM)	1.23 ± 0.23	1.38 ± 0.04
Tm (°C)	61.87±1.06	54.20±1.03*

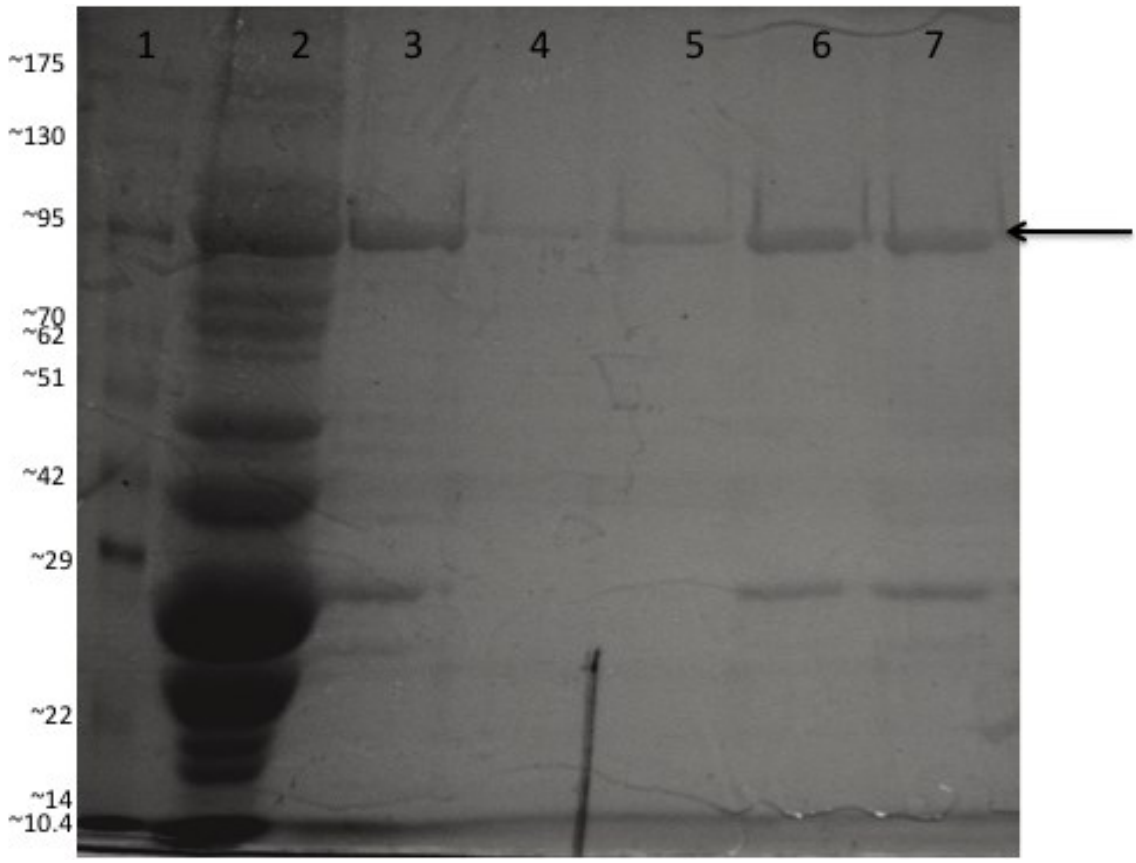
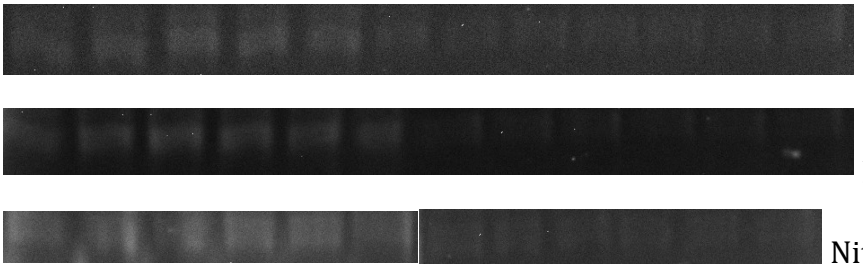


Figure 2.1: Example SDS-PAGE gel showing HK-containing fractions purified from the Cibacron blue column. Lane 1: molecular weight markers with sizes indicated on the left; Lane 2: crude homogenate, Lane 3: pooled DEAE Sephadex elution, Lane 4 & 5: 2 pure HK fractions pooled for use in DSF; Lane 6 & 7: 2 partially pure HK fractions sufficient for use in kinetic studies and western blotting. Arrow shows the HK bands that correspond with the expected molecular mass of the HK monomer of ~100 kD.



p-Ser control on left

p-Thr control on left

Nitro control on left

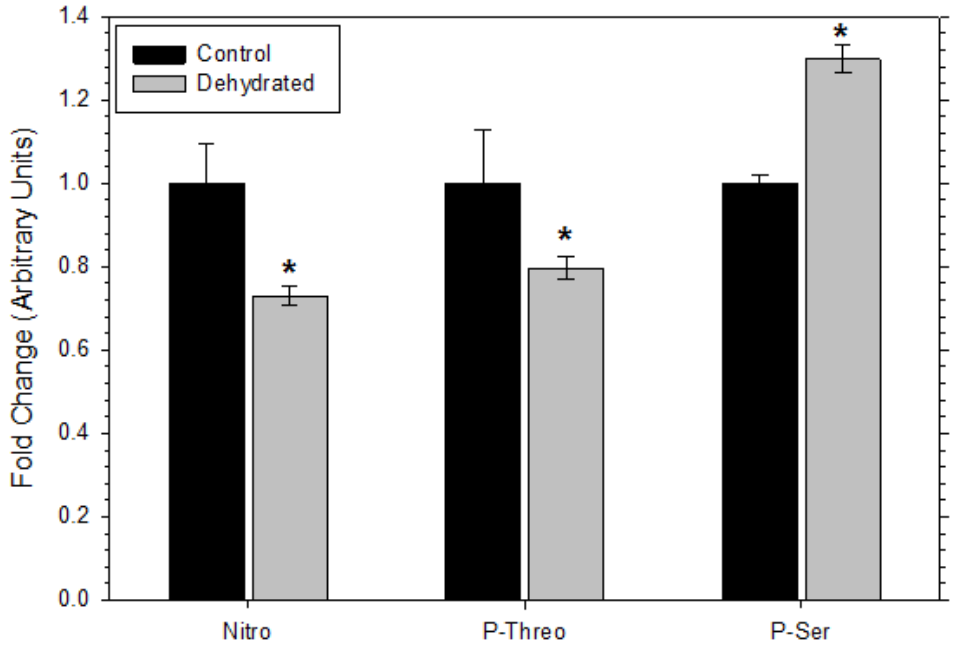


Figure 2.2: Relative post-translational modifications of purified muscle HK from control and dehydrated frogs: nitrosylation, phosphothreonine, and phosphoserine**.** Equal amounts of protein were loaded into each lane of SDS-PAGE gels. Immunoblot band density in each lane was normalized against the density of the same band when re-stained with Coomassie blue. The histogram shows normalized relative band intensities, mean ± SEM, *n* = 6 independent determinations. * - Significantly different from the corresponding control value by the Student's t-test, $P<0.05$.

Chapter 3:

Regulation of lactate dehydrogenase by reversible posttranslational modifications in response to dehydration in the leg muscle from *Xenopus laevis*

3.1 Introduction:

In order to counteract the gradual loss of water as the mud of their ponds dries, *X. laevis* undergo some classic adaptations known to aestivators: 1) they build up osmolytes to reduce water loss from their tissues to the drying mud that they are burrowed into; and 2) they enter a state of hypometabolism to reduce the energy cost of their cellular functions during their aestivation. Frog metabolism must remain tightly regulated as they gradually lose body water in order to tolerate the eventual dehydration related hypoxia (Churchill and Storey 1995). One of the main concerns resulting from hypoxia is the capacity of cells to maintain redox balance during ATP synthesis without a functioning electron transport chain. Glycolysis must continue in order to provide produce ATP and therefore NADH must be reconverted to NAD+. As the terminal enzyme of anaerobic glycolysis, LDH converts pyruvate to lactate in an NADH-dependent reaction that regenerates NADH. The M4 LDH isoform in skeletal muscle (the prominent form) is typically poised to favor the pyruvate reductase direction, which converts pyruvate to lactate (Eventoff et al., 1977). This process regenerates the NAD+ that is needed to sustain glycolytic flux under anaerobic conditions (Hochachka and Mommsen 1983), an important process for redox balance when the mitochondria are not functioning optimally. As such, the glycolytic pathway is almost universally employed for ATP production by animals under low oxygen (hypoxia, anoxia) conditions (Storey and Storey 2004).

Reversible protein phosphorylation is a prominent mechanism for the posttranslational modification and regulatory control of enzymes/proteins and is involved in virtually every aspect of cell life. For example, phosphorylation or

dephosphorylation of an enzyme can influence its activity, kinetic parameters, thermal stability, and protein–protein or subunit–subunit interactions, often modifying enzyme function in response to different environmental or physiological stresses (Cohen 2002). Multiple studies by our lab and others have shown that reversible enzyme phosphorylation is an important regulatory mechanism in animal response to environmental stress (Storey and Storey 2004, 2007). The present study investigates the physical, kinetic, and regulatory properties of *X. laevis* skeletal muscle LDH in response to dehydration to 30% of total body water lost.

3.2 Methods

3.2.1. Animals and chemicals

Animal care, holding and experimentation was essentially as described in Chapter 2 although frogs were larger (80-128 g) and the final percent body water lost for the dehydrated group was 28.0 ± 1.6 % (n=8).

3.2.2. Sample Preparation

Frozen thigh muscle samples were crushed into small pieces under liquid nitrogen and then homogenized 1:10 w:v, using a Diamed Pro 200 homogenizer, in cold homogenization buffer containing 25 mM Tris-HCl, 15 mM 2-mercaptoethanol, 2.5 mM EGTA, 2.5 mM EDTA, 25 mM ß-glycerol phosphate and 10% v:v glycerol, pH 8.0. A few crystals of phenylmethylsulfonyl fluoride (PMSF) were added at the time of the homogenization. Homogenates were centrifuged for 30 min at 13,500 × g at 4°C. The resulting supernatant was decanted and held on ice until use.

3.2.3. Purification of LDH

LDH was purified from muscle of both control and dehydrated frogs using a combination of ion exchange and affinity chromatography. A sample of crude muscle extract was applied to a DEAE Sephadex G50 column (1.5 cm × 8 cm) equilibrated in homogenization buffer. The column was then washed with 15 ml of the same buffer; LDH did not bind to this column, but was eluted in the wash and collected in 3.0 mL fractions. Aliquots from each fraction were diluted by 100-fold, assayed and peak fractions were identified. The corresponding undiluted peak fractions were pooled and applied to a Cibacron blue column (1.5 cm × 5 cm) equilibrated in homogenization buffer. The Cibacron blue column was washed with 30 ml of homogenization buffer and then eluted with a 25 ml of homogenization buffer containing 3 mM NADH and 3 mM pyruvate. Fractions of 1.5 mL were collected and assayed and aliquots of peak fractions were loaded individually onto lanes of a 10% SDS-polyacrylamide gel. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out essentially as in Xiong and Storey (2012) running the gels for 45 min and using non-concentrated protein. Subsequently, the gels were stained with Coomassie blue or silver nitrate. Fractions that contained both NADH reducing activity and showed a strong band at the molecular mass of LDH subunits (with little contamination by other proteins) were pooled (usually the first 4 fractions showing activity). Pooled fractions were loaded onto a second Cibacron blue column (1.5 cm × 5 cm) equilibrated in homogenization buffer. This column was washed with 30 ml of homogenization buffer and then eluted with 25ml of a 0 to 2 M KCl gradient in buffer. Fractions of 1.5 ml were collected and assayed and those with the most

activity were pooled for analytical studies. For some procedures, purified LDH was 8-fold concentrated using a centricon (Ultracel-10K regenerated cellulose 10,000 NMWL, REF: UFC801024) before use.

3.2.4. Assay

LDH activity was assayed using a Thermo Labsystems Multiscan Spectrum microplate spectrophotometer and Multiscan software (with a reading interval of 12 sec). LDH activity was measured by monitoring the consumption or production of NADH at 340 nm. Optimum assay conditions for the forward (NADH using) reaction for muscle LDH from both control and dehydrated frogs were determined to be 1 mM pyruvate, 0.1 mM NADH, 20 mM Tris-HCl pH 8.0 or 20 mM HEPES buffer pH 7.2 in a total volume of 200 µl with 15 µl of purified muscle extract used per assay. The optimum assay conditions for the reverse (NADH synthesizing) reaction for muscle LDH from both control and estivating frogs were 2 mM NAD, 4 mM L-lactate, 20 mM Tris-HCl pH 8.0 or 20 HEPES buffer, pH 7.2 in a total volume of 200 µl with 40 µl of pure muscle extract used per assay. Activity was measured in mU/mg soluble protein and routine assays were run at room temperature (21°C). For K_m and V_{max} determinations, assays were run at constant, saturating co-substrate concentrations.

Soluble protein concentrations were determined using the Coomassie blue G-250 dye-binding method with the Bio-Rad Laboratories prepared reagent and bovine serum albumin as the standard.

3.2.5. Differential Scanning Fluorimetry

DSF was conducted as described in Ch. 2.

3.2.6. Dot blots

Dot blots were used to analyze and identify protein posttranslational modifications using the method of Dawson et al., (2013). Purified LDH was applied to nitrocellulose membranes using a Bio-Dot microfiltration apparatus that was pre-washed twice with TBS (20 mM Tris base, 140 mM NaCl, pH 7.6). In brief, samples passed through the apparatus by gravity and were then washed twice with TBST (TBS containing 0.05% v/v Tween-20). The membrane was subjected to three 5 min washes with TBST, blocking with 1 mg/ml polyvinyl alcohol in TBST for 30 s, and then washing again with TBST. The membrane was then divided (with a razor blade) into segments containing control and stress dots for probing with different antibodies. The cut membranes were incubated with the different primary antibodies overnight at 4°C. Essentially all the antibodies used in this study were made in rabbits except for the anti-phospho tyrosine which was mouse. All were diluted 1:1000 v:v in TBST before use: (1) rabbit anti-phosphoserine (Cat # 618100, Invitrogen, Carlsbad, CA, USA); (2) rabbit anti-phosphothreonine (Cat. # 718200, Invitrogen, Carlsbad, CA, USA); (3) mouse anti-phosphotyrosine (Cat # 700286, Invitrogen, Carlsbad, CA, USA); (4) rabbit anti-ubiquitin (ab19247, Abcam, Cambridge, UK); (5) anti-acetyl (Santa Cruz Biotechnology; cat. # sc 8663-R,); (6) anti-methyl arginine (Covalab; cat. # mab0002-0); (7) anti-methyllysine (Biosciences Inc.; cat. # SPC-158F); (8)anti-SUMO 1 and 2/3 (generous gift from Dr. JM Hallenbeck, NINDS, NIH, Bethesda, MD); or (9)and anti-nitrosyl (Abcam; cat. #

ab50185). Unbound primary antibody was removed with three 5 min washes with TBST and the membrane was incubated with HRP-conjugated anti-rabbit (or anti-mouse) secondary antibody (BioShop, diluted 1:4000 v:v in TBST) for 30 min at room temperature, followed by three 5 min washes with TBST. Membranes were then developed using Western Lighting Chemiluminescence Plus reagents (NEN, Perkin Elmer) following manufacturer's protocols, washed three times for 5 min and signal was detected using enhanced chemiluminescence (ECL). Detection used the ChemiGenius Bioimaging System (Syngene, MD) and dot densities were quantified using GeneTools software (v3.00.02). Subsequently, membranes were re-stained for 5 min with Coomassie blue (25% w/v Coomassie Brilliant Blue R in 50% v/v methanol, 7.5% v/v acetic acid) and destained for 10 min with destaining mix (50% v/v methanol, 10% v/v acetic acid in distilled deionized H_2O (ddH_2O) LDH dot intensities were normalized against the corresponding Coomassie blue stained dots to correct for any variations in sample loading.

3.2.7. ProQ Diamond phosphoprotein stain

Dot blots were also used to analyze and identify protein phosphorylation using Pro-Q diamond phosphorylation stain. The enzyme was dotted onto the nitrocellulose membrane and treated essentially as described above. In order to prepare the membrane for Pro-Q staining the membrane was placed face down in fixing solution (7% acetic acid, 10% methanol and 83% ddH_2O) and incubated for 10 minutes. Fixed membrane was washed 3 times in ddH_2O) for 5 minutes each. The membrane was then immersed in 25 mL of Pro-Q® Diamond phosphoprotein blot stain (1000x diluted in ProQ phosphoprotein dot buffer) for 15 minutes face down.

After staining the membrane was washed in destain solution (15 mL acetic acid, 75 mL isopropanol, 300 mL ddH_2O) three times for 5 minutes. Following this the membrane was washed again three times in ddH_2O for 5 minutes. The membrane was then allowed to dry (face up) at which point it was placed in the Chemi-Genius imager (face up); to visualize the dots the instrument was set to the 254 nm UV light and the red UV filter was inserted.

3.2.8. Data and statistical analysis

Procedures were carried out as described in Chapter 2.

3.3. Results

3.3.1. Purification of X. laevis LDH

LDH from hind leg skeletal muscle was purified from control and dehydrated (30% total body water loss) African clawed frogs using a three-step method. Table 3.1 shows a typical purification for LDH from muscle of dehydrated frogs, which was the same protocol used for control animals. Figure 3.1 shows a representative SDS-PAGE gel for purified LDH from dehydrated muscle; control muscle was purified using the same procedure. The first purification step was a DEAE ion exchange column. LDH eluted in the wash, but many other proteins were bound and separated from LDH. The second step was elution of LDH from a Cibacron blue affinity column using a mixture of pyruvate and NADH. The third step was elution off a second Cibacron blue affinity column using a KCl gradient. For dehydrated muscle the DEAE column gave a 1.5-fold purification and a yield of 84.9%. The first Cibacron blue column gave a 10.6- fold purification and a yield of 48.3% whereas the second

column gave a 61.6-fold purification and a 39.1% yield. SDS-PAGE analysis showed single bands of LDH protein for all purified preparations (Table 3.1). The purified *Xenopus* LDH was very close in size to the bovine LDH standard, which has a molecular weight of 36.6 kD (http://www.uniprot.org/uniprot/P19858), falling just below it between the molecular weight markers of 42kD and 29kD (Fig 3.1).

3.3.2. Activities and kinetic analysis of purified LDH from control and dehydrated muscle:

Multiple kinetic parameters of purified LDH were compared between control and dehydrated states (Table 3.2). V_{max} values for purified LDH were measured at both pH 7.2 and pH 8.0 at 22°C, the optimum pH values for the forward and reverse reactions, respectively, determined from an initial analysis of velocity versus pH for both directions. For the forward (pyruvate reducing) direction the control V_{max} at pH 7.2 was 3.97±0.14 mU/mg for purified LDH while the LDH from dehydrated tissue had a 74% lower V_{max} of 1.05±0.04 mU/mg. Similarly, the V_{max} for the forward reaction of LDH from dehydrated tissue at pH 8.0 was 3.72± 0.078 mU/mg that was 77% lower than for LDH from control frogs that was 0.84 ± 0.05mU/mg. In the reverse (lactate oxidizing) direction the V_{max} of control muscle LDH at pH 7.2 and pH 8.0 were 0.79 ±0.005 and 1.09±0.01 mU/mg, respectively, while the V_{max} for LDH from dehydrated muscle decreased to 0.17± 00.002 and 0.26 ± 0.0132 mU/mg revealing a decrease in V_{max} of 78% and 76% for the enzyme from dehydrated muscle. Hence, the activity of frog muscle LDH in both directions was lower in dehydrated muscle. Interestingly, the ratio of the V_{max} activity between the forward and reverse reaction did not change during dehydration. In control at pH 8.0 the

forward reaction was 3.4 fold greater than the reverse V_{max} while in dehydration the forward reaction V_{max} was 3.2 fold faster. At pH 7.2 the control forward reaction V_{max} was 5 fold greater than the reverse reaction V_{max} while for the enzyme from dehydrated frogs the forward reaction Vmax was 6.3 fold greater that of the reverse V_{max} (Table 3.2).

3.3.3. Kinetic parameters for the LDH forward reaction

In the forward direction the K_m for pyruvate was measured at two pH values that are the pH optima for the forward (pH 7.2) and reverse (pH 8.0) reactions. At pH 7.2 the K_m pyruvate for LDH from dehydrated frog skeletal muscle (0.21 ± 0.04 mM) was 30% lower than that of control muscle LDH (0.30 ± 0.03 mM) whereas at pH 8.0 the K_m pyruvate for dehydrated muscle LDH (0.64 ± 0.09 mM) was not significantly different from that of LDH from control muscle (0.66 ± 0.11 mM) (Table 3.2). The K_m pyruvate was significantly different between the two different pH conditions with both enzyme conditions demonstrating significantly lower Kms in the pH 7.2 condition. The control muscle enzyme had a 54% ($p<0.05$) decrease from ph8.0 to pH 7.2 and the enzyme from dehydrated frogs had a 67% decrease in Km from pH8.0 to pH7.2 ($p<0.005$).

3.3.4. Kinetic parameters for the LDH reverse reaction

In the reverse direction the K_m for lactate was measured at the same two pH values. At pH 7.2 the K_m lactate for LDH from dehydrated muscle LDH (20.0 ± 0.81 mM) was 67% higher than that of control muscle LDH (13.39 ± 0.65 mM) whereas at pH 8.0 the K_m lactate for dehydrated muscle LDH (23.0 ± 2.29 mM) was 69% higher

(P<0.001) than that of control muscle LDH (15.8 ± 0.86 mM) (Table 3.2). The K_m lactate was not significantly different between the two different pH conditions for the enzyme from dehydrated frogs, whereas the K_m of the control enzyme increased by 15% (p=0.042) at pH 8.0 as compared with pH 7.2 (Table 3.2).

3.3.5. Structural Stability of LDH

DSF was employed to assess the thermal stability of purified LDH from control versus dehydrated frogs. T_m values, the temperature at which 50% of protein is unfolded, were measured. The T_m value for LDH from dehydrated frogs was significantly higher (57.1 ± 0.08˚C) by 4.3°C than that of the control enzyme (52.8 ± 0.12˚C) (p<0.001, n=4 for both) (Figure 3.2).

The effects of two additives (urea and KCl) on the T_m at pH 7.0 were also evaluated for LDH purified from control and dehydrated frogs (Figure 3.2). Both urea and KCl stabilized the enzyme from the control and the dehydrated states, the melting temperatures being significantly increased when either was added. Control enzyme T_m increased by 1˚C (54.02 ± 0.33˚C) in the presence of 300 mM urea and by 2˚C (55.16 ± 0.11˚C) with 300 mM KCl added whereas the T_m for LDH from dehydrated frogs was increased by 4˚C in the presence of 300mM urea (61.25 ± 0.23˚C) and 300mM KCl (61.29 ± 0.45˚C) (Figure 3.2).

3.3.6. ProQ diamond phosphoprotein staining:

Potential differences in the phosphorylation state of LDH between control and dehydrated conditions were investigated by staining the purified enzyme with ProQ Diamond phosphoprotein stain, which detects covalently bound phosphate on

proteins. Intensities of ProQ Diamond stained dots were standardized against the intensities of the same dots after they were re-stained with Coomassie blue. The data showed that the standardized relative intensity of phospho-LDH dots in samples from dehydrated muscle were reduced by ~25% ($p<0.05$, $n=8$) as compared with LDH from muscle control frogs (Figure 3.3).

3.3.7. Dot blots for protein posttranslational modifications

To support the results obtained by ProQ staining, dot blotting was used for further assessment of differential PTMs on LDH purified from control versus dehydrated frogs. Substantial amounts of phospho-threonine and nitrosylation of LDH were detected but other PTMs (phospho-serine, acetyl, methyl arginine, methyl lysine, and SUMO 1 and 2/3) were only weakly detected. Dot blots using antibodies that detected two different PTMs on LDH are shown in Figure 3.4. The relative levels of phospho-threonine and nitrosylation of LDH differed strongly between control and dehydrated muscle LDH. LDH from dehydrated skeletal muscle showed a significantly lower level of phospho-threonine (a 93% decrease) whereas relative nitrosylation of LDH decreased by 80% ($p < 0.001$, $n = 6$ for all) (Figure 3.4). The decrease in threonine phosphorylation correlated with the reduction in relative total protein phosphorylation between the two state detected by Pro-Q diamond staining (Figure 3.3).

3.4. Discussion

In this study, the effect of high dehydration (~30%) on the function and regulation of skeletal muscle LDH of *X. laevis* was examined. The dehydration

related hypoxia that these animals experience causes them to rely on anaerobic pathways to support ATP production and maintain redox balance during their later stages of aestivation. It has been demonstrated in other low oxygen animals that lactate levels rise during low oxygen stress even when metabolic pathways are globally suppressed (Hermes-Lima and Storey 1998; Storey and Storey 2004). The results of this study suggest that when the animals are experiencing the higher levels of tolerable dehydration their LDH is less able to bind lactate since its affinity for the molecule is decreased while at the same time LDH from dehydrated frogs is more poised to bind pyruvate as its affinity for the forward direction substrate is increased. This suggests that the frog muscle enzyme is poised during dehydration to react more readily with pyruvate and less readily with lactate. When *Xenopus* suffers from dehydration-related hypoxia, ATP production by the mitochondria would be reduced as oxygen levels decrease. LDH must continue in the pyruvate reductase direction in order to keep glycolysis running, first by replenishing NAD+ and second by removing pyruvate. The kinetic shifts demonstrated in this study suggest that LDH is regulated to promote the use of the anaerobic glycolytic pathway during hypoxic stress and contribute to cell survival during dehydration.

The stability of LDH purified from control and dehydrated muscle was also compared. LDH from dehydrated tissue had a significantly higher melting temperature compared to the control enzyme when unfolded in regular DSF buffer. Physiological levels of urea and KCl affected LDH from both control and dehydrated tissue when they were added, but the effects on LDH from dehydrated tissue were much stronger with a substantial significant increase in T_m when either urea or KCl

was added (Figure 3.2). This increase in enzyme stability indicates that these additives contribute to enhancing the thermal stability of LDH and have a greater effect on the dehydrated enzyme versus the control.

These stability changes and the changes to LDH binding affinities between LDH from control and dehydrated frogs were correlated with changes in PTMs between LDH from control and dehydrated tissue. Reversible phosphorylation has been shown to be a crucial regulatory mechanism of metabolic rate depression in aestivation and multiple other hypometabolic states (Storey 2002). Recent studies on LDH from other systems have shown that the enzyme is also subject to a variety of other PTMs that can change between control and hypometabolic states. For example, liver LDH from the turtle, *Trachemys scripta elegans*, showed increased phosphorylation and acetylation in response to anoxia (Xiong and Storey 2012). Skeletal muscle LDH from the wood frog, *Rana sylvatica,* showed reduced acetylation and enhanced ubiquitinylation of the enzyme isolated from muscle of dehydrated frogs compared with controls (Abboud and Storey 2013). However, wood frog LDH did not show large kinetic changes between the two states; the K_m and V_{max} remained nearly stable between the conditions.

The present study shows that *X. laevis* muscle LDH undergoes a change in phosphorylation and nitrosylation state in response to dehydration (Fig. 3.3 and 3.4). There was a concomitant reduction in nitrosylation on cysteine residues and phosphorylation on threonine residues between the control and dehydrated frogs that correlates with the functional changes to the enzymes' binding affinities. Phosphorylation is well known to mediate changes in enzyme function and

nitrosylation has been demonstrated to also mediate changes in protein activity, localization, stability, and interactions and is sensitive to redox changes such as those that would occur during hypoxia (Beavo and Krebs 1979; Benhar et al., 2009). Nitrosylation has been demonstrated to be an activating PTM and, therefore, the reduced level of nitrosylation on LDH from dehydrated skeletal muscle could potentially be responsible for the lowered binding affinities for substrates of the reverse reaction. Further experimentation to demonstrate if these PTM changes are causing the alterations to LDH binding affinities is required. These could include incubating the enzyme in the presence of protein phosphatases or kinases to modify the enzyme phosphorylation state followed by re-assessing kinetic constants. Incubations in the presence versus absence of added redox enzymes, such as thioredoxins, which have recently been shown to catalyze denitrosylation on caspase-3, could be effective to assess the effects of reversible nitrosylation on enzyme properties (Benhar et al., 2009, 2008).

Table 3.1. Purification of dehydrated *X. laevis* muscle LDH.

	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Fold Purification	Yield (%)
Crude	13.6	12.3	0.9		
DEAE Sephadex	7.6	10.5	1.4	1.5	84.9
Cibacron Blue NAD^+/Pyruvate	0.6	6.0	9.6	10.6	48.3
Cibacron Blue KCl	0.1	4.8	56.0	61.6	39.1

Table 3.2. Kinetic parameters of purified muscle LDH from control and dehydrated *X. laevis*.

Enzyme parameter	**Control**	**Dehydrated**
Forward reaction (pyruvate → lactate)		
V_{max}, pH 8, 22°C (mU/mg)	3.72±0.0.078	0.836±0.052*
V_{max}, pH7.2, 22°C (mU/mg)	3.97±0.14	1.05±0.043*
K_m pyruvate, pH 8, 22°C (mM)	0.66 ± 0.12	0.64 ± 0.09
K_m pyruvate, pH 7.2, 22°C (mM)	0.30 ± 0.03	0.21 ± 0.04*
Reverse reaction (lactate → pyruvate)		
V_{max}, pH 8, 22°C (mU/mg)	1.09±0.011	0.26±0.0.0132*
V_{max}, pH7.2, 22°C (mU/mg)	0.79±0.005	0.166±0.002*
K_m lactate, pH 8, 22°C (mM)	15.8 ± 0.86	23.0 ± 2.29 *
K_m lactate, pH 7.2, 22°C (mM)	13.4 ± 0.65	20.0 ± 0.81*

Data are means ± SEM, n = 5 trials on different preparations of purified enzyme.

* -Significantly different from the corresponding control value using the Student's t-test, $P<0.05$.

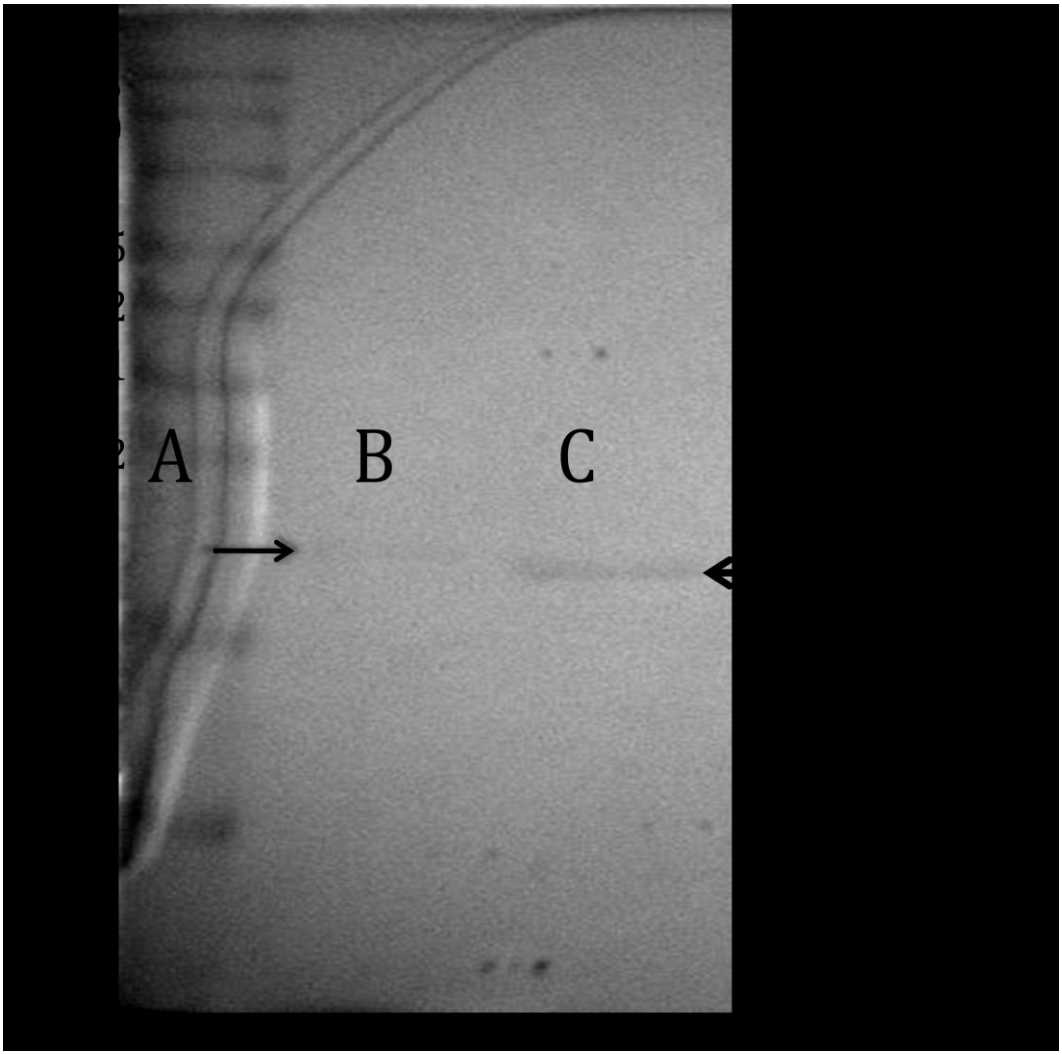


Figure 3.1: Purified muscle LDH from dehydrated *X. laevis*. Coomassie brilliant blue stained gel shows (A) FroggaBio protein ladder with kD values shown to the left, (B) bovine M-type LDH standard (left arrow) and (C) purified *X. laevis* LDH. Arrow on the right shows the position of frog LDH subunit at ~35 kDa.

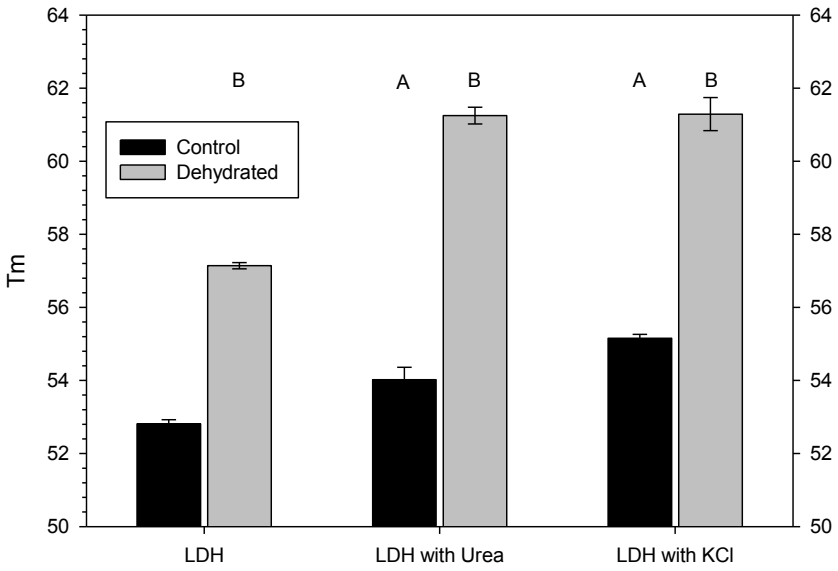


Figure 3.2: The effect of osmolytes on the T_m of purified LDH from muscle of control and dehydrated frogs. Concentrations of additions were 0.3 M urea and 0.3 M KCl. Data are mean ± SEM, n = 5. A – significantly different from the control value without additives, $P < 0.05$; B – significantly different from the corresponding control value containing the same additive, $P < 0.05$.

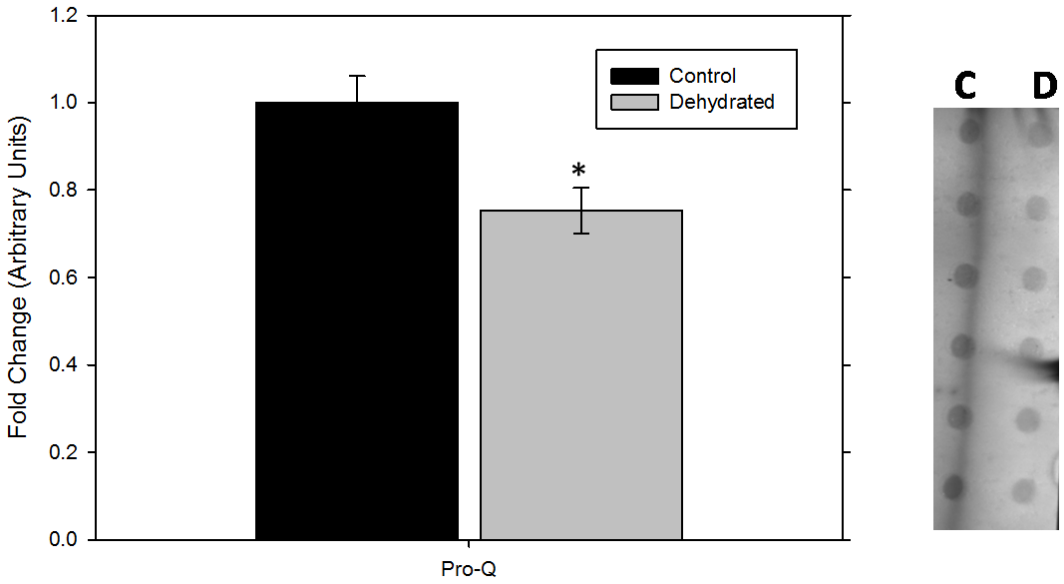


Figure 3.3: Pro-Q Diamond dot blot analysis of relative phosphorylation of purified muscle LDH from control and dehydrated *X. laevis*. Stained blot is shown to the right with control (C) dots on the left, dehydrated (D) on the right. Histogram shows mean relative intensity ± SEM, n = 6 repeats of one prep for each of control and dehydrated. * – significantly different from the corresponding control value, $P < 0.05$.

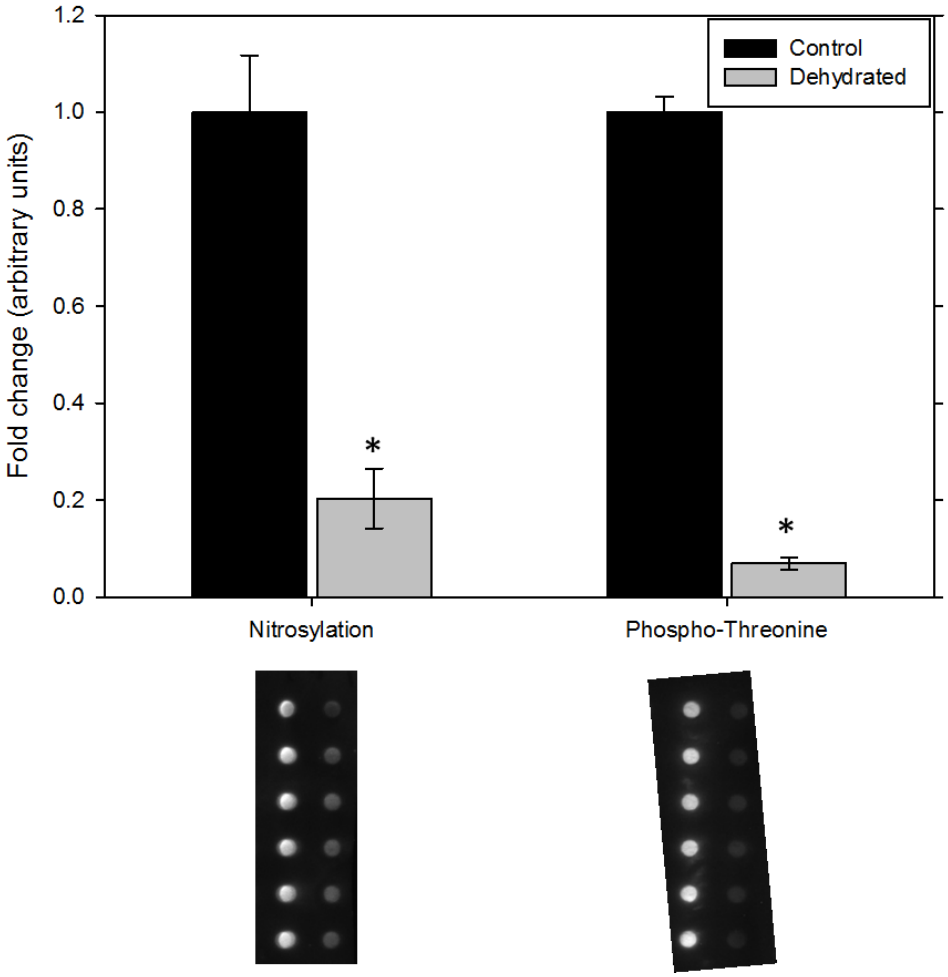


Figure 3.4: Dot blot analysis of posttranslational modifications (nitrosylation, and phosphothreonine) of purified *X. laevis* muscle LDH from control and dehydrated frogs. Chemiluminescent images are shown below the corresponding histogram bars (control on the left, dehydrated on the right). Data are relative intensities, means ± SEM, n = 6 repeats of one preparation for each condition. * – significantly different from the corresponding control value, $P < 0.05$.

Chapter 4:

General Discussion

There are many organisms that inhabit environments where they experience varying degrees of dehydration. Dehydration eventually leads to a reduction in oxygen delivery to peripheral tissues as the blood become thick and circulation slows (Hillman et al., 2009). Animals that can survive various degrees of dehydration therefore must depend on their ability to adapt metabolically to endure the stress of water loss and the subsequent oxygen stress that often follows.

A capacity to withstand a high degree of water loss and the resulting dehydration related hypoxia is a critical factor in the life of many anuran species. Frogs and toads have very permeable skin and therefore are sensitive to changes in environmental moisture. Many dehydration tolerant frogs as well as freeze tolerant frogs use metabolic rate depression as a component of their survival strategy during stress conditions. The spadefoot toad, *Scaphiopus couchii*, is a well-documented terrestrial aestivator, reduces its metabolic rate to only 20% of its resting metabolism in order to survive 9-10 months beneath the desert soil. During this time it can lose 50% of its total body water and builds up 300 mM urea in its cells as an osmolyte (Cowan and Storey 2002). The wood frog, *R. sylvatica*, is a freeze tolerant forest dwelling species that can also withstand 50-60% total body water loss into extracellular ice and minimizes the further loss of cell volume by accumulating extremely high concentrations of glucose as an osmolyte and cryoprotectant. The leopard frog, *R. pipiens*, is an aquatic frog that can withstand high dehydration during which time lactate levels in its blood rise indicating the stimulation of anaerobic energy production to help maintain cellular energetics (Hermes-Lima and Storey 1998). Anaerobic energy metabolism has been

demonstrated to be critical for these animals to survive during dehydration with elevated blood lactate levels characterizing extensive dehydration (Churchill and Storey 1993). The African clawed frog, *Xenopus laevis*, a largely aquatic frog, undergoes aestivation to survive the drying of its habitat. *Xenopus* accumulates urea similar to the spadefoot toad, as demonstrated by a 33-fold increase in blood urea and an increase in urea cycle enzyme activities (Malik and Storey 2009). In order to survive as long as possible, frogs are thought to reduce their metabolic rate and, when dehydration begins to affect oxygen distribution to tissues, they recruit anaerobic glycolysis to meet ATP demands (Malik and Storey 2009). It has further been demonstrated that *X. laevis* liver enzymes are modified in response to dehydration in order to adjust their function to the new metabolic demand and to ensure that when urea levels are high the enzymes retain their functionality (Katzenback et al. 2014).

As animals aestivate a coordinated suppression of many cellular processes begins, reducing the rate of ATP turnover by up to 80-90% in many cases. This reduction in ATP usage is accomplished by reprioritizing the ATP-using pathways so that homeostasis is established at a reduced rate of ATP turnover, allowing the animals to extend the use of fuel reserves over the months of not eating. Reversible protein phosphorylation has been shown to be a major regulatory mechanism operating to achieve metabolic depression in many phyla (Storey and Storey 1990; Storey and Storey 2007). During dehydration-induced hypoxia, the anaerobic glycolytic pathway is a main source of ATP since mitochondrial oxidative phosphorylation is compromised. Hence, control over glycolysis is critical for the

organism's survival. Accordingly, HK and LDH, which are key enzymes of the glycolytic pathway that represent the first and last steps of the pathway when glucose is the substrate, have been demonstrated in this thesis to be regulated via reversible phosphorylation. Comparable findings have recently become available for other species that withstand oxygen deprivation such as marine mollusks, freshwater turtles and crayfish (Storey and Storey 2012; Xiong et al., 2012; Dawson and Storey 2012). However, reversible phosphorylation is no longer the sole PTM considered to be involved in regulation; this thesis shows that nitrosylation is also a PTM that changed between control and dehydrated conditions for both HK and LDH.

In animal models that do not survive sustained hypoxia or anoxia, the first response to hypoxia is to boost glycolytic rate in an attempt to maintain the supply of ATP to meet the normal demands of cellular ATP-utilizing processes (a compensation strategy). However, this cannot be sustained for long without seriously depleting the supply of fermentable fuel (glycogen, glucose). In animal models that do survive hypoxia and anoxia for sustained periods a conservation strategy takes over when oxygen falls below a critical value to support long-term survival. Cellular reorganization occurs to lower ATP demand to a level that can be sustained by glycolytic ATP production alone and, indeed, metabolic arrest can be strong enough in some cases, that glycolytic rate itself is also suppressed. However, glycolytic rate needs to continue in the forward direction and this cannot continue without a means to regenerate the NAD+ that is needed by the glyceraldehyde-3-phosphate step of glycolysis (Storey and Storey 1990). If oxygen delivery to the cell becomes compromised pyruvate cannot be broken down by the TCA cycle and

therefore its conversion to lactic acid solves two problems – regeneration of NAD+ and a metabolic sink for accumulating pyruvate. However, lactate subsequently becomes more concentrated in cells and blood due to poor circulation (Hermes-Lima and Storey 1998). Since aestivators typically enter into metabolic rate depression before dehydration occurs, cells may already be in a sufficiently hypometabolic state when dehydration related hypoxia begins to immediately be able to function on glycolysis alone, further extending the survival time of the frog during aestivation.

As the first committed step of glycolysis, glucose is phosphorylated by HK to form G6P which then allows the hexose phosphate to be directed into at 3 different pathways; glycolysis, pentose phosphate pathway, or glycogen synthesis (Wilson 2003). By decreasing HK affinity for glucose the production of G6P from carbohydrate fuel reserves such as blood glucose is not poised to occur, which can force fuel to be drawn from other reserves. This fits what is known about other frogs such as spadefoot toad metabolism, with the majority of energy during the aestivation period being drawn from lipids and amino acid metabolism (Storey and Storey 1990). Since *Xenopus* also displays a similar process of producing urea as an osmolyte it is likely that *Xenopus* has a similar fuel breakdown during its period of inactivity, preferentially decreasing the entry of carbohydrates into the glycolytic pathway when other fuels can be used. Protein metabolism is an effective fuel when combating increasing dehydration stress since it provides the nitrogen materials required for the production of urea. Accordingly it has been shown that in spadefoot toads protein catabolism occurs in proportion to urea requirements but only begins

once the animals enter a negative water balance later in aestivation (Storey and Storey 1990).

4.1. Enzyme regulation during aestivation

In analyzing the kinetics of *Xenopus* muscle LDH from control and dehydrated frogs, it was evident that there were significant differences between the two enzyme states. LDH from dehydrated muscle had a lower V_{max} in both the forward and reverse directions when compared to the corresponding control muscle LDH V_{max}. The K_m for the reverse reaction, the lactate oxidizing reaction, of LDH from dehydrated muscle was significantly increased while the K_m for the forward reaction, the pyruvate reduction direction, was lowered. These changes in binding affinities poise LDH would favor the pyruvate reducing direction in dehydrated frogs and aid in the removal of the final product of glycolysis under oxygen compromised conditions. The regulation of LDH to convert pyruvate to lactate even in high lactate concentrations would ensure that the glycolytic pathway continues to provide ATP and regenerate NAD+. The LDH reaction is therefore regulated to continue to regenerate the NAD+ needed for the G3PDH reaction and maintain the cytosolic redox potential to promote forward glycolytic flux.

Through the analysis of HK kinetics it was found that HK from dehydrated frog muscle had a lower affinity for its glucose substrate, potentially resulting in a slower production of G6P from glucose. However, since glucose concentrations in *Xenopus* blood increase from 2.52 mM to 5.81 mM during dehydration it is possible that the K_m of glucose increased in proportion with blood glucose levels in order to

maintain a steady rate or decrease the rate of glucose conversion to G6P (Malik and Storey 2009). Regulation of HK is therefore key in gating glucose entry into glycolysis slowing it down as plasma glucose levels rise. This is also seen in *R. sylvatica*, a freeze and dehydration tolerant frog, where both stresses cause extreme hyperglycemia. Glucose levels rise in the liver of *R. sylvatica* by the time 10% total body water was lost (Churchill et al., 1994). Skeletal muscle HK from *R. sylvatica* was also found to have a lowered affinity for glucose after freeze exposure, an event known to increase cell glucose concentrations (Dieni and Storey 2011).

4.2. Role of posttranslational modifications

Reversible posttranslational modifications allow enzymes to be quickly modified in rapidly changing environments and just as quickly reversed when conditions return to their optimal range. In this study of HK and LDH, the kinetic alterations of the enzymes all occurred along with covalent modifications to enzyme structure. LDH and HK from dehydrated muscle were both significantly dephosphorylated at threonine sites and also showed a significant decrease in the amount of S-nitrosylation, as compared with control enzymes. Posttranslational modifications are known to influence enzyme function. The loss of phosphorylation in conjunction with the reduced affinity for lactate suggests a causal link between the dephosphorylation event and the kinetic result. However, nitrosylation has also been demonstrated to influence enzyme activity and therefore it could also be the removal of NO moieties that is hampering the interaction between the enzyme and the substrate (Hess et al., 2005).

It was demonstrated by Katzenback et al., (2014) that in the presence of physiological concentrations of urea, all the substrate K_m values of liver LDH from dehydrated *X. laevis* were restored to control values. This allows the enzyme to continue functioning normally as urea levels rose over the course of dehydration. The present thermal denaturation studies of muscle LDH also demonstrated that urea has a stabilizing influence on LDH from dehydrated muscle. In the presence of urea, LDH from dehydrated tissue showed a higher T_m than the values for either LDH from control tissue or LDH from dehydrated tissue alone as well as a higher T_m than LDH from control tissue under the same urea condition (Figure 3.2). This suggests that the PTMs made to the enzyme allow the enzyme to remain stable in the face of physiologically increased concentrations of a chemical denaturant. The same influence was found with the addition of physiological levels of potassium chloride, suggesting that the dehydrated enzyme is altered to endure increased solute concentrations in general. HK demonstrated a constant resistance to urea between control and dehydrated conditions, as there was no change in the I_{50} value for urea between conditions. However, dehydrated HK showed weaker thermal stability when compared to control HK. HK is known to bind to the mitochondrial membrane (Dawson and Storey 2012) but this binding interaction loses its advantage as the mitochondria become compromised by hypoxia. The dehydration-responsive changes in PTMs may therefore allow the enzyme to dissociate from the membrane and remain stable in the face of urea while becoming structurally less stable without the hydrophobic interaction with the mitochondrial membrane.

4.3. Conclusion

Although estivation typically relies mainly on lipid catabolism as a main fuel, when dehydration rises into the higher ranges that *Xenopus* can survive, lactate accumulates due to hypoxia arising from impaired circulation. Hence, the regulation of glycolysis is of increased importance in the dehydration stressed state. The results in this thesis indicate that controls on glycolysis at both its initial and terminal enzymes contribute to regulating dehydration/hypoxia responsive carbohydrate catabolism. Both HK and LDH showed altered kinetic properties, and structural stabilities concurrently with changes to PTMs in response to dehydration, which supports the predictions made in section 1.4. This suggests that HK and LDH are modified to suit the altered metabolic conditions and demands of carbohydrate metabolism associated with dehydration. For instance, the altered binding affinities of LDH from dehydrated muscle appear to poise the enzyme to continue in the forward direction to support continued glycolytic flux by maintaining redox balance under conditions of dehydration-induced hypoxia. The molecular mechanism of the altered properties may be changes in enzyme PTMs that could alter both kinetic properties and structural stability of the enzymes under dehydration stress as well as compensate for the effects of rising urea concentrations.

4.4. Future Directions

The studies described in this thesis identified the effects of phosphorylation and nitrosylation as a potential means of regulating two glycolytic enzymes to prepare glycolysis for stress conditions. However, these studies also revealed new

directions, which may be pursued to clarify the overall impact of the changes noted. Naturally, there is a significant amount of work that can be further performed on the regulation of glycolytic enzymes, from a more in depth investigation of the enzymes studied in this thesis to the investigation of other glycolytic enzymes both from other tissues and other animal models that use hypometabolism to survive a variety of environmental stresses.

Future studies on the enzymes from this thesis could include an analysis of the phosphatases and kinases that are thought to act on the glycolytic enzymes. The aim of these studies would be to determine which protein kinases and protein phosphatases are involved in the regulation of *Xenopus* glycolytic enzymes. For example, in *R. sylvatica* PKA and PKC and protein phosphatases 1, 2A and 2C are involved in regulating HK during freeze tolerance (Dieni and Storey 2011) and could likely be responsible for the phosphorylation and dephosphorylation of *Xenopus* HK as well. Dephosphorylation of phosphorylated enzymes is a major player in the regulation of HK and LDH from *Xenopus* and is likely also a major regulatory factor for many other glycolytic enzymes from different species (Storey and Storey 2002). Phosphatases and kinases are known to work in concert to regulated entire pathways. Incubation studies of glycolytic enzymes simulating conditions in vivo and stimulating individual phosphatases or kinases followed by kinetic analysis to determine the resulting changes to enzyme properties, could provide a causal mechanism for reversible phosphorylation in the regulation of LDH and HK as well as other glycolytic enzymes. Studies such as this could also be used to separate the effects of reversible phosphorylation from other forms of PTM such as reversible

nitrosylation. Some information is known about the removal of the NO moiety from enzymes and therefore incubation tests could potentially be designed to assess the effects of conditions that should promote the addition or removal of the NO moiety on target enzymes. The phosphorylation state of the enzyme could be held constant (by the presence of kinase/phosphatase inhibitors) while the nitrosylation state is altered. Either method will determine the influence of each PTM individually.

Aside from additional studies on the enzymes of this thesis, there are several future studies that are related to the present research that could also be pursued. As stated previously, there are other branch points in carbohydrate metabolism that could be regulated to influence fuel use during aestivation. The pentose phosphate pathway, for example, also uses G6P to generate NADPH and sugar phosphates for biosynthesis. NADPH in particular would be valuable for hypometabolism because of its key involvement in antioxidant defense, which is important for long-term survival in aestivators. Hence, control of the distribution of G6P between glycolysis and the pentose phosphate pathway could be crucial. Analysis of the regulation of G6PDH, the enzyme that gates the pentose phosphate pathway, could help to determine if there are changes in the relative demands of these two pathways between control and dehydrated states. In the estivating snail *Otala lactea*, it was shown that G6PDH is a phosphoprotein and that the phosphorylation of G6PDH enhances the rate of the pentose phosphate cycle, which was hypothesized to help maintain antioxidant defense (Ramnanan and Storey 2006).

Alternatively, since it has been shown that dehydration increases the levels of plasma amino acids even under starvation conditions in *Xenopus,* an increase in

the enzymes involved amino acid catabolism could be expected (Balinksy et al., 1967). With an increase in amino acid metabolism a concomitant change in glutamate dehydrogenase (GDH) activity could be expected, GDH being a main generator of NH_4^+ for urea biosynthesis as well as an entry point for the catabolism of the carbon skeletons of various amino acids (Frick et al., 2008). An analysis of the regulation of GDH could help to determine how amino acid catabolism is controlled in response to dehydration and whether this regulation changes (e.g. GDH inhibited) under hypoxia stressed high dehydration when amino acid oxidation by the TCA cycle could be compromised. Analysis of the potential PTM controls on key enzymes of the urea cycle and how these changes in response to dehydration would also be a novel area for study.

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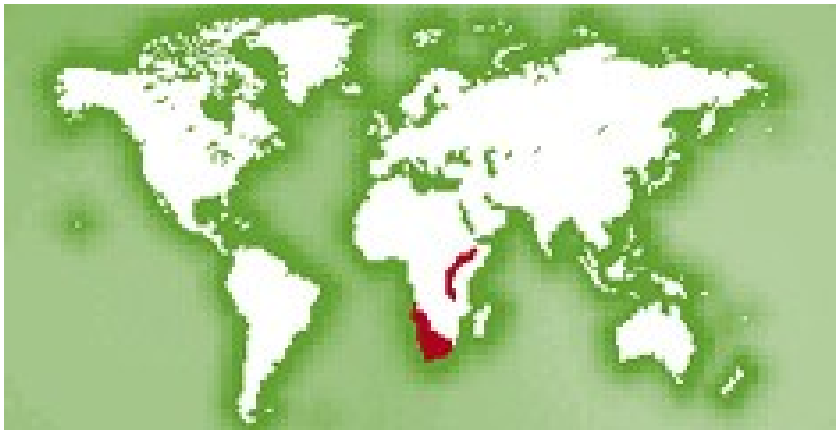
Appendix 1: Native Geographical Range of *X. laevis* in Africa

http://www.robinsonlibrary.com/science/zoology/reptiles/anura/clawedfrog.htm



http://www.edmonton.ca/attractions_events/edmonton_valley_zoo/animals/african-clawed-frog.aspx