REGULATION OF ENZYME FUNCTION IN FREEZE- TOLERANCE

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Abstract

The wood frog (*Rana sylvatica*) is one of the few vertebrate species that can survive whole-body freezing during the cold winter months. The frog endures the freezing of 65-70% of total body water as extracellular ice and, while frozen, shows no respiration, heart beat, or brain activity. Consequently, the frogs experience anoxia and ischemia throughout the freeze followed by oxidative stress when oxygen is reperfused. Enzymes, the biochemical catalysts of cells, must be appropriately controlled to ensure survival. This thesis explores the properties and regulation of key enzymes of adenylate metabolism (AMP-deaminase, AMPD; creatine kinase, CK) and glucose metabolism (glucose-6-phosphate dehydrogenase, G6PDH; hexokinase, HK). The studies showed that changes in pH, temperature, inhibitor and activator concentrations, and binding to myofibrils are involved in regulating these enzymes in the transition to the frozen state. Moreover, reversible protein phosphorylation appears to be a key regulatory mechanism, altering enzyme activity and substrate affinity to suit physiological needs during freezing. Analysis of kinetic parameters showed an increase in enzyme activity for CK and decreased activity for HK. Affinity of CK for one of its substrates, creatine, increased, whereas HK, G6PDH, and myofibril-bound AMPD showed reduced substrate affinity in the transition to the frozen state. These changes in kinetic parameters were the result reversible protein phosphorylation; bound AMPD and CK both increased in phosphorylation state in frozen frogs, whereas G6PDH and HK both decreased in phosphorylation state. Changes in enzyme activity as a result of reversible phosphorylation were analyzed by *in vitro* stimulation of endogenous protein kinase and

protein phosphatase activities. Native phosphorylation states of these enzymes, and changes between control and frozen frogs were further confirmed by elution profiles off DEAE-Sephadex ion-exchange columns that were interconverted between the two physiological states, as well as SDS-PAGE studies that compared phosphoprotein levels to total protein levels. Though phosphorylation states of these enzymes changed, protein levels remained constant in the transition to the frozen state. Overall, these studies showed that multiple mechanisms of enzyme regulation, particularly reversible protein phosphorylation, control enzyme function and the reorganization of metabolic pathways in freeze-tolerance.

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Having no biological siblings of my own, I am fortunate to be blessed with nevertheless true brothers in the Tau Kappa Epsilon fraternity. One stands far above the rest: my own big brother, Pat Gordon, who always had the patience to listen to my endless rants about, as he *incorrectly* referred to it, “*Ranus sylvaticus*, the tree frog.” Though he did it just to annoy me (and mission accomplished Pat!), his otherwise impeccable support has always meant the world to me.

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List of Abbreviations

ADP	Adenosine diphosphate
AEC	Adenylate energy charge
AMP	Adenosine monophosphate
AMPD	AMP deaminase
AMPK	AMP dependent protein kinase
ATP	Adenosine triphosphate
CaM	Calmodulin
CaMK	Calcium-calmodulin dependent protein kinase
cAMP	Cyclic AMP
cGMP	Cyclic GMP
CK	Creatine kinase
Cr	Creatine
CREB	cAMP response element binding protein
EDTA	Ethylenediamine-tetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid
F1,6P ₂	Fructose-1,6-bisphosphate
F2,6P ₂	Fructose-2,6-bisphosphate
F6P	Fructose-6-phosphate
G6P	Glucose-6-phosphate
G6PDH	Glucose-6-phosphate dehydrogenase
GDH	Glutamate dehydrogenase

GMP	Guanosine monophosphate
GP	Glycogen phosphorylase
GTP	Guanosine triphosphate
HK	Hexokinase
I_{50}	Concentration of inhibitor that lowers enzyme velocity by 50%
IMP	Inosine monophosphate
IPA	Insoluble protein A
K_a	Concentration of activator that produces half maximal activation
kDa	Kilo Dalton
K_m	Michaelis-Menten constant (substrate affinity constant)
LDH	Lactate dehydrogenase
MAPK	Mitogen activated protein kinase
MAPKAP-K2	Mitogen activated protein kinase-activated protein kinase-2
MOPS	2-(N-morpholine)-proanesulfonic acid
NAD	β -nicotinamide adenine dinucleotide
NADH	β -nicotinamide adenine dinucleotide, reduced form
NADP	β -nicotinamide adenine dinucleotide phosphate
NADPH	β -nicotinamide adenine dinucleotide phosphate, reduced form
PAGE	Polyacrylamide gel electrophoresis
PCr	Phosphocreatine
PFK-2	6-phosphofructo-2-kinase
PK	Pyruvate kinase
PKA	Protein kinase A

PKB	Protein kinase B / Akt
PKC	Protein kinase C
PKG	Protein kinase G
PKK-1	6-phosphofructo-1-kinase
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenylmethylsulfonyl fluoride
PP1	Protein phosphatase type-1
PP2A	Protein phosphatase type-2A
PP2B	Protein phosphatase type-2B
PP2C	Protein phosphatase type-2C
PPase	Protein phosphatase
PVDF	Polyvinylidene difluoride membrane
ROS	Radical oxygen species
$S_{0.5}$	Half-saturation constant (substrate affinity constant)
SDS	Sodium dodecyl sulfate
TBS	Tris-buffered saline
TBST	TBS containing the detergent Tween-20
Tris	Tris(hydroxymethyl)aminomethane
V_{max}	Maximal enzyme velocity
β GP	B-glycerophosphate
β ME	B-mercaptoethanol

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CHAPTER 1

GENERAL INTRODUCTION

Freeze tolerance

Temperatures below 0°C can be rapidly lethal to organisms encountering them. For all its advantages, the human species cannot withstand prolonged and unprotected exposure to freezing conditions. Without a means of insulation, hypothermia quickly sets in, eventually followed by death. Some mammals are able to survive cold temperatures by mechanisms such as hibernation. This combines a coordinated decrease in core body temperature to near 0°C with strong metabolic rate depression, and allows animals to survive the cold temperatures and limited food availability over the winter months (Carey et al., 2003). The unique physiology of ectotherms allows some species to take a different approach to survival during the cold months. First reported almost 70 years ago, the concept of freeze-tolerance is such that some ectotherms can actually undergo whole body freezing; water in extracellular or extra-organ spaces freezes out as ice (Luyet and Gehenio, 1940; Ramløv, 2000; Packard and Packard, 2003; Wharton, 2003; Storey and Storey, 2004b; 2004c; Storey, 2006). Among vertebrates, for example, the survivable limit on ice accumulation can be as low as 21% in the Italian wall lizard, *Podarcis sicula* (Burke et al., 2002), 50% in the marsh frog, *Rana ridibunda* (Voituron et al., 2005), and as high as 65% in the wood frog, *Rana sylvatica* (Storey and Storey, 1992). Heart beat and respiration are undetectable, and anoxia and ischemia are persistent over the days, weeks or even months that animals remain frozen (Storey and Storey, 2004b). With the arrival of spring, these animals thaw and resume their normal life cycle, without any obvious injury or impaired function as a result of the months spent frozen.

Most freeze-tolerant animals have a common strategy for survival: cryoprotection. High concentrations of an osmolyte, typically a sugar or polyhydric alcohol (glycerol

being the most common), are synthesized and delivered in high intracellular concentrations to all tissues (Storey, 1997; Yancy, 2005). These osmolytes, known in the context of freeze tolerance as cryoprotectants, order intracellular water and limit or prevent two forms of damage i) intracellular freezing and any resulting physical damage to subcellular structures, and ii) cellular dehydration and extreme volume reduction caused by the osmotic outflow of cell water to join growing extracellular ice crystals. Most cryoprotectants are derived from carbohydrates and are synthesized from large stores of glycogen that most animals have in their liver (Storey, 1997). For example, freeze tolerant terrestrially-hibernating frogs typically synthesize glucose as a cryoprotectant (Storey and Storey, 1992; Layne, 1999; Steiner et al., 2000). Glucose is synthesized in three steps from glycogen: glycogen $\rightarrow$ glucose-1-phosphate $\rightarrow$ glucose-6-phosphate $\rightarrow$ glucose. Freeze-tolerant animals thus need to reorganize the regulation of carbohydrate metabolism to maintain high levels of cryoprotectants and ensure survival.

The adenylate energy charge and energetics of freeze tolerance

Hydrolysis of ATP, the universal energy currency of the cell, allows biochemical work to be done and is essential to survival. ATP hydrolysis produces ADP and if the rate of ATP use exceeds the rate of synthesis (e.g. during burst muscular work), ADP accumulates. The adenylate kinase reaction ($2 \text{ ADP} \rightarrow \text{ATP} + \text{AMP}$) allows some ATP to be recovered but leads to accumulation of AMP, the ultimate end product of ATP hydrolysis. AMP is an important signalling molecule capable of activating catabolic pathways (Brenman and Temple, 2007; Röckl et al., 2008). Derivatives of AMP including adenosine, inosine and IMP also have signalling functions.

Since ATP has the highest energy release upon hydrolysis (Gajewski et al., 1986), it is necessary to maintain high levels of ATP relative to the other two adenylates. Decreasing levels of ATP and increasing levels of ADP and AMP indicate a reduced cellular energy state. The ratio of ATP and half of ADP, to the total adenylate pool, is known as the adenylate energy charge (AEC) (Pradet and Raymond, 1983):

$$\text{AEC} = \frac{[\text{ATP}] + \frac{1}{2} [\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

When ATP levels drop and the AEC declines, metabolism is reordered to raise the AEC. Catabolic pathways are activated and stored fuels, such as glycogen and triglycerides, are consumed through glycolysis, fatty acid oxidation, and ultimately the citric acid cycle and oxidative phosphorylation to synthesize ATP. There are other ways as well to raise the AEC. Stored phosphagens such as phosphocreatine are mobilized to transfer high-energy phosphate groups to ADP, replenishing ATP (Wallimann et al., 1992). Adenylate kinase can also restore some ATP while maintaining a constant-sized adenylate pool but at the expense of AMP accumulation which affects the AEC (Atkinson, 1968). If all of these options are exhausted, a high AEC can also be achieved by draining AMP from the adenylate pool by reactions such as AMP deaminase. Although this does not restore [ATP], it elevates the relative amount of ATP and effectively raises the AEC (Chapman and Atkinson, 1973).

In response to freezing, most physiological and biochemical processes are strongly suppressed or even turned off. ATP-dependent ion-motive forces are reduced (Hemmings and Storey, 2001). Gene transcription and protein synthesis are virtually halted (Storey and Storey, 2004c). Exceptions to this are genes and proteins that are part of common metabolism and promote survival, as well as some that are unique to

individual animals (Cai et al., 1997; Cai and Storey, 1997a; 1997b; Churchill and Storey, 1994; McNally et al., 2002; 2003; Storey et al., 1992; 1997; Wolanczyk et al., 1990). However, any crucial transcription and translation that takes place during freezing will be a drain on cellular energetics. Any enzyme catalytic activity that is ATP-dependent will also drain that resource. Although overall energetic demands are greatly lowered, some demand will still be present in the frozen state. In addition, there will be a high energy demand upon thawing associated with restarting physiological functions (e.g. heart beat, breathing), reversing waste accumulation or cellular damage done during freezing, and dealing with issues such as a rapid increase in oxygen free radical generation associated with the resumption of oxygen perfusion of tissues (Joannis and Storey, 1997). The result is a potentially severe energy challenge presented to the frozen and/or thawing animal. With undetectable respiration and no oxygen intake, all metabolism in the frozen state is limited to anaerobic pathways (Storey and Storey, 1986; Storey, 1987b). Fatty acid oxidation, the citric acid cycle, and oxidative phosphorylation cannot function in the frozen state. Animals must therefore rely exclusively on anaerobic glycolysis to satisfy energetic means. However, one must also consider the need to maintain cryoprotectant pools, which are carbohydrates or carbohydrate derivatives, in the frozen state. This restricts the availability of substrates for even anaerobic glycolysis.

Reversible protein phosphorylation

The large-scale changes in metabolic rate and the reprioritization of ATP use by different cell functions during freezing implies that pathway flux must be regulated at key control points. Metabolism can be regulated by coarse control by altering mRNA or

protein levels. However, this is a wasteful process when energy levels are at a premium. The regulation of an existing pool of enzymes/proteins, without altering synthesis or degradation, is an economical mechanism for shutting off (or activating) metabolic pathways. Enzyme activity is often effectively regulated by signal transduction pathways and post-translational modification (MacDonald, 2004), the most common modification being reversible protein phosphorylation (Pawson and Scott, 2005).

Reversible phosphorylation is already known to participate in biochemical regulation during freezing, studies to date having focused mainly on enzymes of carbohydrate metabolism. Enzymes controlled in this manner include glycogen phosphorylase, phosphofructokinase-1 and phosphofructokinase-2 in wood frogs (Crerar et al., 1988; Vazquez Illanes and Storey, 1993), pyruvate kinase in the marine periwinkle, *Littorina littorea* (Russell and Storey, 1995) and the sodium-potassium ATPase in larvae of the gall fly, *Eurosta solidaginis* (McMullen and Storey, 2008).

Model organism: the freeze-tolerant wood frog, *Rana sylvatica*

Since the first modern report of freezing survival by frogs 26 years ago (Schmid, 1982), the freeze-tolerant wood frog has been the subject of intense scrutiny and study. As winter approaches, these frogs nestle under the layer of dead leaves on the forest floor and are eventually covered by snowfall. This insulation protects the frog against the brute harshness of winter, but the frogs can nevertheless encounter subzero temperatures for weeks or months at a time. In damp surroundings, the frogs will freeze whenever temperature drops below about -0.5°C whereas under drier conditions they can cool to -2 to -3°C before nucleating. The frogs can endure the freezing of 65-70% of total body

water as extracellular ice and, while frozen, show no respiration, heart beat, or brain activity. As a consequence of the interruption of breathing and heart beat, the frogs experience anoxia and ischemia throughout the freeze followed by oxidative stress when oxygen is reperused during thawing (Storey and Storey, 2004b; 2004c). Wood frogs use glucose as their natural cryoprotectant; levels as high as 200-300 mM accumulate in core organs (control values are ~5 mM) as a result of glucose synthesis from liver glycogen (Storey and Storey, 1985; 1986; Storey, 1987a, Crerar et al., 1988).

Wood frogs are known to activate a suite of genes and proteins that enable to survive the freeze/thaw process (Cai et al., 1997; Cai and Storey, 1997a; 1997b; Churchill and Storey, 1994; McNally et al., 2002; 2003; Storey et al., 1992; 1997; Wolanczyk et al., 1990). Energy metabolism is based on anaerobic carbohydrate catabolism with L-lactate and L-alanine accumulating as end products but with extended freezing times ATP levels drop and the AEC begins to decline in liver (and likely other core organs as well) (Storey and Storey, 1984; Storey, 1987b). At least over the short term (up to 2 weeks frozen), this decline was not seen in skeletal muscle, probably due to the consumption of creatine phosphate reserves but energetics could be compromised with longer freezing once phosphagen is consumed.

The focus of this study is the enzymatic regulatory mechanisms that allow for a delicate balance of energy production, glucose homeostasis, and antioxidant defence in the freeze-tolerant wood frog, *Rana sylvatica*.

Hypotheses and course of study

The wood frog must survive the multiples stresses imposed on it by freezing.

Coordinated regulatory mechanisms are needed for global responses as well as enzyme-specific mechanisms that attune individual enzymes/pathways to the functions needed in the frozen state. I have made the following predictions about the research to come:

- i) A dominant need that must be satisfied is the maintenance of high levels of cryoprotectant. Thus, metabolic pathways will be regulated in such a way that promotes the preservation of large pools of free glucose. Hypothesis: Hexokinase, the enzyme that gates glucose entry into metabolic pathways, is closely regulated to inhibit the consumption of cryoprotectant in the frozen state.
- ii) To maintain long term viability while frozen, organ ATP levels and the AEC must be supported. This may include regulated control of ATP production from energy reserves and adjustments to the adenylate pool size to sustain a high AEC. Hypothesis: Regulatory controls on creatine kinase gate the use of creatine phosphate reserves to support ATP levels in muscle of frozen frogs. Hypothesis: Control mechanisms acting on AMP deaminase serve to adjust adenylate pool size in frozen tissue by regulating AMP catabolism.
- iii) Frogs show no sign of damage from the oxidative stresses encountered during freezing and thawing. Antioxidant levels appear to be kept high throughout the freezing process and, critical to this, is the provision of NADPH reducing power. Hypothesis: Glucose-6-phosphate dehydrogenase, that gates entry in the pentose phosphate pathway, is subject to freeze-specific controls to regulate NADPH production in the

frozen state.

- iv) In line with a need to minimize energy use in the frozen state, control mechanisms that modify the activities or properties of existing enzymes should be favoured over changes in enzyme levels brought about by protein synthesis or degradation. Hypothesis: Reversible protein phosphorylation, due to protein kinases and protein phosphatases, has a central role in enzyme regulation during freeze/thaw transitions.

Studies on four enzymes are presented in this thesis in order to examine the regulation of metabolism and the crosstalk between cellular energetics, glucose homeostasis, and antioxidant defence.

5'-Adenosine monophosphate deaminase (AMPD; EC 3.5.4.6) is a catabolic enzyme and part of adenylate degradation. It irreversibly deaminates AMP to inosine monophosphate, thereby removing AMP from the adenylate pool, and decreasing the overall size of the pool. Chapter 2 presents a study of skeletal muscle AMPD examining multiple mechanisms of enzyme control to determine how the enzyme regulates AMP catabolism during freezing-induced energy stress.

Creatine kinase (CK; EC 2.7.3.2) catalyzes the interconversion of ADP and PCr with ATP and Cr, allowing metabolism to draw upon high energy phosphagen reserves to support ATP levels in skeletal muscle. This enzyme is best known for its role in supporting ATP levels during the first few seconds of burst exercise in muscle before anaerobic glycolysis is fully activated. Chapter 3 presents a study of frog skeletal muscle CK evaluating multiple potential regulatory mechanisms to determine how the enzyme is controlled in the frozen state.

Glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.49) irreversibly oxidizes G6P to 6-phosphoglucono- δ -lactone, as the first step of the pentose phosphate pathway, and in doing so, produces NADPH. This pathway is the main supplier of NADPH reducing power for reductive biosynthesis, xenobiotic metabolism, and antioxidant defence in cells. However, G6PDH uses G6P derived from the hydrolysis of glycogen, and thereby competes for G6P with the pathway of glucose cryoprotectant synthesis. Chapter 4 evaluates G6PDH regulation in wood frog liver and in particular analyzes a newly recognized mechanism of G6PDH regulation, reversible protein phosphorylation.

Hexokinase (HK; EC 2.7.1.1) irreversibly phosphorylates glucose to G6P, rendering it incapable of crossing the cell membrane, and priming it for several possible fates in cellular metabolism. G6P can be converted to UDP-glucose and polymerized into glycogen. It can also be shunted through the pentose phosphate pathway to generate NADPH and precursors for nucleotide biosynthesis or catabolised by glycolysis and the Krebs cycle to generate ATP. However, in frozen frogs, HK should predictably be inhibited to prevent draining of the free glucose pool that provides cryoprotection. Chapter 5 explores the kinetic and regulatory properties of wood frog muscle HK.

CHAPTER 2

REGULATION OF 5'-ADENOSINE MONOPHOSPHATE DEAMINASE IN THE FREEZE-TOLERANT WOOD FROG, *RANA* *SYLVATICA*

INTRODUCTION

5'-Adenosine monophosphate deaminase (AMPD; EC 3.5.4.6) catalyzes the irreversible hydrolysis of AMP to produce inosine monophosphate (IMP) and ammonium ion (NH_4^+). AMPD is distributed in animal tissues, but skeletal muscle has the highest activity (Lowenstein, 1972). AMPD removes AMP from the adenylate pool (Hancock et al., 2006), and this action can lead to either a temporary drain of AMP (with IMP accumulating) or to a more permanent change if IMP is channeled into catabolic pathways leading ultimately to uric acid excretion. In skeletal muscle, the enzyme is well-known to be key to stabilizing the AEC during high-intensity muscle work in a range of vertebrate species (Rundell et al., 1992b; Rundell et al., 1993; Lushchak and Storey, 1994; Lushchak et al., 1997; Hellsten et al., 1999; Krause and Wegener, 1996; Fishbein et al., 1993). The regulation of AMPD is complex and includes activation or inhibition by ions and metabolite effectors (Raffin, 1984; Ashby and Frieden, 1978; Merkler and Schramm, 1990), reversible binding to myosin (Rundell et al., 1992b), oligomerization (Szydlowska et al., 2004), and covalent modification (Thakkar et al., 1993). Moreover, regulation by one of these factors can influence subsequent control by others. For example, binding of AMPD to myosin subunits alleviates allosteric inhibition of the enzyme from some sources, in addition to influencing substrate affinity and activity (Ashby and Frieden, 1998). Phosphorylation of AMPD, in turn, may either influence or succeed binding, and has not yet been clearly elucidated (Lushchak and Storey, 1994).

The freezing of 65-70% of total body water in extracellular and extra-organ spaces in the freeze-tolerant wood frog, *Rana sylvatica*, halts heart beat and breathing and

rapidly leads to conditions of anoxia and ischemia (Storey and Storey, 2004b; Hermes-Lima and Zenteno-Savin, 2002). These conditions place energy stress on cells and over time the tissues of frozen frogs build up glycolytic end products (lactate, alanine) and deplete ATP (Storey and Storey, 1986). This reduces the Adenylate Energy Charge (AEC): $AEC = ([ATP] + 0.5[ADP]) / ([ATP] + [ADP] + [AMP])$. However, viability is aided if AEC can be maintained at a high value for as long as possible and so mechanisms are in place, particularly in skeletal muscle, to stabilize the AEC. The action of adenylate kinase ($2 ADP \leftrightarrow ATP + AMP$) allows some ATP to be resynthesized from ADP as it accumulates but this elevates [AMP]. However, if AMP is removed from the adenylate pool, the AEC ratio can be kept at a high value at the expense of an overall reduction in adenylate pool size. This mechanism is commonly seen in working skeletal muscle and also under a variety of environmental stress conditions that compromise energy reserves such as hypoxia, ischemia (Lushchak et al., 1998b; Hohl, 1999), freezing (Storey and Storey, 1986) or during torpor in hibernating mammals (English and Storey, 2000).

The present study examines the regulation of AMPD in wood frog skeletal muscle. The enzyme from control (5°C acclimated) and frozen animals was compared to look for freeze-responsive changes in enzyme activity, kinetic parameters, phosphorylation state, temperature effects, and response to low molecular weight effectors.

MATERIALS AND METHODS

Animals and Biochemicals

Male wood frogs were captured from spring breeding ponds in the Ottawa area. Animals were washed in a tetracycline bath, and placed in plastic containers with damp sphagnum moss at 5°C for two weeks prior to experimentation. Control frogs were sampled directly from this condition. For freezing exposure, frogs were placed in closed plastic boxes with damp paper toweling on the bottom, and put in an incubator set at -3°C. A 45 min cooling period was allowed during which the body temperature of the frogs cools to below -0.5 °C (the equilibrium freezing point of wood frog body fluids) and nucleation is triggered due to skin contact with ice crystals formed on the paper toweling (Storey and Storey, 1985). Subsequently, timing of a 24 h freeze exposure began. Both control and experimental frogs were sacrificed by pithing, followed by rapid dissection, and freezing of tissue samples in liquid nitrogen. Frozen tissues were stored at -80°C until use. Conditions for animal care, experimentation, and euthanasia were approved by the Carleton University Animal Care Committee in accordance with guidelines set down by the Canadian Council on Animal Care. Biochemicals and coupling enzymes were purchased from Sigma Chemical Co. (St. Louis, MO) or Boehringer-Mannheim (Montreal, PQ).

Preparation of tissue extracts

Samples of frozen skeletal muscle were homogenized 1:5 w:v in ice-cold buffer A to prevent *in vitro* phosphorylation changes (50 mM MOPS, 2 mM EDTA, 2 mM EGTA, 50 mM β -glycerophosphate, 10 mM β -mercaptoethanol, pH 7.0), with a few crystals of the protease inhibitor, phenylmethylsulfonyl fluoride, added immediately prior to homogenization with a Polytron homogenizer at 50% of full power. Metal ion chelators,

EDTA and EGTA, prevent protein kinase and phosphatase activation, and β GP is a competitive inhibitor of protein phosphatases. Homogenates were centrifuged in a Hermle Z 360 K centrifuge for 30 min at 10,000 x g and the supernatant was removed.

In cases where ion or metabolite effects on AMPD were being investigated, crude extracts were then centrifuged through small columns of Sephadex G-50 to remove endogenous enzyme effectors. To do this, 5 ml syringe barrels were plugged with glass wool and packed with Sephadex G-50 equilibrated in buffer A. Packed syringes were centrifuged in a bench-top IEC clinical centrifuge at full power for 1 min and eluant was discarded. A 500 μ l aliquot of supernatant was then layered onto the column followed by a second centrifugation as above. The eluant was collected and stored on ice.

For experiments where bound AMPD activity in the pellet was assessed, the pellet from the original homogenate was resuspended in a volume of buffer A equal to that used for the original homogenization. The resuspension was then centrifuged at 10,000 x g for 30 min and the supernatant was decanted. The procedure was repeated twice more and then the final pellet was resuspended in homogenization buffer, and used for assay.

Enzyme assay

Enzyme activity was assayed according to the method of Chapman and Atkinson (Chapman and Atkinson, 1973) in a 200 μ l final volume using a Thermo Labsystems Multiskan Spectrum microplate reader. Standard assays were conducted at 25°C. Optimal assay conditions for AMPD were 50 mM MOPS buffer (pH 7.2), 5.0 mM AMP, and 20 μ l of enzyme extract, monitored at 285 nm. One unit of enzyme activity is defined as the amount that produced 1 μ mol of IMP per minute at 25°C. For assays of bound AMPD, in

which a resuspension of the pellet was assayed, a glutamate dehydrogenase (GDH)-coupled assay was used. Optimal conditions were 50 mM MOPS buffer (pH 7.2), 5.0 mM AMP, 0.15 mM NADH, 7.5 mM α -ketoglutarate, 1 unit of GDH and 10 μ l of resuspended pellet, monitored at 340 nm. One unit of enzyme activity is defined as the amount that consumed 1 μ mol of NADH per minute at 25 °C.

In vitro incubations to stimulate endogenous kinases and phosphatases

To assess the effect of reversible phosphorylation on the bound form of AMPD, incubations were prepared under conditions that stimulated the activities of endogenous protein kinases or protein phosphatases and the resulting effects on AMPD were assayed. Pellets containing bound AMPD were prepared and washed as above and then resuspended in 50 mM MOPS, pH 7.2, and 10 mM β -mercaptoethanol, aliquoted into different tubes, and incubated under the following conditions, in a final volume of 1 mL:

- (A) *“Stop” conditions:* 50 mM β -glycerophosphate, 2 mM EDTA and 2 mM EGTA to inhibit both protein kinase and phosphatase activities.
- (B) *Stimulation of endogenous protein kinases:* 5 mM Mg-ATP, 50 mM β -glycerophosphate and either (1) 1 mM cAMP to stimulate PKA; (2) 1 mM cGMP to stimulate PKG; (3) 1.3 mM CaCl_2 + 7 μ g/mL phorbol myristate acetate to stimulate PKC; (4) 1 mM AMP to stimulate AMPK; (5) 1 U of calmodulin + 1.3 mM CaCl_2 to stimulate CaMK; (6) all of these components to stimulate all kinases together.
- (C) *Stimulation of endogenous protein phosphatases:* 5 mM MgCl_2 and 5 mM CaCl_2 to stimulate all protein phosphatases.

Samples were incubated for 4 hours at 4°C. Incubations were then centrifuged at

10,000 x g for 30 minutes, and the supernatant was discarded. The pellet was resuspended in 1 mL of buffer A, used for the original tissue homogenization. The resuspension was then centrifuged at 10,000 x g for 30 min and the supernatant was decanted. The procedure was repeated twice more (to ensure removal of all low molecular weight effectors) and then the pellet was resuspended in homogenization buffer, and used for assay.

Kinetic parameters

Enzyme kinetic constants were determined using a nonlinear least-squares regression computer program (Brooks, 1992); substrate affinity data were fitted to Hill plots. See also Appendix for additional information.

RESULTS

Activity and kinetic parameters of AMPD in crude extracts of skeletal muscle

AMPD is known to bind to myosin and a portion of total activity remains bound in the pellet after normal homogenization and centrifugation. The total activity of AMPD in wood frog muscle was 22-28 U/gww and the majority of activity was in the soluble fraction (Table 2.1). The percentage of AMPD in the bound form was 20.3 ± 0.13 % in control frogs and increased significantly to 35.4 ± 0.09 % in muscle from frozen frogs. Free AMPD showed a sigmoidal relationship between velocity and [AMP] with values for the Hill coefficient (n_H) that were 1.76 ± 0.06 in muscle extracts from control frogs and 2.76 ± 0.10 in frozen frogs (Table 2.2). By contrast, bound AMPD displayed velocity versus substrate concentration profiles that were essentially hyperbolic (n_H values of

1.03-1.12). Affinity of free AMPD for its substrate did not change between control and frozen states. However, $S_{0.5}$ AMP of the bound enzyme was both significantly lower than the corresponding value for the free enzyme and changed between control and frozen states, $S_{0.5}$ AMP of bound AMPD from muscle of frozen frogs being 3-fold higher than the value for control frogs. Oligomeric states of AMPD are not known, however, AMPD is known to exist in various oligomeric states from dimers to octomers (Szydlowska et al., 2004)

Endogenous effectors of AMPD

Soluble fractions of crude muscle extracts were centrifuged through small columns of Sephadex G-50 to remove ions and low molecular weight metabolites present in the crude extract. Desalting reduced $S_{0.5}$ values for AMP by 23-35%, but values for the enzyme from control and frozen frogs remained similar (Table 2.3). The n_H value for AMPD from control frogs also increased significantly but was similar to the value for frozen frogs.

Allosteric effectors of AMPD

Sephadex G-50 filtered AMPD was assayed in the presence of potential effectors, to determine if these would affect activity. ATP only activated the free (soluble) form of AMPD, whereas ADP activated both free and bound enzyme forms (Table 2.4). The K_a ATP decreased significantly by 69% for AMPD from muscle of frozen frogs, compared with the value for control frogs. ATP activated AMPD from control frogs by 1.53-fold, whereas in frozen frogs, the fold activation was 2.69. Similarly, the K_a ADP for both free

and bound forms of AMPD was lower than the corresponding values for the enzyme from control frogs. The K_a ADP for bound AMPD in control frogs was significantly higher than the corresponding free value for the enzyme from either control or frozen frogs. ADP activated free AMPD in control frogs by 2.17-fold, whereas in frozen frogs, this increased to 5-fold. A similar increase was noted for the bound forms of AMPD. ADP activated bound AMPD in control frogs by 1.25-fold and this increased to 3.42-fold in frozen frogs.

GTP was a very strong inhibitor with I_{50} values of just 0.08-0.15 mM (Table 2.5). I_{50} GTP of free AMPD was about 50% lower for the enzyme from frozen frogs but the value for bound AMPD was unaffected by the transition to the frozen state. IMP is the product of the AMPD reaction. IMP inhibited bound AMPD from muscle of frozen frogs with an I_{50} of 0.23 ± 0.02 mM but interestingly, did not inhibit the enzyme in any other situation at the IMP concentrations tested (up to 10 mM). Salts also inhibited AMPD; all three salts tested had Cl^- as the anion so differences in inhibitory effects are due to cation effects. Na^+ and K^+ were very weak inhibitors of free AMPD with I_{50} values in the range of 650-970 mM but both cations had much stronger effects on bound AMPD with I_{50} values between 130-160 mM. NH_4^+ , another product of the AMPD reaction, inhibited the free enzyme with I_{50} values in the 250-275 mM range. The effects of NH_4^+ on the bound enzyme could not be tested because NH_4^+ is the product being measured by the GDH-coupled assay used for the bound enzyme.

Temperature and cryoprotectant effects

AMPD activity was assayed in the presence versus absence of glucose, the

cryoprotectant that is present in high intracellular concentrations in tissues of frozen frogs. Activity was also assessed at high (25°C) and low (5°C) temperatures. Addition of high glucose (250 mM) significantly decreased the $S_{0.5}$ AMP for the enzyme from both control and frozen frogs when assayed at high temperature, the effect being particularly strong (84% reduction) for the enzyme from frozen frogs (Table 2.6). In contrast, $S_{0.5}$ AMP increased significantly when the enzyme was assayed at a cold temperature (5°C), close to the freezing temperatures when frogs would naturally experience high glucose. The combined effects of cold temperature and high glucose together resulted in 1.9- and 1.4-fold increases in $S_{0.5}$ AMP for the enzyme from control and frozen frogs, respectively. The $S_{0.5}$ AMP for AMPD from frozen frogs under these conditions was still markedly higher (by 2.5-fold) than the corresponding value for the enzyme from control frogs.

Reversible phosphorylation

Many enzymes are targets of covalent modification by protein kinases and protein phosphatases. To determine whether reversible phosphorylation affected bound AMPD, incubations were set up in which enzyme phosphorylation or dephosphorylation was promoted by stimulating the activities of endogenous protein kinases and phosphatases. Incubations that stimulated all protein kinases resulted in a significant 2-fold increase in $S_{0.5}$ AMP of bound AMPD from muscle of control frogs (Figure 2.1). Similarly, $S_{0.5}$ AMP of control AMPD increased significantly when the activities of PKA, PKC, CaMK, or AMPK (but not PKG) were stimulated individually. However, stimulation of these protein kinases had no effect on the substrate affinity of AMPD from muscle of frozen

frogs.

When incubations stimulated protein phosphatase activities instead the opposite effect was seen. The $S_{0.5}$ AMP of muscle AMPD from frozen frogs decreased significantly by 49 % after stimulation of phosphatase activities. Conversely, stimulation of protein phosphatases had no noticeable effect on $S_{0.5}$ AMP of the enzyme from control frogs.

Table 2.1 AMPD maximal activity (V_{max}) and partitioning between free and bound forms in skeletal muscle from control (5°C-acclimated) and 24 h frozen frogs.

	V_{max} (U/gww)	% of total activity
Control		
Free AMPD	17.6 ± 1.53	79.7 ± 6.46
Bound AMPD	4.5 ± 0.03	20.3 ± 0.13
Total	22.1 ± 1.56	100.0
Frozen		
Free AMPD	17.8 ± 0.69	64.6 ± 2.07^a
Bound AMPD	9.8 ± 0.03^a	35.4 ± 0.09^a
Total	27.6 ± 0.72^a	100.0

Data are expressed as units per gram wet weight (U/gww), means $\pm$ SEM, for determinations on muscle preparations from $n = 8$ individual frogs. ^a Significantly different from the corresponding value in control muscle by the Student's *t*-test, $P < 0.05$; percentage data were evaluated after arcsine $\sqrt{}$ transformation of the raw data.

Table 2.2 Kinetic parameters of AMPD in crude extracts of skeletal muscle from control (5°C-acclimated) and 24 h frozen wood frogs.

	$S_{0.5}$ AMP (mM)	n_H
Control		
Free AMPD	2.16 ± 0.11^b	1.76 ± 0.06^{bc}
Bound AMPD	0.46 ± 0.03^{ac}	1.03 ± 0.07^{ac}
Frozen		
Free AMPD	2.19 ± 0.15^b	2.76 ± 0.10^{ab}
Bound AMPD	1.50 ± 0.19^{abc}	1.12 ± 0.09^a

Data are means $\pm$ SEM, for determinations on muscle preparations from $n = 4$ individual frogs $S_{0.5}$ is the substrate concentration that produces half-maximal enzyme velocity; n_H is the Hill coefficient. Significantly different from ^a the corresponding value for free AMPD from control frogs, ^b the corresponding value for bound AMPD from control frogs, or ^c the corresponding value for free AMPD from frozen frogs using the Student's t test, $P < 0.05$.

Table 2.3 Kinetic parameters for free AMPD before and after removal of low molecular weight ions and metabolites by centrifugation through Sephadex G50 columns

	Before spun columns		After spun columns	
	$S_{0.5}$ AMP (mM)	n_H	$S_{0.5}$ AMP (mM)	n_H
Control	2.16 ± 0.11	1.76 ± 0.06	1.35 ± 0.08^a	2.37 ± 0.17^a
Frozen	2.19 ± 0.15	2.76 ± 0.10^a	1.68 ± 0.21^a	2.76 ± 0.27

Data are means $\pm$ SEM, for determinations on muscle preparations from $n = 4$ individual frogs. ^a - Significantly different from the corresponding free control value prior to desalting by the Student's t -test, $P < 0.05$.

Table 2.4 Adenylate activation of wood frog skeletal muscle AMPD from control versus frozen frogs

	Control		Frozen	
	Free AMPD	Bound AMPD	Free AMPD	Bound AMPD
K_a Mg·ATP, mM	1.90 ± 0.03	NE	0.63 ± 0.04^a	NE
Mg·ATP fold-activation	1.53 ± 0.09	NE	2.69 ± 0.20^a	NE
K_a Mg·ADP, mM	1.12 ± 0.15^b	3.75 ± 0.37	0.54 ± 0.02^{ab}	1.14 ± 0.10^a
Mg·ADP fold-activation	2.17 ± 0.29	1.25 ± 0.21	5.01 ± 0.69^a	3.42 ± 0.24^a

Data are means $\pm$ SEM, for determinations on Sephadex G50-filtered muscle extracts from $n = 4$ individual frogs. Significantly different from ^a the corresponding value in control frogs, ^b the corresponding value for bound AMPD in control frogs, by the Student's *t*-test, $P < 0.05$. NE, no effect. K_a is the activator concentration that produces half-maximal activation; values were determined at 0.5 mM AMP. K_a and fold activation values were calculated using a nonlinear least-squares regression kinetics program (Brooks, 1992).

Table 2.5 Inhibitor effects (I_{50} values) on AMPD from skeletal muscle of control versus frozen wood frogs

	Control		Frozen	
	Free AMPD	Bound AMPD	Free AMPD	Bound AMPD
Mg·GTP, mM	0.15 ± 0.02	0.08 ± 0.01	0.08 ± 0.01^a	0.10 ± 0.01
IMP, mM	NE	NE	NE	0.23 ± 0.02^a
KCl, mM	660 ± 44	148 ± 34	702 ± 21^a	162 ± 24
NaCl, mM	973 ± 20	141 ± 31	824 ± 26^a	133 ± 21
NH ₄ Cl, mM	276 ± 5.0	ND	254 ± 10	ND

Data are means $\pm$ SEM, for determinations on Sephadex G50-filtered muscle extracts from $n = 4$ individual frogs. I_{50} is the inhibitor concentration that reduces enzyme velocity by 50%. ^a - Significantly different from the corresponding value for muscle from control frogs as assessed by the Student's t -test, $P < 0.05$. NE, no effect. ND, not determined. I_{50} values were measured at an AMP concentration of 0.5 mM.

Table 2.6 Effects of assay temperature and glucose on the $S_{0.5}$ AMP for bound AMPD from skeletal muscle of control versus frozen wood frogs.

	$S_{0.5}$ AMP (mM)	
	Control	Frozen
25°C, no glucose	0.46 ± 0.03	1.50 ± 0.19
25°C, 250 mM glucose	0.30 ± 0.04^a	0.24 ± 0.03^a
5°C, no glucose	0.70 ± 0.03^a	1.77 ± 0.03^a
5°C, 250 mM glucose	0.87 ± 0.04^a	2.14 ± 0.04^a

Data are means $\pm$ SEM, for determinations on Sephadex G50-filtered muscle extracts from $n = 4$ individual frogs. ^a - Significantly different from the corresponding value at 25°C without glucose as assessed by the Student's t -test, $P < 0.05$.

Figure 2.1 Effect of reversible phosphorylation on the substrate affinity for AMP of bound AMPD from skeletal muscle of control (black bars) and frozen (gray bars) wood frogs. Data are means $\pm$ SEM, $n = 4$ determinations on separate preparations of enzyme. Stars indicate kinetic parameters that are significantly different from the corresponding value for incubations in stop buffer as assessed by the Student's t test, $P < 0.05$.

Discussion

Freezing imposes long term ischemia upon wood frog organs and while frozen, organ ATP levels decline and so can the AEC (Storey and Storey, 1986). Freeze-thaw cycles accelerate the decline in AEC, and it can be expected that several freeze-thaw cycles must be endured over a typical winter (Storey, 1987b). Studies of wood frog liver and leg muscle showed decreasing [ATP] in both organs during freezing but whereas a noticeable drop in AEC occurred in liver, no appreciable change was noted in leg muscle over several freeze-thaw cycles. This implies that skeletal muscle has a metabolic strategy in place to support AEC at high values for as long as possible (Storey, 1987b). This strategy appears to involve the action of AMPD in draining AMP from the muscle adenylate pool and hence, the present study addressed the regulation of skeletal muscle AMPD, comparing enzyme properties from unfrozen versus frozen wood frogs.

An initial analysis of the maximum activity (V_{max}) and apparent substrate affinity ($S_{0.5}$) of wood frog AMPD in soluble extracts of skeletal muscle found no change in these enzyme parameters between control and frozen states (Tables 2.1 and 2.2). Removal of endogenous low molecular weight effectors using Sephadex G50 spun columns reduced the $S_{0.5}$ for AMP of the free enzyme from both control and frozen muscle by similar amounts and altered the n_H value for control AMPD (Table 2.3). This indicates that one or more endogenous inhibitors (e.g. GTP) are present in crude extracts. Further analysis of the responses by free AMPD to activators and inhibitors showed differences between the enzyme from control and frozen states (Tables 2.4 and 2.5). The enzyme from muscle of frozen frogs had significantly lower K_a values for ATP and ADP and the I_{50} GTP was also reduced by about 50%. Overall, then free AMPD in muscle of frozen frogs was more

sensitive to high energy phosphates than in the control state. Given that the levels of these high energy phosphates also fall *in vivo* in muscle during freezing, these changes in the properties of the enzyme could possibly maintain the relative sensitivity of the enzyme to regulatory control by high energy phosphates in the frozen state.

A comparison of bound AMPD from muscle of control versus frozen frogs showed that control AMPD had much higher affinity for AMP than did the enzyme from frozen muscle (Table 2.2). However, bound AMPD from frozen frogs had a lower K_a ADP (just 30% of the value for control frogs) which would increase bound AMPD sensitivity to the product of ATP hydrolysis in frozen muscle and potentially link it more closely to muscle energetics. Interestingly, IMP exclusively inhibited the bound form of AMPD from frozen frogs, but did not inhibit free forms of the enzyme, or bound AMPD in control frogs. The unique inhibitory effect of IMP on bound AMPD from frozen frogs suggests that this form of the enzyme exists in a distinct state, where it can be tightly controlled by key metabolites (adenylates, GTP, IMP) that reflect the energy status of skeletal muscle while frozen.

Binding to myosin is a major regulatory feature of AMPD that has been extensively explored (Rundell et al., 1992b; Rundell et al., 1993; Lushchak and Storey, 1994; Lushchak et al., 1997; Ashby and Frieden, 1978; Rundell et al., 1992a). The amount of AMPD that was bound in wood frog muscle increased from 20% in control frogs to 35% in frozen animals suggesting that a greater association of AMPD with myofibrils benefited metabolism in frozen muscle. This response may be triggered by the anoxic/ischemic conditions that accrue as freezing progresses. Low oxygen stress is known to enhance AMPD binding in muscle from other species (Lushchak et al., 1998b).

Indeed, Rundell et al. (Rundell et al., 1992a) reported that muscle AMPD remained bound as long as ischemic conditions persisted. Bound AMPD had a greater affinity for AMP substrate than free AMPD in both control and frozen frogs and bound AMPD also showed hyperbolic AMP kinetics compared with the sigmoidal relationship seen for free AMPD. Bound AMPD was less sensitive to ADP activation than was its comparable free form but was much more strongly inhibited by Na^+ and K^+ ions in both control and frozen frogs.

Not only is AMPD distributed between free and bound states but myofibril-bound AMPD is also subject to covalent modification by protein kinases and phosphatases (Lushchak and Storey, 1994). Indeed, cardiac AMPD was phosphorylated by at least one major protein kinase, PKC (Thakkar, 1993). Protein kinases and phosphatases, although often soluble, can be compartmentally restricted through anchoring and scaffolding proteins, allowing them access to phosphorylate and dephosphorylate bound enzymes such as AMPD (Lester and Scott, 1997; Coghlan et al., 1995). To determine whether wood frog muscle AMPD was subject to reversible phosphorylation, preparations of bound AMPD from control frogs were incubated under conditions that stimulated the action of endogenous protein kinases (PKA, PKC, CaMK, or AMPK). After protein kinase treatment, the enzyme consistently showed reduced affinity for AMP ($S_{0.5}$ increased by about 2-fold) (Fig. 2.1). Conversely, conditions that promoted the dephosphorylation of bound AMPD from frozen frogs caused an increase in AMP affinity ($S_{0.5}$ decreased by about half). This implies that wood frog muscle AMPD is regulated by reversible phosphorylation when bound, and that in the transition from the control to frozen states, the phosphorylation state is modified. The data are consistent

with a low phosphate form of bound AMPD in control frogs and a high phosphate form in frozen frogs and with one of the actions of phosphorylation being to reduce the affinity of the bound enzyme for AMP. Enhanced phosphorylation of bound AMPD may also be responsible for other changes in the kinetic properties of the bound enzyme from frozen muscle such as the change in sensitivity to ADP activation, the unique inhibition by IMP, and the response of the enzyme to high glucose.

Freezing alters cytosolic conditions in frog cells, in particular by greatly elevating the concentrations of glucose which acts as a cryoprotectant, preventing cytosolic freezing. Hence, effects of high glucose on enzymes need to be considered as well as potential interactions between cryoprotectants and temperature. High concentrations of glucose increased the substrate affinity for AMP of bound AMPD when the enzyme was assayed at 25°C but had relatively little effect on $S_{0.5}$ at low assay temperatures. By contrast, low temperature assay at 5°C decreased AMP substrate affinity of the enzyme from both control and frozen frogs. Overall, the additive effects of high glucose and low temperatures led to a substantial reduction in AMPD affinity for its substrate compared with the situation at high temperature without glucose with increases in $S_{0.5}$ AMP of 2-fold for the bound enzyme from control frogs and ~25% for AMPD from frozen frogs. Interestingly, both of these factors coupled together led to a combined decrease in substrate affinity of AMPD. Hence, both temperature change and high glucose could influence the function of AMPD between unfrozen and frozen states.

Many previous studies have focused on AMPD in working skeletal muscle and found that the enzyme is more active and more bound under exercising conditions. By contrast, the present study suggests that in the frozen state, bound AMPD is less active

with reduced substrate affinity. This agrees with a study on AMPD in muscle of hibernating mammals that found that substrate affinity was reduced at low temperatures in the torpid state (English and Storey, 2000). AMPD was also inhibited in rabbit heart experiencing hypoxia (Gustafson et al., 1999). In that study, AMP deamination was high early in the ischemic period, and this served to preserve the AEC. After prolonged ischemia, however, AMPD was stably inhibited and IMP accumulation was implicated in AMPD inhibition. Hence, it can be recognized that AMPD is involved in two forms of energy stress in muscles – that caused by burst muscle exercise (e.g. active frogs jumping) and that caused by ischemia/hypoxia due to oxygen limitation. In both cases, an initial decrease in ATP and increase in ADP and AMP leads to an increase in the amount of bound AMPD localized physically with the subcellular area(s) where AMP is accumulating. However, whereas energy stress due to exercise is typically short-lived until fatigue sets in and then quickly reversed, energy stress due to hypoxia/ischemia can be of indefinite term (e.g. days or weeks of freezing for wood frogs). Therefore, it appears that species that are tolerant of freezing (or of anoxia or of deep torpor) allow their total adenylate pool to be partially depleted as an early response to stress (using AMPD to drain the pool) but then need to arrest the process and re-establish homeostasis in a hypometabolic state that can be sustained over the long term. Phosphorylation of bound AMPD may be the key in this phase. Bound AMPD in 24 h frozen frogs is a high phosphate form with relatively low affinity for AMP and strongly inhibited by IMP. Hence, it is likely that enzyme activity *in vivo* is low over the long term in frozen muscle. Effects of low temperature and high glucose (both conditions in frozen muscle) on $S_{0.5}$ AMP support the same conclusion. Numerous studies with multiple animal systems of

diverse phylogenetic origins have shown that one of the key mechanisms that regulates animal transitions into hypometabolic states is protein phosphorylation (Storey and Storey, 1990). The present data suggest that AMPD activity is similarly integrated into the needs of the hypometabolic, frozen state by phosphorylation of the bound enzyme.

Reversal of these processes during thawing, including enzyme dephosphorylation, temperature rise and glucose decrease, will all contribute to a return by AMPD to normal function, as in control muscle. Recovery of other enzymatic systems will also allow a recovery of muscle high energy reserves (e.g. total adenylate and creatine phosphate pools), the clearance of glucose and glycolytic end products, the re-establishment of aerobic metabolism, and the reactivation of a variety of energy-expensive cell functions that are typically strongly suppressed in hypometabolic states including the activities of ion motive ATPases (needed for muscle contractile activity), transcription and translation (Storey and Storey, 1990; Storey and Storey, 2007). Given the variety of metabolic functions that must recover (or be repaired) after thawing, it is perhaps not surprising that hind leg reflex contractions took more than 4 hours to return in frogs thawed at 6-8°C (Layne and First, 1991) and that isolated wood frog skeletal muscles still showed marked impairment of locomotor endurance up to 96 h post-freeze (Storey and Storey, 1985).

Overall, this study shows that skeletal muscle AMPD from control and frozen frogs has significantly different kinetic parameters that would alter enzyme function during freezing. Binding, allosteric effectors, glucose, temperature and reversible phosphorylation all play roles in regulating wood frog AMPD and adjusting enzyme function for optimal action in the regulation of cellular adenylate levels in ischemic frozen muscle. For example, phosphorylation of bound AMPD may be responsible for

some of the unique properties of the bound enzyme in frozen muscle such as the change in sensitivity to ADP activation, inhibition by IMP, and the response of the enzyme to high glucose. Reversible phosphorylation control of AMPD could also have a critical role in coordinating AMPD function with respect to other enzymes of muscle energy metabolism to minimize overall energy use while frozen and achieve maximum extension of viability.

CHAPTER 3

REGULATION OF CREATINE KINASE IN THE FREEZE- TOLERANT WOOD FROG, *RANA SYLVATICA*

INTRODUCTION

Creatine kinase (CK; EC 2.7.3.2) catalyzes the transfer of a phosphate group from ATP to creatine (Cr) to produce phosphocreatine (PCr) and ADP, and *vice versa*. As an enzyme responsible for buffering energy reserves, CK is found in tissues with high and/or fluctuating ATP demands such as skeletal muscle, heart, and brain (Wallimann et al., 1992). For example, CK constitutes ~20% of the total soluble protein of skeletal muscle (Lipskaya, 2001). Two cytosolic isozymes of CK are present in organ-specific dimers: an MM-CK homodimer in skeletal muscle (M is the muscle-specific monomer), a BB-CK homodimer in brain (B is the brain-specific monomer), and an MB-CK heterodimer in heart (McLeish and Kenyon, 2005). The main role of CK in skeletal or cardiac muscle is to maintain energy homeostasis at sites of high ATP turnover (typically as a result of contraction). In this function, CK uses the phosphagen reservoir, PCr, to buffer fluctuations in the intracellular ATP/ADP ratio (McLeish and Kenyon, 2005). In brain, CK-BB has been implicated in maintaining high levels of ATP for ATP-driven ion homeostasis and neurotransmitter trafficking (Wallimann and Hemmer, 1994). Mitochondrial CK isozymes also exist; these are octomers, with Miu-CK being ubiquitous whereas Mis-CK is present in sarcomeric mitochondria (McLeish and Kenyon, 2005). The discovery of mitochondrial isozymes gave rise to the idea of a phosphotransfer network wherein a Cr/PCr shuttle, involving mitochondrial and cytosolic CK isozymes, moves high energy phosphate between sites of ATP synthesis (mitochondria) and ATP consumption (Dzeja and Terzic, 2003; McLeish and Kenyon, 2005).

Changes in the activity of CK can have an effect on available ATP and the

Adenylate Energy Charge (AEC): $AEC = ([ATP] + 0.5[ADP])/([ATP] + [ADP] + [AMP])$. Possible mechanisms for the regulation of CK under energy stress have been studied, and protein phosphorylation is known to be a post-translational regulatory mechanism. For example, AMP-dependent protein kinase (AMPK) phosphorylation has variable effects on CK both *in vitro* and in differentiated muscle cells (Ponticos et al., 1998; Ingwall, 2002). Inhibition of protein kinase C (PKC) also decreased CK activity in brain (Chida et al., 1990a; Chida et al., 1990b). Calcium-calmodulin dependent protein kinase CaMK has been shown to phosphorylate CK in some cases but had no apparent effect on activity (Singh et al., 2004). Protein kinase A (PKA), protein kinase G (PKG), PKC and AMPK have also been shown to phosphorylate skeletal muscle CK in ground squirrels, with PKA, PKG, and PKC all affecting activity (Abnous and Storey, 2007).

Winter survival for a large number of terrestrially-hibernating ectothermic organisms relies on freeze tolerance, the ability to endure the conversion of 65-70% of total body water into extracellular ice. To survive, animals strongly suppress their energy-consuming physiological needs and undergo a series of metabolic changes that provide cryoprotection (Crerar et al., 1988; Costanzo et al., 1993; King et al., 1993; Swanson et al., 1996; Storey et al., 1992; Layne et al., 1989; Storey and Storey, 2004b). One consequence of freezing is the interruption of heart beat and breathing that causes anoxia and ischemia, and places a consequent energy stress on organs including a drop in ATP levels and the AEC (Storey, 1987b). A mechanism is needed to stabilize cellular energetics and help maintain viability in skeletal muscle over long term freezing ischemia. The pool of PCr and appropriate regulation of CK could accomplish this; indeed, consumption of PCr fully sustained the adenylate pool and AEC of skeletal

muscle in wood frogs (*Rana sylvatica*) frozen for 4 days but organs without significant phosphagen reserves (e.g. liver) showed a large decline in adenylates and AEC over the same time (Storey and Storey, 1984). The present study analyzes the mechanisms of CK regulation that could be involved in enzyme control with respect to the energetic needs for freezing survival, using as our model skeletal muscle CK from the freeze-tolerant wood frog.

MATERIAL AND METHODS

Animals and biochemicals

Male wood frogs (*Rana sylvatica*) were treated as previously described in Chapter 2. All chemicals came from the same sources as described in Chapter 2.

Preparation of tissue extracts

Frozen muscle samples were mixed 1:10 w:v in ice-cold buffer A (50 mM MOPS, 2 mM EDTA, 2 mM EGTA, 50 mM β -glycerophosphate, 10 mM β -mercaptoethanol, pH 7.0), with a few crystals of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) added and then immediately homogenized and centrifuged as described in Chapter 2. The supernatant was removed and stored on ice until assay.

Enzyme assays and determination of kinetic parameters

Enzymes were assayed as previously described (Abnous and Storey, 2007) at 340 nm in a 200 μ l final volume using a Dynatech MR5000 microplate reader. Optimal assay conditions for CK were determined to be 50 mM Tris buffer (pH 8.6), 10 mM Mg^{2+} •ATP,

12.5 mM creatine, 4 mM phosphoenolpyruvate (PEP), 0.225 mM NADH, 2.5 mM Mg^{2+} , 1 U each of pyruvate kinase (PK) and lactate dehydrogenase (LDH), and 10 μl of enzyme extract diluted 20-fold immediately prior to assay. One unit (U) of CK activity is defined as the amount that consumed 1 μmol of NADH per min at 25°C. Enzyme kinetic constants were determined using a nonlinear least-squares regression computer program (Brooks, 1992); substrate affinity data were fitted data to Michaelis-Menten plots.

In vitro incubations to stimulate endogenous kinases and phosphatases

To assess the effect of reversible phosphorylation on HK, aliquots of enzyme extract were incubated under conditions described in Chapter 2. Samples were incubated overnight at 4°C. Following incubations, all samples were diluted 20-fold in ice-cold buffer A assayed under optimal conditions.

To confirm that the treatments used to stimulate protein kinases actually resulted in the phosphorylation of CK, tissue extracts (1:5 w:v) were prepared in buffer A as above. Extracts were then treated to fully dephosphorylate CK. To do this, extracts were first centrifuged through small columns of Sephadex G-50 to remove endogenous enzyme effectors and various components (EDTA, EGTA) of buffer A. Five ml syringe barrels were plugged with glass wool and packed with Sephadex G-50 equilibrated in 50 mM MOPS, pH 7.0 and 10 mM β -mercaptoethanol followed by centrifugation in a bench-top IEC clinical centrifuge at full power for 1 min. The eluant was discarded and a 500 μl aliquot of supernatant was then layered onto the column followed by a second centrifugation. The final eluant was collected, and then MgCl_2 and CaCl_2 were added to final concentrations of 5 mM each to stimulate endogenous protein phosphatases.

Samples were incubated at 4°C overnight and then incubations designed to stimulate specific protein kinases were set up as above but this time with the additional presence of 10 µCi radiolabeled γ -³²P-ATP. After overnight incubation, 60 µl of insoluble protein A (IPA) (Sigma, P7155) was added to each sample, incubated overnight, and then samples were centrifuged at 2,000 x g and the supernatant was removed; this treatment removed proteins that bind non-specifically to IPA. Samples were then incubated with 1 µg of MM-CK goat polyclonal IgG (Santa Cruz) for one h and then 60 µl IPA was added and incubated overnight to form CK-antibody-IPA complexes. Pellets were collected by centrifugation at 2,000 x g following by washing 7 times with 100 µl 50 mM Tris buffer, pH 7.5. Aliquots (5 µl) of the suspension were spotted on P81 paper and exposed to a phosphorscreen. The radioactivity in each spot was quantified using a Personal Molecular Imager-FX (BioRad).

DEAE ion exchange chromatography

Ion exchange chromatography was used to separate multiple forms of CK. Muscle extracts were prepared 1:5 w:v in buffer B (12.5 mM MOPS, 0.5 mM EDTA, 0.5 mM EGTA, 12.5 mM β -glycerophosphate, 2.5 mM β -mercaptoethanol, pH 7.0) with homogenization and centrifugation as above. A 200 µl aliquot of supernatant was applied to a DEAE Sephadex G50 column (1.5 cm x 5 cm) equilibrated in buffer B. The column was then washed with 10 mL of buffer B, followed by a 10 mL linear KCl gradient (0-600 mM) in buffer B; 1 mL fractions were collected and assayed.

Western blotting

Skeletal muscle extracts from both control and frozen frogs were prepared in Buffer A as described earlier. Soluble protein concentration in each sample was determined by the BioRad protein assay and then protein concentration was adjusted to 4 $\mu\text{g}/\mu\text{l}$ with extraction buffer. Aliquots (100 μl) of each extract were mixed with 100 μl 2X sample 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 fresh 2-mercaptoethanol) and the mixture was boiled for 5 min, cooled on ice and frozen at -80°C . Equal amounts of protein (5 μg) were loaded into each lane of 10% SDS-PAGE gels, and then electrophoresis was carried out at 120 V for 80 min. Proteins were transferred to polyvinylidene difluoride membrane (PVDF) at 3 mA for 90 min. Membranes were blocked in 5% skim milk in Tris-buffered saline containing the detergent Triton-X (TBST) for 2 h, and then washed three times with TBST. Membranes were then incubated with primary antibody raised against muscle CK (CK-M goat polyclonal IgG) (Santa Cruz) overnight at 4°C . After washing with TBST three times, membranes were incubated with secondary antibody (mouse anti-goat IgG) for 15 min and then washed. Immunoreactive bands were visualized by enhanced chemiluminescence using a ChemiGenius (SynGene, MD, USA) and band intensities were quantified using the associated Gene Tools program.

Structural stability of creatine kinase using pulse proteolysis

The method of Park and Marqusee was used (Park and Marqusee, 2005). Aliquots of 20 μl (~ 400 μg total protein) from crude muscle extract (1:5 w:v homogenization in buffer A without PMSF) from control and frozen frogs were incubated overnight at room temperature with 100 μl of buffer A containing different concentrations of urea. After

incubation, pulse proteolysis was initiated by adding thermolysin (Sigma) to a final concentration of 0.40 mg/ml (stock thermolysin was prepared in 2.5 M NaCl + 10 mM CaCl₂). After a 10 min incubation, the reaction was quenched by adding 18 µl of 50 mM EDTA (pH 8.0). Western blotting was then used to quantify the amount of folded protein remaining.

RESULTS

Activity and kinetic parameters of CK in crude extracts of skeletal muscle

Initial studies of CK from skeletal muscle of *Rana sylvatica* optimized homogenization conditions for retrieving maximum CK activity. Inclusion of protein kinase inhibitors (EDTA/EGTA) or the protein phosphatase inhibitor (β-glycerophosphate) in the homogenization buffer (buffer A) did not affect the measurable amount of CK but the presence of NaF, another phosphatase inhibitor, reduced CK activity by ~30%; hence, standard homogenization conditions omitted NaF. Tissue extracts of CK prepared as described in Methods and Materials and stored at 4°C were stable for up to a week. The pH optimum of the CK reaction (for the creatine + ATP direction) was determined to be 8.6 ± 0.1 ($n = 4$ determinations on separate preparations of enzyme). However, the peak was rather broad with over 90% of activity retained at pH values 7.8 and 9.0. Free magnesium concentration was also optimized; maximum activity was achieved at 2.5 mM, and was subsequently used for enzyme assays. Concentrations above 2.5 mM reduced activity below optimal, whereas concentrations of 7.5 mM and above inhibited CK relative to the absence of magnesium.

The maximum activity of CK in soluble extracts of skeletal muscle from control

frogs was 4.51 ± 0.19 U/mg protein (equivalent to 584 U/gram wet mass) and was ~35% higher in muscle of frozen frogs (Table 3.1). Velocity versus substrate concentration relationships were hyperbolic for both creatine and ATP. The K_m creatine was ~29% lower and significantly different ($P < 0.05$) for the enzyme from frozen frogs, compared with control CK (Table 3.1). However, there was no significant difference in K_m ATP between the two states.

Reversible phosphorylation of CK

Stable kinetic differences in the properties of an enzyme between two physiological states are often the result of state-dependent reversible phosphorylation catalyzed by protein kinases and protein phosphatases. To determine if this mechanism was responsible for the differences in CK properties between control and frozen states, tissue extracts were incubated overnight under conditions that stimulated the activities of endogenous protein kinases or protein phosphatases, followed by measuring V_{max} and K_m creatine. Incubations that promoted total kinase action generally increased both activity (V_{max} increased by 35%), and apparent creatine affinity (K_m decreased by ~19%) of CK from control frogs (Figure 3.1). In extracts from frozen frogs, stimulation of protein kinases increased the V_{max} by ~10%. Subsequent incubations to examine specific protein kinases indicated that conditions that facilitated AMPK or CaMK elevated CK activity in extracts from both control and frozen frogs (Figure 3.2a), whereas conditions that facilitated PKA, PKC, and PKG had no significant effect on activity (data not shown).

Comparable incubation studies were used to examine protein phosphatase effects on CK. Incubation under conditions that stimulated the total endogenous protein

phosphatases activities generally produced a significant decrease ($P < 0.05$) in CK activity and substrate affinity. For CK in control extracts, K_m creatine increased by ~13%, whereas CK in frozen extracts showed an increase in K_m creatine of ~36% and a decrease in V_{max} of ~55% (Figure 3.1). Stimulation of specific phosphatases indicated that conditions that facilitated PP2B and PP2C activities reduced CK activity (Figure 3.2b), whereas those facilitating PP1 and PP2A did not (data not shown).

To confirm that the incubations that stimulated protein kinases actually resulted in the phosphorylation of CK, muscle extracts from both control and frozen CK were first treated to dephosphorylate enzymes and were then incubated under phosphorylating conditions but this time in the presence of γ - ^{32}P -ATP. Radiolabeled CK was then immunoprecipitated and quantified. These studies showed that incubations that stimulated PKA, PKC, PKG, CaMK, and AMPK all resulted in high levels of ^{32}P labelling of the precipitated CK-antibody-IPA complex. Relative to the control incubation without added stimulators of protein kinases, the mean increases in ^{32}P labelling of CK were 2.0-, 1.9-, 1.6-, 1.8-, and 1.8-fold in incubations that stimulated PKA, PKC, PKG, CaMK, or AMPK, respectively (Figure 3.3).

High and low phosphate forms of enzymes can frequently be separated by ion exchange chromatography because phosphorylation alters the net charge on the enzyme. CK from muscle of control frogs eluted in two peaks, with the majority of activity (~60%) in peak II, eluting with added KCl (Figure 3.4). CK in muscle extracts from frozen frogs also eluted in two peaks, but in this case the majority of activity (~65%) was in peak I, eluting without any added KCl. The data are consistent with two forms of CK having different net charges. In control frogs, CK exists predominantly in a form that

binds more strongly to DEAE, whereas in frozen frogs, the dominant form does not bind to DEAE.

CK protein levels

Relative levels of CK protein were determined by Western blotting. The mammalian antibody cross-reacted with a strong band on the PVDF membrane at the expected molecular weight of ~42 kDa. Total CK protein levels did not change significantly in the transition from the control to the frozen state.

Structural stability of CK

The effect of urea on CK unfolding was assessed using the pulse proteolysis method. Urea-induced unfolding abolishes enzyme activity and makes the enzyme more sensitive to thermolysin proteolysis, and hence the amount of CK protein detected on Western blots at the 42 kDa subunit size declines when proteolysis occurs. Activity of control CK was unaffected by urea up to concentrations of ~3 M but activity decreased rapidly at higher urea values (Figure 3.5). The calculated urea concentration for loss of half of the activity (I_{50} urea) was 3.65 ± 0.02 M. CK from frozen frogs showed the same profile of response to urea with an I_{50} urea of 3.70 ± 0.03 M. Structural integrity of CK, as assessed by sensitivity to thermolysin degradation of the enzyme, appeared to be maintained to a higher urea concentration since Western blotting showed strong bands of CK protein at the 42 kDa subunit size up to about 3.5 M urea. However, at higher urea levels, CK protein became much more sensitive to thermolysin degradation and the intensity of the CK band at 42 kDa decreased until it was no longer detectable at 4.25 M

urea in extracts from in control frogs or at 4.5 M urea for CK from frozen frogs.

Table 3.1 Kinetic parameters of CK in crude extracts of skeletal muscle from control (5°C-acclimated) and 24 h frozen wood frogs.

	K_m Creatine (mM)	K_m ATP (mM)	V_{max} (U/mg protein)
Control	4.52 ± 0.19	1.76 ± 0.07	4.51 ± 0.19
Frozen	3.22 ± 0.09^a	1.67 ± 0.03	6.06 ± 0.06^a

Assays were conducted with crude extracts of muscle at 25°C and parameters were determined using an enzyme kinetics package that fitted data to the Michaelis-Menten equation. Data are means $\pm$ SEM, $n = 8$ independent preparations of enzyme from individual frogs.

^a Significantly different from the corresponding untreated control value as determined by the Student's t -test, $P < 0.05$.

Figure 3.1 Effect of in vitro incubations promoting the action of protein kinases or protein phosphatases on CK activity in soluble extractions of frog skeletal muscle. (A) Effect on K_m creatine of incubations that inhibited both kinase and phosphatase action (stop), promoted total kinase action (kinases), or promoted total phosphatase action (phosphatases). (B) Effect of the same incubation conditions on CK maximal activity. Enzyme extracts were incubated overnight at 4°C, under the conditions outlined Materials and methods, followed by dilution and assay. Data are means $\pm$ SEM, $n = 4$ independent preparations of enzyme from individual frogs. ^a Significantly different from the corresponding control extract in stop buffer (no additions) using the Student's *t*-test, $P < 0.05$; ^b significantly different from the corresponding frozen extract in stop buffer, $P < 0.05$.

Figure 3.2 Effect of in vitro incubations promoting the action of specific protein kinases or protein phosphatases on CK activity in soluble extractions of frog skeletal muscle. (A)

Incubations that promote endogenous protein kinase activities including AMPK and Ca^{2+} /CaMK.

(B) Incubations that promote endogenous phosphatases including PP2B and PP2C. Enzyme extracts were incubated overnight at 4°C, under outlined in the materials and methods, followed by dilution and assay. Data are means $\pm$ SEM, $n = 4$ independent preparations of enzyme from individual frogs. ^a Significantly different from the corresponding control extract in stop buffer (no additions) using the Student's *t*-test, $P < 0.05$; ^b significantly different from the corresponding frozen extract in stop buffer, $P < 0.05$.

Figure 3.3 Effect of in vitro incubations promoting the action of specific protein kinases on CK radiolabeling in soluble extractions of frog skeletal muscle. Enzyme extracts were incubated overnight at 4°C with γ -³²P-ATP, as outlined in Material and methods, followed by immunoprecipitation, spotting, and phosphorimaging. Data are means $\pm$ SEM, $n = 3$ independent preparations of enzyme from different frogs. ^a Significantly different from the corresponding control extract in stop buffer (no additions) using the Student's *t*-test, $P < 0.05$; ^b significantly different from the corresponding frozen extract in stop buffer, $P < 0.05$.

Figure 3.4 DEAE Sephadex ion exchange chromatography of frog skeletal muscle CK. The column was eluted with column buffer (first 10 fractions), followed by a linear KCl gradient (0-600 mM) in the same buffer (last 10 fractions). Data show representative profiles for single preparations but the same separations were achieved in multiple ($n = 3$) trials.

Figure 3.5 Structural stability of muscle CK from control versus frozen frogs assessed by susceptibility to urea denaturation. Crude extracts were incubated for 24 h with different concentrations of urea. Aliquots were assayed for activity, or subjected to pulse proteolysis to degrade denatured CK (10 min incubation with thermolysin) and then Western blotting to measure the amount of native folded CK remaining. Activity data are means $\pm$ SEM, $n = 4$ independent preparations of enzyme from different frogs. The Western blot is representative of $n = 3$ independent preparations of enzyme from individual frogs.

Discussion

The maintenance of muscle energetics during freezing is important not just to long-term viability but it is also critical to the arousal process, as thawing requires significant ATP consumption in muscle to resume energetically expensive cell functions such as the re-establishment of ATP-dependent ion motive pumps (needed for muscle contractile activity), transcription and translation. For example, ATP-dependent calcium transport increased by 2.32-fold in skeletal muscle sarcolemma of thawed frogs. In skeletal muscle sarcoplasmic reticulum, calcium uptake rebounded to 47% of control values during thawing, after being suppressed to 8% of control values in the frozen state (Hemmings and Storey, 2001). High PCr reserves have been implicated in a metabolic strategy to maintain a constant energy charge in skeletal muscle during freezing, whereas other tissues that lack PCr pools, such as liver, showed a strong decline in ATP levels and energy charge over time frozen, and over multiple freeze-thaw cycles (Storey and Storey, 1984; Storey, 1987b). In addition to buffering ATP demands during muscle exercise, PCr reserves and CK activity have been shown to help preserve a high energy state in muscle under stresses imposed by hypoxia (Richards et al., 2007; Overgaard et al., 2007a) and hibernation (Abnous and Storey, 2007). The present research shows that skeletal muscle CK from control versus frozen wood frogs has different kinetic properties, and appears to be interconverted between two forms by reversible phosphorylation, predictably regulating it for optimal function in the two physiological states.

Both maximal CK activity and enzyme affinity for creatine substrate increased significantly during freezing, as compared with control muscle. Maximal activity increased by 35% despite no comparable increase in CK protein content (as determined

by immunoblotting). Kinetic properties of muscle CK also changed significantly between control and frozen states. K_m values for creatine were significantly lower (substrate affinity was higher) for the enzyme from frozen muscle, compared with controls. This increase in activity in the frozen state, coupled with the increase in creatine substrate affinity, suggests that the enzyme undergoes a stable modification in the frozen frog, one that may promote CK action in stabilizing muscle energetics in the frozen state.

The stable changes in CK activity and properties documented above implicated post-translational modification of the enzyme as the probable underlying mechanism responsible for the freeze-induced changes in the enzyme. Prior evidence for the phosphorylation of CK has been found. CK autophosphorylation of threonine residues near the active site has been suggested as a means to regulate the activity and direction of the reversible reaction (Stolz et al., 2002). The BB-CK (brain) isoform of CK is phosphorylated and inactivated by PKC (Childa, 1990a; Childa, 1990b), whereas a flagellar form of CK in sea urchin sperm is phosphorylated by PKA (Su et al., 2005). In rats, the skeletal muscle-specific form of calcium-calmodulin dependent protein kinase ($\text{CaMKII}\beta_M$) assembles with the glycolytic complex, and several other enzymes at the sarcoplasmic reticulum and phosphorylates CK, although this had no effect on CK activity (Singh et al., 2004). Other studies showed MM-CK phosphorylation by AMPK leading to inactivation of MM-CK *in vitro* (Ponticos et al., 1998; Neumann et al., 2003). Subsequent studies, however, suggested that CK phosphorylation by AMPK was dependent on physiological state. Thus, CK from rat hearts show a positive linear relationship between [AMP], AMPK velocity and CK velocity in normoxia but, under hypoxic conditions, CK velocity fell, while there was a modest increase in AMPK

(Ingwall, 2002). Moreover, stimulation of AMPK *in vitro* had no apparent effect on the activity of CK in muscle extracts from either euthermic or hibernating ground squirrels (Abnous and Storey, 2007). This suggests that the consequence of AMPK phosphorylation of CK is more complex than the simple decrease in activity that was initially proposed. Indeed, when we used the Minimoto Miner application from the University of Connecticut (<http://sms.engr.uconn.edu/servlet/SMSSearchServlet>) (Balla et al., 2006) to analyze the amino acid sequences of MM-CK from human, mouse, and rat we found that the enzyme contained putative consensus sequences for the phosphorylation sites of PKA, PKG, PKC, AMPK and CaMK. Incubations under conditions that stimulated AMPK or CaMK significantly increased soluble CK activity from both control and frozen wood frog muscle, but stimulation of PKA, PKC, and PKG did not change activity or K_m creatine. However, incubations done in the presence of ^{32}P -ATP found that stimulation of all five of these kinases increased ^{32}P -labeling of CK, showing that CK can indeed be phosphorylated by multiple protein kinases. Thus, phosphorylation by PKA, PKC, or PKG may affect parameters other than those assessed in the present study. The physiologically important protein kinase related to CK function in muscle of frozen frogs may be AMPK since we have found increased AMPK activity in frozen frogs (Rider et al., 2006), which could lead to increased phosphorylation of CK in the *in vivo* frozen state.

Frog CK also responded to treatment with protein phosphatases. Incubations promoting total protein phosphatases, as well those specifically stimulating PP2B and PP2C, all led to significant decreases in soluble CK activity in extracts of frozen frogs (31-55% decrease). Stimulation of total protein phosphatases also increased K_m creatine

of both control and frozen CK to values higher than the K_m of untreated control. The pattern of response by frozen CK to protein kinases (activity increases) and protein phosphatases (activity decreases) suggests that soluble CK in frozen muscle is the high phosphate form and must be dephosphorylated to revert to the control condition. Several phosphatases are known to reverse the effects AMPK and CaMK, both of which increase CK activity by phosphorylation. PP2A and PP2C inactivate AMPK (Davies et al., 1995) and by inhibiting AMPK may prevent phosphorylation of AMPK targets. Downstream target sites phosphorylated by AMPK can be dephosphorylated by PP2A and PP2B (Abel et al., 1995; Blume et al., 2007). PP2B, which is notably regulated by lower calcium/calmodulin levels than are required to stimulate CaMK, and PP2C also dephosphorylate downstream targets of CaMK (Colbran, 2004; Crabtree, 1999). PP2C dephosphorylated rabbit MM-CK that had been phosphorylated by purified rabbit AMPK (Ponticos et al., 1998). In hibernating ground squirrels, conditions that stimulated all protein phosphatases, all serine/threonine protein phosphatases, PP1, and PP2B all led to CK dephosphorylation (Abnous et al., 2007). Over long-term freezing, total PP1 activity decreases in wood frog skeletal muscle (MacDonald and Storey, 2002), and if other phosphatases behave similarly, this would suggest that active protein kinases phosphorylate CK in frozen frogs, and with suppressed protein phosphatase activities, CK will remain in a high phosphorylation state throughout the freezing episode until thawing allows a reversal.

To identify differentially-charged populations of CK, we used ion-exchange chromatography on DEAE Sephadex G50 to evaluate the elution profiles of CK from control and frozen frogs, and found that a mixture of high phosphate and low phosphate

CK forms was present in both control and frozen frogs. Control frog muscle contained an abundance of the peak II enzyme that eluted at high salt concentration whereas muscle from frozen frogs had a greater abundance of the peak I form. Hence, we conclude that soluble CK in wood frog muscle exists as a mixed population of low and high phosphate forms. In the control situation, a greater population of low phosphate form is present, along with a smaller population of highly-phosphorylated enzyme. In frozen muscle, the situation is reversed with a greater content of the high-phosphate enzyme form. This elution profile may seem contrary to what might be expected (highly phosphorylated enzymes should bind more tightly to DEAE), but it is possible that phosphorylation of CK causes conformational changes, exposing more basic side chains and increasing the net positive charge of the enzyme, as has been shown as the effect of phosphorylation of the cAMP response element-binding protein (CREB) transcription factor (Bullock and Habener, 1998). Based on the changes in CK kinetic parameters following *in vitro* phosphorylation, and their resemblance to *in vivo* kinetic parameters, the data indicate that CK is predominantly dephosphorylated in the control state, and phosphorylated in the frozen state.

Analysis of CK stability with respect to urea denaturation revealed that the structure of ground squirrel CK was disrupted and rendered susceptible to thermolysin proteolysis at concentrations of 3-4 M urea and that treatment with 5 M urea caused >90% denaturation. Greater flexibility of CK from a mammalian hibernator, compared with nonhibernating mammals, has previously been shown to aid the enzyme in maintaining its function over a wide range of body temperatures that are naturally encountered, from high values (~37°C) in euthermia to lows of 0-5°C during winter

hibernation (Abnous and Storey, 2007). Since denaturation occurs at similar urea concentrations for CK from both control and frozen frogs, CK can be said to have similar structural flexibilities in both phosphorylation states. Structural stability and flexibility is a desirable property in the case of freeze tolerant frogs, where conditions of extensive cellular dehydration and extreme hyperglycaemia in the frozen state may lead to compaction of the enzyme. Indeed, previous studies on rabbit CK demonstrated a high retention of activity under glycerol compaction (Fen and Yan, 2008), and similar flexibility and stability (as documented by resistance to urea denaturation) by frog CK may help the enzyme resist temperature and compaction changes as a result of intracellular hyperglycaemia and dehydration during freezing (Churchill and Storey, 1993).

The energy stress caused by hypoxia/ischemia during winter freezing episodes may last for days or weeks. Enzymes such as CK, which are crucial to muscle energy metabolism, have often been studied in contracting muscle of exercising animals, and less commonly in hypoxia/ischemia tolerant model systems. To date, however, little is known about enzyme regulation in freeze-tolerance or, indeed, other forms of natural hypometabolism. We recently analyzed AMP-deaminase (AMPD), another important enzyme in skeletal muscle energy metabolism, from wood frog muscle and was found that it was uniquely regulated (Dieni and Storey, 2008). AMPD action allows the total adenylate pool size to be reduced during freezing, and by draining AMP, contributes to maintaining a high AEC. Previous studies have shown that inhibition of CK activity by creatine analogues or in knockout models alters the energy state, and leads to phosphorylation and altered kinetics of AMPD. The altered kinetics of AMPD activates

deamination to a higher degree within physiological concentrations of AMP (Tullson et al., 1996; 1998). NMR studies have shown that energy metabolism in frozen frogs draws upon PCr stores, depleting them during the first day of rapid freezing before ATP levels began to decline. During slow freezing, the ATP pool remained stable and PCr declined modestly over seven days (Layne and Kennedy, 2002). Thus, CK may work in tandem with AMPD in freeze tolerance; in order to minimize the need for deamination of the adenylate pool to maintain a high AEC, CK may draw upon PCr reserves and ADP to first replenish ATP levels before deamination becomes necessary.

Overall, the present study shows that muscle CK from control and frozen frogs displays significantly different kinetic parameters and phosphorylation states, which may optimize enzyme function for action in frozen, energy-stressed versus control states. The active regulation of CK advocates an important role in muscle energy metabolism under physiological stress.

CHAPTER 4

REGULATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN THE FREEZE-TOLERANT WOOD FROG, *RANA SYLVATICA*

INTRODUCTION

Glucose-6-phosphate dehydrogenase (G6PDH) catalyzes the rate-limiting step in the pentose phosphate pathway (PPP) by oxidizing glucose-6-phosphate (G6P) to 6-phosphoglucono- δ -lactone, while reducing nicotinamide adenine dinucleotide phosphate (NADP⁺) to generate NADPH. The PPP has several important functions in cells including the production of pentose phosphates for nucleotide biosynthesis and 3-7 carbon sugars or sugar phosphates for a variety of anabolic uses. In addition, the PPP is a major source of NADPH which has a wide range of uses as a biological reducing agent for reductive biosynthesis and antioxidant defence (Carvalho et al., 1993; Kletzien et al., 1994). A well-known human pathology, and in fact the most common human enzyme defect, is based on decreased activity of G6PDH; this deficiency is the result of X-linked mutations in the G6PDH gene, with functional results that can result in symptoms ranging from neonatal jaundice, to severe haemolytic anaemia (Cappellini and Fiorelli, 2008). G6PDH has also been implicated in important roles in cell growth and development (Tian et al., 1998) as well as preventing radical oxygen species (ROS)-induced cell death (Tian et al., 1999).

Protein phosphorylation is a predominant and widespread mechanism of metabolic control in many cellular pathways (Pawson and Scott, 2005), extending to fuel and energy metabolism (Kahn et al., 2005) and stress response (Keyse, 2008), among others. G6PDH itself is regulated by reversible phosphorylation; it is known to be phosphorylated in aortic endothelial cells, kidney cortex, and macrophages (Cosa Rosa et al., 1995; Xu et al., 2005; Zhang et al., 2000). Most recently, analysis of G6PDH in estivating desert land snails showed that regulation of the enzyme by reversible

phosphorylation was a metabolic response to external environmental stresses (Ramnanan and Storey, 2006).

Freezing places a number of demands on carbohydrate metabolism in wood frog liver. On the one hand, huge amounts of glycogen are converted to G6P and then to glucose to act as a cryoprotectant, and this glucose (200-300 mM in liver) must be maintained as the free sugar in all organs throughout the freeze (Crerar et al., 1988; King et al., 1993). Another “stream” of G6P must feed anaerobic glycolysis to fuel ATP production in the ischemic frozen state. A third “stream” of G6P is needed by the PPP to support NADPH generation for antioxidant defenses. Good antioxidant defenses are important for sustaining long term viability in hypometabolic states and also to deal with a burst of reactive oxygen species generation when oxygen is reperfused into organs after thawing (Joannis and Storey, 1996). Thus, the task in frog liver is to control and balance these three options for G6P metabolism in order to (i) produce and sustain glucose as an intracellular cryoprotectant, (ii) fuel ATP production by glycolysis, and (iii) sustain NADPH production for antioxidant defences. To accomplish this, it is necessary to regulate enzymes at or near the G6P branch point. Strong regulation of glycogen phosphorylase via freeze-responsive reversible phosphorylation regulates the initial entry of carbohydrate into metabolism (Crerar et al., 1988; Storey and Storey, 1988) but regulation of other enzymes is then needed to direct the disposition of G6P to multiple fates. Multiple other metabolic enzymes, as well as protein kinases involved in signal transduction are also regulated by freeze-induced phosphorylation including AMP-deaminase (Dieni and Storey, 2008), AMP-dependent kinase (AMPK) (Rider et al., 2006), and several mitogen-activated protein kinases (Greenway and Storey, 2000).

In the present study, I hypothesized that G6PDH would be differentially regulated in liver during natural freezing to help control and direct the use of G6P for NADPH production by the PPP to support antioxidant defence.

MATERIALS AND METHODS

Animals and Biochemicals

Male wood frogs (*Rana sylvatica*) were treated as previously described in Chapter 2. All chemicals came from the same sources as described in Chapter 2.

Preparation of tissue extracts

Samples of frozen liver were quickly weighed and mixed 1:5 w:v in ice-cold buffer A (50 mM MOPS, 2 mM EDTA, 2 mM EGTA, 50 mM β -glycerophosphate, 10 mM β -mercaptoethanol, 10% v/v glycerol, pH 7.0) with a few crystals of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) added and then immediately homogenized and centrifuged as described in Chapter 2. In cases where ion or metabolite effects on soluble G6PDH were being investigated, crude extracts were then centrifuged through small columns of Sephadex G-50 to remove endogenous enzyme effectors, as described in Chapter 2.

Enzyme Assay and Kinetic Parameters

Assays used a Dynatech MR5000 microplate reader at 340 nm, as previously described (Ramnanan and Storey, 2005). Optimal assay conditions for G6PDH were determined to be 50 mM Tris buffer (pH 8.2), 5 mM G6P, 0.5 mM NADP⁺, 10 mM

Mg²⁺, and 5 µl of tissue extract in a 200 µl final volume. One unit of enzyme activity is defined as the amount that produced 1 µmol of NADPH per minute at 25°C. Enzyme kinetic constants were determined using a nonlinear least-squares regression computer program (Brooks, 1992); substrate affinity data were fitted data to Michaelis-Menten plots.

DEAE ion exchange chromatography

Ion exchange chromatography was used to separate multiple forms of G6PDH. Liver extracts were prepared 1:5 w:v in buffer B (12.5 mM MOPS, 0.5 mM EDTA, 0.5 mM EGTA, 12.5 mM β-glycerophosphate, 2.5 mM β-mercaptoethanol, 2.5% v/v glycerol, pH 7.0) as above. A 500 µl aliquot of supernatant was applied to a DEAE Sephadex G-50 column (1.5 cm x 5 cm) equilibrated in buffer B. The column was eluted first with buffer B (15 x 1 mL fractions collected) and then with a 20 mL linear KCl (0-1 M) gradient in buffer B, again with 1 mL fractions collected. Peak fractions were pooled and kinetic analysis was performed on high- versus low-phosphate enzyme forms.

In vitro incubations to stimulate endogenous kinases and phosphatases

To assess the effect of reversible phosphorylation on G6PDH, aliquots of enzyme extract were incubated under conditions described in chapter 2, but used pooled peak fractions from DEAE ion-exchange chromatography instead of crude homogenates. Samples were incubated overnight at 4°C. Following incubation, samples were centrifuged through spun columns of Sephadex G-50, as previously described.

Western blotting

Liver extracts from both control and frozen frogs were prepared from control and frozen frogs, and 10 μg of protein was electrophoresed, and transferred to a PVDF membrane as described in Chapter 2. Membranes were blocked in 5% skim milk in TBST for 2 h, and then washed three times with TBST. Membranes were incubated with primary rabbit antibody raised against yeast G6PDH (Sigma) overnight at 4°C. After washing with TBST three more times, membranes were incubated with secondary antibody (mouse anti-rabbit IgG) for 1 h and then washed. Immunoreactive bands were visualized with the enhanced chemiluminescence assay using a ChemiGenius (SynGene, MD, USA) and band intensities were quantified using the associated Gene Tools program.

Phosphoprotein staining

SDS-PAGE gels were run as described above, except that 1 μg of total protein was loaded in each well. Gels were stained with the Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes), according to the manufacturers' instructions. Phosphoprotein bands were visualized using UV transillumination with the ChemiGenius and band intensities were quantified.

Structural stability of G6PDH using pulse proteolysis

The method of Park and Marqusee (2005) was used. Aliquots of 20 μl (~400 μg total protein) from crude liver extracts (1:5 w:v homogenization in buffer A without PMSF) were incubated overnight at room temperature with 100 μl of buffer A containing

different concentrations of urea, and subjected to pulse proteolysis as described in Chapter 2. Each sample was then subjected to Western blotting as described above to assess the amount of folded protein remaining.

RESULTS

Activity and kinetic parameters of G6PDH in crude liver extracts

Initial studies of G6PDH from liver of *Rana sylvatica* optimized homogenization conditions for retrieving maximum G6PDH activity. Inclusion of protein kinase inhibitors (EDTA/EGTA) or the protein phosphatase inhibitor (β -glycerophosphate) in the homogenization buffer (buffer A) did not affect the measurable amount of G6PDH. The pH optimum of the G6PDH reaction was determined to be 8.2 ± 0.1 ($n = 4$ determinations on separate preparations of enzyme).

The maximum activity of G6PDH in soluble extracts of liver was 75.7 ± 2.3 mU/mg protein (2.13 ± 0.12 U/gram wet mass) in liver from control frogs and a similar value of 78.9 ± 4.8 mU/mg in frozen frogs ($n = 8$) (Table 4.1). The velocity versus [G6P] relationship was hyperbolic for the enzyme from both control and frozen frogs. The K_m G6P was ~23% higher and significantly different ($P < 0.05$) for the enzyme from frozen frogs, compared with control G6PDH (Table 4.1). Similar results were seen for the velocity versus [NADP⁺] relationship: the relationship was hyperbolic and K_m NADP⁺ was ~31% higher and significantly different ($P < 0.05$) for the frozen enzyme.

Ion-exchange chromatography

High and low phosphate forms of enzymes can frequently be separated by ion

exchange chromatography because phosphorylation alters the net charge on the enzyme. DEAE ion exchange chromatography of crude extracts from frog liver showed that G6PDH eluted in two peaks. In the control situation, the majority of activity, ~61%, eluted in peak I (Figure 4.1). G6PDH in extracts from frozen liver also eluted in two peaks, but with a different distribution of activity; in this case, the majority of activity, ~55%, was in peak II, eluting with an added KCl gradient. The data are consistent with two forms of G6PDH in frog liver having different net charges. In control frogs, the majority of G6PDH exists in a form that does not bind to DEAE, whereas in frozen frogs, the dominant form does bind to DEAE.

Kinetic parameters were determined for G6PDH in isolated peaks. Peak I had a K_m G6P of $94.1 \pm 1.1 \mu\text{M}$ and a K_m NADP^+ of $61.2 \pm 3.5 \mu\text{M}$ (Table 4.1). Both of these values are similar to those found in crude liver extracts of control frogs. Peak II had a K_m G6P of $172 \pm 4.3 \mu\text{M}$ and a K_m NADP^+ of $98.2 \pm 3.3 \mu\text{M}$, which more closely resemble (but are overall higher than) the values seen for crude liver extracts from frozen frogs.

Reversible phosphorylation of G6PDH

Stable kinetic differences in the properties of an enzyme between two physiological states are often the result of state-dependent reversible phosphorylation catalyzed by protein kinases and protein phosphatases. To determine if this mechanism might be responsible for the differences in G6PDH properties seen between control and frozen states, pooled fractions from peak I and peak II were incubated overnight under conditions that stimulated either protein phosphorylation or dephosphorylation, followed by spun-columns to remove extraneous metabolites, and then the resulting effects on

G6PDH kinetics were measured. Incubations that promoted kinase action generally increased apparent substrate affinity (ie. decreased K_m G6P) (Figure 4.2). Stimulation of specific protein kinases indicated that the action of PKG, PKC, AMPK and CaMK all facilitated a decrease in K_m G6P in G6PDH from both peaks I and II. For the peak I enzyme, decreases in K_m G6P of 34.2%, 61.9%, 57.4%, and 71.8% were observed as a result of stimulation of PKG, PKC, AMPK and CaMK, respectively. For peak II G6PDH, the effect was less marked with decreases in K_m G6P of 7.9%, 26.9%, 11.7%, and 17.3% as a result of stimulation of PKG, PKC, AMPK and CaMK, respectively

Comparable incubations were set up to stimulate the activity of protein phosphatases, as described in the Materials and Methods. However, none of the conditions used resulted in significant changes to G6PDH kinetic parameters (data not shown). It is possible that phosphatases did not significantly co-elute with G6PDH, or that phosphatases were not stable under purification conditions.

G6PDH total protein and phosphoprotein levels

Relative levels of total G6PDH protein in liver from control and frozen frogs were assessed by Western blotting. A single, strong band was identified at ~60 kDa, the expected subunit molecular weight of the vertebrate enzyme. Total G6PDH protein levels did not change significantly in the transition from the control to the frozen state (Figure 4.3). The amount of phosphoprotein was then assessed with the Pro-Q Diamond phosphoprotein gel stain. The amount of phosphoprotein staining of the 60 kDa band was strongly reduced by 44% in the transition to the frozen state.

Structural stability of G6PDH

The effect of urea on G6PDH unfolding was assessed using the pulse proteolysis method. Urea-induced unfolding of a protein typically causes a loss of enzymatic activity and makes the enzyme more sensitive to thermolysin proteolysis; hence, when subsequently run on Western blots, the amount of G6PDH protein detected at the 60 kDa subunit size declined when proteolysis occurs. As Figure 4 shows, G6PDH protein from both control and frozen frogs was structurally stable up to urea concentrations of ~1 M as evidenced by the strong bands detected on Western blots. At higher urea concentrations, however, detectable protein at 60 kDa decreased greatly. G6PDH activity was much more sensitive to increasing urea concentrations and detectable activity decreased with the addition of as little as 200 mM urea. The calculated urea concentration for loss of half of the activity (I_{50} urea) was 0.49 ± 0.08 M ($n = 3$) for control G6PDH and 0.31 ± 0.08 M ($n = 3$) for the enzyme from frozen frogs (significantly different, $P < 0.05$).

Table 4.1 Kinetic parameters of liver G6PDH from control and frozen frogs and for peaks I and II of G6PDH separated by DEAE chromatography.

	Control	Frozen	Peak I	Peak II
V_{max} (mU/mg protein)	75.7 ± 2.3	78.9 ± 4.8	397 ± 13.5	414 ± 19.5
K_m G6P (μ M)	98.2 ± 3.8	121 ± 5.3^a	94.1 ± 1.1	172 ± 4.3^b
K_m NADP ⁺ (μ M)	65.5 ± 2.3	89.1 ± 4.8^a	61.2 ± 3.5	98.2 ± 3.3^b

Assays were conducted at 22°C and data are means $\pm$ SEM, $n = 8$ for values from control and frozen frogs, $n = 6$ for values for peaks separated by DEAE chromatography. Given the same elution characteristics, Peak I G6PDH was isolated from both control and frozen extracts and pooled for assay; peak II was prepared likewise.

^a Significantly different from the corresponding control value as determined by the Student's t -test, $P < 0.05$; ^b Significantly different from the corresponding Peak I value, $P < 0.05$.

Table 4.2 Effect of assay temperature and glucose on the K_m G6P for G6PDH separated by DEAE chromatography

	K_m G6P (μ M)	
	Peak I	Peak II
25°C, no glucose	94 $\pm$ 1.1	172 $\pm$ 4.3
25°C, 250 mM glucose	123 $\pm$ 9.0 ^a	222 $\pm$ 7.4 ^a
5°C, no glucose	104 $\pm$ 1.5 ^a	189 $\pm$ 2.3 ^a
5°C, 250 mM glucose	131 $\pm$ 3.3 ^a	245 $\pm$ 5.3 ^a

Data are means $\pm$ SEM, for determinations on DEAE chromatography-resolved peaks from $n = 4$ individual frogs. ^a Significantly different from the corresponding value at 25°C without glucose as assessed by the Student's t -test, $P < 0.05$.

Figure 4.1 DEAE Sephadex ion exchange chromatography of frog liver G6PDH. The column was developed using buffer B (first 15 fractions), followed by a linear KCl gradient (0-1 M) in the same buffer (last 25 fractions), as described in the Methods and Materials. Data show representative profiles for single preparations but the same separations with consistent percent yields were achieved in $n = 3$ trials.

Figure 4.2 Effect of in vitro incubations promoting the action of specific protein kinases or protein phosphatases on K_m G6P in pooled fractions of peak I and peak II G6PDH from DEAE chromatography of frog liver extracts. Incubations promoted endogenous protein kinase activities including PKG, PKC, AMPK, and CaMK. Samples were incubated overnight at 4°C, as outlined in the Materials and Methods, followed by spun-column and assay. Data are means $\pm$ SEM, $n = 4$ independent preparations of enzyme from individual frogs. ^a Significantly different from the corresponding control incubation in stop buffer (no additions) using the Student's *t*-test, $P < 0.05$; ^b significantly different from the corresponding incubation in stop buffer, $P < 0.05$.

Figure 4.3 Relative levels of total G6PDH protein, and the phosphorylated form of G6PDH, in control (5°C acclimated) and frozen (-3°C for 24 h) frogs as determined by SDS-PAGE followed by Western blotting or Pro-Q Diamond Phosphoprotein staining, respectively.

Data are means $\pm$ SEM, for determinations on extracts from $n = 4$ individual frogs. ^a

Significantly different from the corresponding value in control frogs as assessed by the Student's *t*-test, $P < 0.05$.

Figure 4.4 Structural stability of liver G6PDH from control versus frozen frogs assessed by susceptibility to urea denaturation. Crude extracts were incubated for 24 h with different concentrations of urea. Aliquots were assayed for activity, or subjected to pulse proteolysis to degrade denatured G6PDH (10 min incubation with thermolysin) and then Western blotting to measure the amount of native folded G6PDH remaining, as outlined in Methods and materials. Activities are means $\pm$ SEM, $n = 4$ independent preparations; the Western blot is representative of $n = 3$ independent trials.

DISCUSSION

G6PDH from liver of the wood frog, *Rana sylvatica*, shows freeze-induced changes in its properties that could help to regulate enzyme function and control carbon entry into the PPP in response to different metabolic needs. Recent studies have documented regulation of G6PDH by altering protein levels, as well as modifying enzyme functional or regulatory properties, in response to a number of other stresses/conditions including cold-hardening in insect larvae, hibernation in frogs, estivation in land snails, and hyperthermia in goldfish (Storey et al., 1997; Bagnyukova et al., 2003, 2006; Ramnanan and Storey, 2005). With these prior findings, and as a liver enzyme near a focal point in antioxidant defence, detoxification, energy production, and biosynthesis, G6PDH was an attractive enzyme for detailed study in the freeze tolerant wood frog.

Several properties of liver G6PDH were notably different between control and frozen frogs. The K_m G6P was significantly higher in liver from frozen frogs, compared with controls. When liver homogenates from both control frogs and frozen frogs were applied to ion exchange chromatography, the presence of two distinct peaks of enzyme activity was revealed. The properties of each peak were similar in both control and frozen extracts. However, the relative proportions of each peak differed between control and frozen frogs. Liver from control frogs showed a dominance of peak I G6PDH. This peak had both a lower K_m G6P and K_m NADP, and mirrored the values determined in crude extracts from control frogs. Conversely, liver extracts from frozen frogs showed a dominance of peak II. This peak displayed a K_m G6P and K_m NADP that were even higher values than those found for crude G6PDH from liver of frozen frogs. Thus, the

kinetic parameters determined in crude homogenates were influenced by the presence of a mixture of the two enzyme forms, as revealed by ion exchange chromatography. A dominance of the higher-affinity peak I in the liver of control frogs with a K_m G6P of 94.1 μ M and K_m NADP of 61.2 μ M, led to a resulting K_m G6P of 98.2 μ M and K_m NADP of 65.5 μ M in crude extracts. A dominance of the lower-affinity peak II in frozen frogs, with a K_m G6P of 171 μ M and K_m NADP of 98.2 μ M, resulted in a net K_m G6P of 121 μ M and K_m NADP of 89.1 μ M in crude extracts.

Additionally, since these two peaks resolved independently on ion-exchange, it appears that freezing triggers a shift in the relative levels of differentially charged enzyme populations. Electrophoresis-based studies confirmed that total G6PDH protein levels did not change from the control to the frozen state, but that phospho-G6PDH levels decreased in frozen frogs. Therefore, the shift in relative proportions of the differentially-charged peaks observed in ion-exchange chromatography is probably due to reversible phosphorylation of G6PDH. This was further confirmed by incubations driving the *in vitro* phosphorylation of G6PDH. Phosphorylation by protein kinases caused a decrease in the K_m G6P (an increase in affinity) in G6PDH isolated from both peak I and peak II. This agrees well with results seen *in vivo*, where G6PDH in control frogs is present in a high phosphate form, as shown by phosphoprotein gel staining, and has high affinity for its substrates. Hence, G6PDH appears to be regulated by reversible phosphorylation, resulting in altered affinity for its substrates. In control frogs, G6PDH is predominantly present in a high-phosphate form and has high affinity for its substrates. The transition to the frozen state results in an overall decrease in the phosphorylation state of G6PDH, without an alteration in G6PDH levels, and a decreased affinity for its substrates.

Previous studies have documented reversible phosphorylation of G6PDH. Phosphorylation by PKA inhibits G6PDH in bovine aortic endothelial cells (Zhang et al., 2000). Other studies agree that phosphorylation by PKA leads to inhibition of mammalian G6PDH (Cosa Rosa et al., 1995; Xu et al., 2005), but a study on estivating snails showed that stimulation of PKA activated the enzyme (Ramnanan and Storey, 2006). In the current study, incubations that stimulated PKG, PKC, AMPK and CaMK all led to a decrease in K_m G6P for G6PDH for both the peak I and II enzymes isolated from ion-exchange chromatography, though PKA stimulation had no apparent effect on substrate affinity. In frozen frogs, however, a shift in population of G6PDH to the low-phosphate form is seen in phosphoprotein gel staining and ion-exchange chromatography. Phosphatases are active in frog liver during the transition to the frozen state. The % active PP-1 rose from 45.9% in control frogs to as high as 78.8% after 1 h of freezing, and began to level off at 66.0% after 12 h of freezing; PP-2A activity also increased by 70.6% after 12 h of freezing (MacDonald and Storey, 1999). Thus, there is a high potential for these phosphatases to dephosphorylate G6PDH.

High concentrations of glucose also had a direct effect on G6PDH activity by inhibition, possibly through an allosteric or competitive mechanism. High glucose (250 mM, similar to *in vivo* amounts in frozen liver) decreased the affinity for G6P for the enzyme from both control and frozen frogs by similar values of ~30% (Table 4.2). Additionally, enzyme assays conducted at low temperature (5°C) showed an increase in K_m G6P of G6PDH from both peaks. The effect of temperature itself is interesting, as a previous study that assessed the affinity of G6PDH for its substrates at different temperatures showed no significant temperature-mediated differences in K_m G6P for

human G6PDH or two Antarctic fish species, *D. mawsoni* and *C. hamatus*, and only K_m NADP from *D. mawsoni* decreased with lowered temperatures (Ciardiello et al., 1997).

Urea-dependent unfolding and inhibition of activity gives a measure of the conformational stability of a protein and this differed between control and frozen frogs. G6PDH from frozen frogs was more susceptible to urea-induced denaturation than was the control enzyme, based on G6PDH activity measurements. However, both enzymes showed similar patterns of urea-dependent unfolding when assessed by their sensitivity to thermolysin degradation (as detected by Western blotting). In frozen frogs, then, structural changes caused by possible dephosphorylation make the enzyme slightly weaker and more easily unfolded by urea, enough to perturb the active site and decrease activity at a lower urea concentration than the control. However, this effect does not seem to be large enough to be reflected in differences in the gross unfolding of the protein at high urea values that provides enough denaturation to expose proteolytic cleavage sites.

Decreases in affinity for G6P by G6PDH, as well as G6PDH stability and activity as a whole, could have important physiological implications in freeze tolerance, since G6P represents an important branch point in glucose homeostasis, and can be used by other pathways preferentially if not consumed by G6PDH and oxidized in the PPP. G6P can be shunted through anaerobic glycolysis to produce ATP as a fuel for sustaining cellular metabolism in the ischemic frozen state. This is particularly necessary in liver which lacks phosphagen reserves that can buffer ATP levels. However, as evidenced by the decrease in liver ATP levels and AEC over cycles of freezing and thawing, either little G6P is being used in anaerobic glycolysis, or it is not enough to compensate for the decline in the AEC (Storey, 1987b). Indeed, in liver, accumulations of both G6P and F6P

are seen, indicating that a clamp on glycolysis occurs at the next step in glycolysis, phosphofructokinase-1 (PFK-1). Furthermore, levels of fructose-2,6-bisphosphate (F2,6P₂), an allosteric activator of PFK-1, decreased, as did the activity and affinity of PFK-2 that synthesizes F2,6P₂ (Storey, 1987a; Vazquez Illanes and Storey, 1993). This would suggest a substantial decrease in G6P utilization by glycolysis; likely the glycolytic clamp observed on PFK ensures that the high G6P levels produced by glycogenolysis are not used in liver glycolysis.

If G6P use by glycolysis is restricted, and the decreased affinity of G6PDH for its substrate also lowers use of G6P by the PPP, then restriction of all these routes would have a net effect of facilitating another fate for G6P – that is dephosphorylation by glucose-6-phosphatase (G6Pase) to make the cryoprotectant, glucose, for export. High concentrations of cryoprotectant are necessary to prevent intracellular freezing in tissues. G6Pase transcript levels increased during freezing (Storey, 2004), and an earlier study showed that liver G6Pase enzyme activity increased by 144% in frozen frogs (Storey and Storey, 1984). Hence, restriction of other fates for G6P coupled with an enhancement of G6Pase would effectively target the bulk of G6P into the pool of cryoprotectant.

The remaining question is whether there is a need for G6P to be oxidized by the PPP during freezing, and this can be examined from several perspectives. Unlike most insect species that use polyhydric alcohols (e.g. glycerol, sorbitol) as cryoprotectants and make these using NADPH generated by the PPP (Storey et al., 1991), wood frogs make glucose as their cryoprotectant by simply dephosphorylating G6P. Hence, the PPP is not needed to support cryoprotectant synthesis by liver. Furthermore, anabolic processes (e.g. biosynthesis, growth, proliferation) are strongly suppressed in the frozen state and so the

need for carbon flow through the PPP to produce five-carbon sugars for nucleotide biosynthesis is also reduced (Storey and Storey, 2004a). However, wood frogs do incur oxidative stress during thawing when blood reperfuses the organs and rapidly reintroduces oxygen and, to deal with this, antioxidant defences are up-regulated in an anticipatory or preventative manner during freezing. This is achieved through increased protein levels and catalytic activities of important antioxidant enzymes (Joanisse and Storey, 1996). Total glutathione peroxidase (GPox) activity increased significantly in muscle, liver, kidney, brain, and heart of frozen frogs, whereas glutathione-S-transferase (GST) activity increased in liver. GPox uses NADPH directly as a substrate, whereas GST uses reduced glutathione that has been produced by the NADPH-dependent glutathione reductase reaction. Sustained production of NADPH is therefore needed in the frozen state to optimize antioxidant potential in preparation for oxygen reperfusion upon thawing. It might seem counterintuitive then that the affinity of G6PDH for G6P is reduced in frozen liver (Table 4.1). However, it is important to remember that, as a result of high rates of glycogenolysis, G6P levels rise drastically to ~7 times the concentration found in control animals over the first hour of freezing, and remain at high levels for at least seven hours (Storey and Storey, 1986; Storey, 1987). Despite lowered G6P affinity, physiological concentrations of substrate are well above those needed for optimal activity, ranging from 0.2 $\mu\text{mol/gww}$ in control frogs, to 1.5 $\mu\text{mol/gww}$ in frozen frogs, and reduced affinity may only be important when G6P levels have begun to drop, as a preventative measure to retain glucose preferentially as a cryoprotectant. Alternatively, it is important to remember that overall differences in affinity arise from shifting the relative populations of the high-affinity versus low-affinity enzyme. Given their

differential phosphorylation states, these two forms of the enzyme could have additional regulatory mechanisms imposed upon them based on phosphorylation state, possibly causing spatial regulation or other factors as well. Further study would be needed to characterize all differences that arise as a result of G6PDH phosphorylation.

In summary, G6PDH was shown to be regulated in the liver of freeze-tolerant frogs. Reversible phosphorylation may be an important regulatory mechanism, as relative proportions of differentially-charged enzymes shift in the transition to the frozen state. Phosphoprotein detection showed G6PDH to be in a lower phosphorylation state in frozen frogs, and assays of both crude tissue extracts and ion-exchange resolved enzyme peaks showed the population of low-phosphate G6PDH had reduced affinity for its substrates. *In vitro* stimulation of protein kinases tends to push kinetic parameters back toward a higher-affinity state. Cold temperatures and high glucose also inhibited G6PDH activity, and G6PDH from frozen frogs showed reduced stability against denaturants. Combining the slight, but significantly decreased affinity for its substrates, inhibition by cold temperature and glucose, and decreased structural stability, this argues for an overall decreased action of G6PDH in frozen frogs.

CHAPTER 5

REGULATION OF SKELETAL MUSCLE HEXOKINASE IN THE FREEZE-TOLERANT WOOD FROG, *RANA SYLVATICA*

INTRODUCTION

Carbohydrate metabolism is a key element in survival for all living organisms. Glucose is virtually universally recognized as a central compound in carbohydrate metabolism, and glucose homeostasis as a whole is highly regulated in physiology (Zierler, 1999; Saltiel and Kahn, 2001). An important enzyme in glucose homeostasis is hexokinase (HK; E.C. 2.7.1.1). HK is an ATP-dependent enzyme that phosphorylates glucose to produce glucose-6-phosphate (G6P) in the reaction: $\text{glucose} + \text{ATP} \rightarrow \text{G6P} + \text{ADP}$. G6P, in turn, has multiple fates. G6P can be catabolised via glycolysis and the tricarboxylic acid cycle to meet energetic and anapleurotic needs. Depending on the tissue, G6P can be oxidized via the pentose phosphate pathway to provide sugar phosphate precursors and NADPH for a range of biosynthetic pathways as well as antioxidant defence. G6P can also be converted to UDP-glucose for polymerization into glycogen, the main storage form of carbohydrates in animal physiology. Phosphorylation of glucose is typically the committed step in one of these fates. Indeed, to move sugar between organs or tissues, G6P has to be dephosphorylated back to glucose before export, as cells are impermeable to the phosphorylated sugar.

In addition to these dependencies on glucose, this sugar is also the cryoprotectant needed for survival in the freeze-tolerant wood frog, *Rana sylvatica*. At the onset of freezing, liver glycogenolysis and increased membrane transport capacity raises glucose concentrations in all organs and tissues, as high as 250 times the normal concentration of glucose present in blood (Storey and Storey, 1984; 2004; King et al., 1993). Freezing survival exerts an energetic strain, and over time, the AEC gradually decreases (Storey, 1987b). Even under anaerobic conditions, each mole of glucose can still produce two

moles of ATP, capable of resupplying ATP to decreasing energy reserves so it is possible that a small proportion of the glucose accumulated for cryoprotection is actually catabolised during long term freezing to support cellular energy production.

Hexokinase is an enzyme shown to be regulated by multiple mechanisms in response to potential perturbations to glucose homeostasis. It can be regulated at the transcriptional (Osawa et al., 1996; Kim and Dang, 2003), and post-translational levels (Huber and Cardin, 2004). The enzyme can assemble into complexes with other enzymes of the glycolytic pathway, often in response to anoxia and ischemic stresses, in cancer cells (Bartrons and Caro, 2007), as well as animals that enter torpor in response to these physiological stresses (Brooks and Storey, 1990; Duncan and Storey, 1992; Lushchak et al., 1998a). Signal transduction pathways are also known to regulate hexokinase. Most studies have focused on how signal transduction cascades ultimately regulate HK expression (Long et al., 2005; Thomson et al., 2008), but several studies have shown how the hexokinase enzyme itself can be regulated by reversible phosphorylation (Lewandrowski et al., 2008; Miyamoto et al., 2008; Randez-Gil et al., 1998; Fernández et al., 1988; Abnous and Storey, 2008).

The aim of this study is to determine how HK is regulated to balance cryoprotectant and energetic needs in *Rana sylvatica*.

MATERIALS AND METHODS

Animals and Biochemicals

Male wood frogs (*Rana sylvatica*) were treated as previously described in Chapter 2. All chemicals came from the same sources as described in Chapter 2.

Preparation of tissue extracts

Samples of frozen skeletal muscle were quickly weighed and mixed 1:5 w:v in ice-cold buffer A (50 mM Tris, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 10 mM β -mercaptoethanol, 10% v/v glycerol, pH 7.0) with a few crystals of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF) added and then immediately homogenized and centrifuged as described in Chapter 2. Supernatants were then centrifuged through small columns of Sephadex G-50 to remove endogenous enzyme effectors, as described in Chapter 2.

Enzyme Assay and Kinetic Parameters

Assays used a Dynatech MR5000 microplate reader at 340 nm, as previously described (Abnous and Storey, 2008). Optimal assay conditions were determined to be 50 mM Tris buffer (pH 8.2), 2 mM glucose, 1 mM ATP, 0.5 mM NADP⁺, 10 mM Mg²⁺, 1 U of G6PDH, and 5 μ l of tissue extract in a 200- μ l final volume. One unit of enzyme activity is defined as the amount that produces 1 μ mol of NADPH per minute at 25°C. Enzyme kinetic constants were determined using a nonlinear least-squares regression computer program (Brooks, 1992); substrate affinity data were fitted data to Michaelis-Menten plots.

In vitro incubations to stimulate endogenous kinases and phosphatases

To assess the effect of reversible phosphorylation on HK, aliquots of enzyme extract were incubated under conditions described in Chapter 2. Samples were incubated

overnight at 4°C. Following incubation, samples were centrifuged through spun columns of Sephadex G-50, as previously described, and assayed.

To confirm that the treatments used to stimulate protein kinases actually resulted in the phosphorylation of HK, tissue extracts were radiolabeled and immunoprecipitated as described in Chapter 3. Tissue extracts (1:5 w:v) were prepared in buffer A as above, and then treated to fully dephosphorylate HK as. Incubations were then prepared to stimulate specific HK phosphorylation by specific protein kinases, with the inclusion of γ -³²P-ATP. HK was then immunoprecipitated with the HK II antibody and IPA, and spotted onto P81 paper. The paper was exposed to a phosphor screen, and quantified using a Personal Molecular Imager-FX (BioRad).

Western blotting

Skeletal muscle extracts from both control and frozen frogs were prepared and samples containing 5 µg of protein was electrophoresed, and transferred to a PVDF membrane as described in Chapter 3. Membranes were blocked in 5% skim milk in TBST for 2 h, and then washed three times with TBST. Membranes were incubated with rabbit primary antibody raised against muscle HKII (HK goat polyclonal IgG) (Santa Cruz) overnight at 4°C. After washing with TBST three more times, membranes were incubated with secondary antibody (mouse anti-rabbit IgG) for 1 h and then washed. Immunoreactive bands were visualized with the enhanced chemiluminescence assay using a ChemiGenius (SynGene, MD, USA) and band intensities were quantified using the associated Gene Tools program.

Phosphoprotein staining

SDS-PAGE gels were run as described above, except that 1 μg of total protein was loaded in each well. Gels were stained with the Pro-Q Diamond Phosphoprotein Gel Stain (Molecular Probes), according to the manufacturers' instructions. Phosphoprotein bands were visualized using UV transillumination with the ChemiGenius and band intensities were quantified.

Structural stability of HK using pulse proteolysis

The method of Park and Marqusee (2005) was used. Aliquots of 20 μl (~ 400 μg total protein) from crude muscle extracts (1:5 w:v homogenization in buffer A without PMSF) were incubated overnight at room temperature with 100 μl of buffer A containing different concentrations of urea, and subjected to pulse proteolysis as described in Chapter 3. Each sample was then subjected to Western blotting as described above to assess the amount of folded protein remaining.

RESULTS

Activity and kinetic parameters of HK in crude muscle extracts

Initial studies of HK from muscle of *Rana sylvatica* optimized homogenization conditions for retrieving maximum HK activity. Inclusion of protein kinase inhibitors (EDTA/EGTA) or the protein phosphatase inhibitor NaF in the homogenization buffer (buffer A) did not affect the measurable amount of HK, but inclusion of 50 mM β -glycerophosphate (βGP) decreased HK activity by $\sim 40\%$; thus, βGP was omitted from all buffers. The pH optimum of the HK reaction was determined to be 8.0 ± 0.1 for the

enzyme from control frogs, and 8.4 ± 0.1 for HK from frozen frogs ($n = 4$ determinations on separate preparations of enzyme) (Figure 5.1). However, over 90% of maximal activity was observed in the range of pH 8.1-8.6 for control frogs, and pH 7.9-8.4 for frozen frogs. HK activity decreased gradually at pH values above the optimum, but decreased sharply at pH values below the optimum. At pH 8.8, HK from control frogs retained ~83% of optimal activity, versus ~73% for frozen frogs. However, only ~28-29% of optimal activity remained by pH 7.6 for HK from both control and frozen frogs, and by pH 6.8, HK activity was no longer detectable.

The maximum activity of HK in soluble extracts of skeletal muscle was 86.1 ± 4.0 mU/mg protein (0.23 ± 0.03 U/gww) in control frogs and decreased to 52.2 ± 4.9 mU/mg in muscle of frozen frogs ($n = 8$) (Table 5.1). The velocity versus [glucose] relationship was hyperbolic for the enzyme from both control and frozen frogs. The K_m glucose was ~73% higher and significantly different ($P < 0.05$) for the enzyme from frozen frogs, compared with control HK (Table 5.1). Similar results were seen for the velocity versus [ATP] relationship: the relationship was hyperbolic and K_m ATP was ~33% higher and significantly different ($P < 0.05$) for the frozen enzyme.

Reversible phosphorylation of HK

Stable kinetic differences in the properties of an enzyme between two physiological states are often the result of state-dependent reversible phosphorylation catalyzed by protein kinases and protein phosphatases. To determine if this mechanism might be responsible for the differences in HK properties between control and frozen states described above, crude muscle homogenates from control and frozen frogs were

incubated overnight under conditions that stimulated either protein phosphorylation or dephosphorylation, followed by passage through spun-columns to remove extraneous metabolites, and then the resulting effects on HK kinetics were measured. With one exception, incubations that promoted protein kinase action affected only the enzyme from frozen frogs and increased apparent affinity for both substrates (ie. decreased K_m glucose and K_m ATP) (Figure 5.2A, 5.3A). Stimulation of specific protein kinases indicated that the action of PKA, PKC, and AMPK all facilitated a decrease in K_m glucose and K_m ATP for HK from frozen frogs. Decreases in K_m glucose of 48.2%, 51.8%, and, 52.6% were observed as a result of stimulation of PKA, PKC, and AMPK, respectively (Figure 5.2A). Decreases in K_m ATP of 32.3%, 35.4%, and 34.9% were observed as a result of stimulation of PKA, PKC, and AMPK, respectively (Figure 5.3A). Stimulation of PKC alone had a significant effect on HK from control frogs, decreasing K_m glucose by 21.2%.

Comparable incubations were set up to stimulate the activity of protein phosphatases. Incubations that promoted phosphatase action affected only HK from control muscle and decreased apparent affinity for both substrates (ie. increased K_m glucose and K_m ATP) (Figure 5.2B; 5.3B). Stimulation of specific protein phosphatases indicated that the action of PP1, PP2A and PP1 combined, and PP2B, as well as stimulation of total protein phosphatases, all facilitated an increase in K_m glucose and K_m ATP in HK from control frogs. Stimulation of total protein phosphatases increased K_m glucose by 52.6% and K_m ATP by 32.2%. Increases in K_m glucose of 57.2%, 55.5%, and 60.8% were observed as a result of stimulation of PP1, PP2A/PP1 and PP2B, respectively (Figure 5.2B). Increases in K_m ATP of 45.9%, 38.4% and 35.8% were observed as a result of stimulation of PP1, PP2A/PP1 and PP2B, respectively (Figure 5.3B). Stimulation of

protein phosphatases had no significant effect on HK from frozen frogs.

To confirm that the incubations that stimulated protein kinases actually resulted in the phosphorylation of HK, muscle extracts from both control and frozen HK were first treated to dephosphorylate the enzyme and were then incubated under phosphorylating conditions but this time in the presence of γ - ^{32}P -ATP. Radiolabeled HK was then immunoprecipitated and quantified by phosphorimaging. These studies showed that incubations that stimulated PKA, PKC, and AMPK all resulted in high levels of ^{32}P labelling of the precipitated HK-antibody-IPA complex. Relative to the stop incubation without added stimulators of protein kinases, ^{32}P labelling of HK from control frog muscle was 2.03-, 1.86-, and 2.40-fold higher in incubations that stimulated PKA, PKC, or AMPK, respectively (Figure 5.4). ^{32}P labelling of HK from frozen frog muscle was 2.11-, 1.93-, and 2.48-fold higher in incubations that stimulated PKA, PKC, or AMPK, respectively

HK total protein and phosphoprotein levels

Relative levels of total HK protein in muscle from control and frozen frogs were assessed by Western blotting using a primary antibody raised against muscle HKII. A single, strong band was identified at ~100 kDa, the expected molecular weight of the vertebrate enzyme. Total HK protein levels did not change significantly in the transition from the control to the frozen state (Figure 5.5). The amount of phosphoprotein was then assessed with the Pro-Q Diamond phosphoprotein gel stain. The amount of phosphoprotein staining of the 100 kDa band was strongly reduced by 33% in the transition to the frozen state.

Temperature and cryoprotectant effects

HK activity was assayed in the presence versus absence high concentrations of glucose, the cryoprotectant that is present in high concentrations in tissues of frozen frogs. In the case of HK, glucose is not only the cryoprotectant within frog tissues, but also the substrate of this enzyme. Activity was also assessed at high (25°C) and low (5°C) temperatures. Addition of high glucose (250 mM) at high temperature had no effect on HK activity or substrate affinity from either control or frozen frogs (Table 5.2). At low temperature, K_m ATP increased in both control and frozen frogs by ~50% in each. An interesting effect observed when combining high concentrations of glucose and cold temperatures, was that high concentrations of glucose prevented the increase in K_m ATP that was seen with low temperatures alone.

Structural stability of HK

The effect of urea on HK unfolding was assessed using the pulse proteolysis method. Urea-induced unfolding of a protein typically causes a loss of enzymatic activity and makes the enzyme more sensitive to thermolysin proteolysis; hence, when subsequently run on Western blots, the amount of HK protein detected at the 100 kDa subunit size declined when proteolysis occurs. As Figure 5.6 shows, HK protein from both control and frozen frogs was structurally stable up to urea concentrations of ~4 M as evidenced by the strong bands detected on Western blots. At higher urea concentrations, however, detectable protein at 100 kDa decreased greatly. Control HK was still strongly visible up to 4.4 M urea, but band intensity began to decrease at higher concentrations

and was largely gone at 5.2 M. In frozen frogs, HK activity was high until 4.0 M and then began to drop and was largely gone at 4.8 M. Hence, structural stability against urea was greater for control HK. Figure 5.6 also shows the effects of rising urea on HK activity. HK activity from control frog muscle remained at ~100% with the addition of up to 3 M urea, and began to decline thereafter. In frozen frogs, HK activity was significantly reduced in the presence of 2 M urea, and decreased quickly thereafter. The calculated urea concentration for loss of half of the activity (I_{50} urea) was 5.13 ± 0.09 M ($n = 3$) for control HK and 4.53 ± 0.17 M ($n = 3$) for the enzyme from frozen frogs, values that were significantly different, $P < 0.05$ (Table 5.1).

Table 5.1 Kinetic parameters of skeletal muscle HK from control and frozen frogs.

	Control	Frozen
V_{max} (mU/mg protein)	86.1 ± 0.40	52.0 ± 0.49^a
K_m Glucose (μ M)	144 ± 4.4	248 ± 12.0^a
K_m ATP (μ M)	248 ± 8.5	330 ± 20.9^a
I_{50} Urea	5.31 ± 0.09	4.53 ± 0.17^a

Assays were conducted at 25°C and data are means $\pm$ SEM, $n = 8$ for values from control and frozen frogs.

^a Significantly different from the corresponding control value in skeletal muscle as determined by the Student's t -test, $P < 0.05$.

Table 5.2 Effect of assay temperature and cryoprotectant on the K_m glucose and K_m ATP for muscle HK from control and frozen frogs

	Control		Frozen	
	K_m Glucose	K_m ATP	K_m Glucose	K_m ATP
25°C, no glucose	144 ± 4.4	248 ± 8.5	248 ± 12.0	330 ± 20.9
25°C, 250 mM glucose	ND	239 ± 5.2	ND	342 ± 12.3
5°C, no glucose	139 ± 2.5	364 ± 12.8 ^a	253 ± 8.6	505 ± 18.5 ^b
5°C, 250 mM glucose	ND	257 ± 5.4	ND	336 ± 13.2

Data are means ± SEM from $n = 4$ individual frogs. ^a Significantly different from the corresponding control value at 25°C without glucose as assessed by the Student's t -test, $P < 0.05$; ^b Significantly different from the corresponding frozen value at 25°C without glucose as assessed by the Student's t -test, $P < 0.05$. ND; not determined.

Figure 5.1 Effect of pH on HK activity. HK from crude homogenates was assayed under optimal conditions at varying assay buffer pH. Data are means $\pm$ SEM, for determinations on extracts from n = 4 individual frogs.

Figure 5.2 Effect of in vitro incubations promoting the action of (A) specific protein kinases or (B) protein phosphatases on K_m glucose of HK from frog skeletal muscle extracts.

Incubations promoted endogenous (A) protein kinase activities including PKA, PKG, PKC, AMPK, and CaMK, or (B) protein phosphatase activities including PP-1, PP-2A, PP-2B, and PP-2C. Samples were incubated overnight at 4°C, as outlined in the Materials and Methods, followed by spun-column and assay. Data are means $\pm$ SEM, $n = 4$ independent preparations of enzyme from individual frogs. ^a Significantly different from the corresponding control incubation in stop buffer (no additions) using the Student's *t*-test, $P < 0.05$; ^b significantly different from the corresponding incubation in stop buffer, $P < 0.05$.

A **B**

Figure 5.3 Effect of in vitro incubations promoting the action of (A) specific protein kinases or (B) protein phosphatases on K_m ATP of HK from frog skeletal muscle extracts.

Incubations promoted endogenous (A) protein kinase activities including PKA, PKG, PKC, AMPK, and CaMK, or (B) protein phosphatase activities including PP-1, PP-2A, PP-2B, and PP-2C. Samples were incubated overnight at 4°C, as outlined in the Materials and Methods, followed by spun-column and assay. Data are means $\pm$ SEM, $n = 4$ independent preparations of enzyme from individual frogs. ^a Significantly different from the corresponding control incubation in stop buffer (no additions) using the Student's *t*-test, $P < 0.05$; ^b significantly different from the corresponding incubation in stop buffer, $P < 0.05$.

A **B**

Figure 5.4 Effect of in vitro incubations promoting the action of specific protein kinases on HK radiolabeling in soluble extracts of frog skeletal muscle. Enzyme extracts were incubated overnight at 4°C with γ -³²P-ATP, as outlined in Materials and Methods, followed by immunoprecipitation, spotting, and phosphorimaging. Data are means $\pm$ SEM, $n = 3$ independent preparations of enzyme from different frogs. ^a Significantly different from the corresponding control extract in stop buffer (no additions) using the Student's *t*-test, $P < 0.05$; ^b significantly different from the corresponding frozen extract in stop buffer, $P < 0.05$.

Figure 5.5 Relative levels of total HK protein, and the phosphorylated form of HK, in control (5°C acclimated) and frozen (-3°C for 24 h) frogs as determined by SDS-PAGE followed by Western blotting or Pro-Q Diamond Phosphoprotein staining, respectively.

Data are means $\pm$ SEM, for determinations on extracts from $n = 4$ individual frogs. ^a

Significantly different from the corresponding value in control frogs as assessed by the Student's *t*-test, $P < 0.05$.

Figure 5.6 Structural stability of skeletal muscle HK from control versus frozen frogs assessed by susceptibility to urea denaturation. Crude extracts were incubated for 24 h with different concentrations of urea. Aliquots were assayed for activity, or subjected to pulse proteolysis to degrade denatured HK (10 min incubation with thermolysin) and then Western blotting to measure the amount of native folded HK remaining, as outlined in Methods and materials. Activities are means $\pm$ SEM, $n = 4$ independent preparations; the Western blot is representative of $n = 3$ independent trials.

DISCUSSION

Freeze-tolerance requires numerous metabolic adaptations in order to facilitate survival. As a result of these adaptations, stresses are placed on i) energy metabolism, and ii) glucose homeostasis (Storey and Storey, 2004b; Storey and Storey, 1988). Hexokinase is an interesting enzyme for study, because is at a locus in metabolism that is contributing to balancing energy needs by anaerobic glycolysis, as well as maintaining glucose needed for cryoprotectant levels in freezing.

Results of the current study show that skeletal muscle HK activity and apparent affinity for both its substrates are lower for the enzyme in frozen frogs, than for control HK. This suggests that HK is regulated to limit its activity in the frozen state. Studies of the phosphorylation state of HK, by phosphoprotein gel staining, as well as by *in vitro* incubations to drive reversible phosphorylation, further revealed that after 24 hours of freezing *in vivo*, the HK found in muscle of frozen frogs was in a lower phosphorylation state than control HK. *In vitro* stimulation of endogenous protein phosphatases revealed that PP-1, PP-2B, and possibly PP-2A could potentially dephosphorylate HK and decrease its affinity (elevate K_m) for both of its substrates. Phosphatase treatment affected only HK in control extracts and altered enzyme kinetic parameters to resemble those seen for HK from frozen frogs. The lack of phosphatase effects on frozen HK is consistent with the results from phosphoprotein gel staining indicating that HK in frozen muscle is already in a low phosphate state. Oppositely, stimulation of PKA, PKC, and AMPK increased HK affinity for its substrates (lowered K_m), and altered kinetic parameters of the enzyme from frozen muscle to resemble those found for HK from control frogs. Overall, then, the data demonstrate that HK in control frogs is in a high activity, high

affinity, and high phosphate state whereas in muscle of frozen frogs, HK has lower activity, lower affinity for its substrates, and is present in a lower phosphorylation state.

In addition to different phosphorylation states, activities, and substrate affinities, a differential response to pH was observed. HK from control frog muscle had a higher pH optimum for maximum activity, pH 8.4, compared pH 8.0 for the enzyme from frozen frogs. Interestingly, HK from both animals showed a similar, sharp decline in activity the pH range of 7.0-7.8, the physiological pH range in wood frog skeletal muscle. Although lactate levels have been shown to increase during freezing (Storey and Storey, 1984; Storey, 1987b), studies involving NMR to measure intracellular pH during freezing have shown that pH remains in a range between 7.2-7.6 (Layne and Kennedy, 2002). This was considered to be a stable pH by these authors, but with respect to HK, transient pH changes in this range may have profound effects on enzyme activity in both control and frozen frogs, based on current findings in Figure 1. Local accumulations of lactate, and resulting decreases in pH, within a microenvironment in muscle cells could inhibit HK activity and alter substrate effects. For instance, in flight muscle of *Dipetalogaster maximus*, HK exhibits a optimal activity at a rather high pH (8.0 or above), and pH values of 7.6 and lower have been shown to decrease HK maximal activity, and additionally cause substrate-induced inhibition by ATP (Scaraffia and Gerez De Burgos, 2000).

HK from both control and frozen frogs showed a high degree of stability against urea, a non-compatible osmolyte, remaining stable to at least 2 M before showing any decrease in activity. HK from control frogs had slightly better stability compared to frozen frogs, by about 0.5 M. This high stability could have important implications in

situations where high levels of osmolytes such as urea, ions, or polyols are required to prevent intracellular dehydration and freezing. Dehydration, or compaction caused by high concentration of osmolytes, could cause HK denaturation and loss of activity. Yeast HK was found to have decreased affinity for its substrates and increased maximal activity with the addition of urea, possibly due to increased enzyme flexibility and resulting lowered energy barriers in the catalytic cycle. This was largely negated in the presence of a crowded system of added BSA (Olsen et al., 2007). Interestingly, although the dominant osmolyte in wood frog freeze-tolerance is glucose, high concentrations of glucose (250 mM) seem to have no effect on HK substrate affinity of ATP (Table 5.2). The lack of inhibitory effect of high glucose concentrations is in itself an interesting finding, since the activity of at least one mammalian isozyme of HK, HK III, is inhibited by concentrations of glucose as low as 1 mM (Wilson, 2003). Lack of activity inhibition may be beneficial in this unique physiology, as HK appears to function even under hyperglycaemic conditions. The lack of inhibition by glucose may have structural stability implications as well; polyols have been shown to stabilize HK against denaturation by high temperature and by non-compatible osmolytes (Tiwari and Bhat, 2006). Thus, glucose may protect HK against a build-up of other, non-compatible osmolytes caused by intracellular dehydration.

Additionally, high intracellular glucose may counteract any inhibition of HK caused by cold temperatures. Other enzymes studied in this thesis showed decreases in substrate affinity with decreasing temperature. In these previous cases, high glucose either independently contributed to decreases in substrate affinity, or potentially compounded the effects of low temperature to decrease affinity further. Cold temperature

caused reduced the ATP affinity of HK from muscle of both control and frozen frogs. However, in the presence of high concentrations of glucose, kinetic parameters of HK did not change when assayed at cold temperature (Table 5.2). This suggests that in addition to glucose not inhibiting HK, the sugar can preserve HK activity and substrate affinity at the cold temperatures encountered during freezing.

HK has been shown previously to be regulated by reversible phosphorylation, particularly as a result of subcellular organization with the mitochondrial membrane, as evidenced in rat brain mitochondria (Lewandrowski et al., 2008). This can have the effect of causing conformational changes, and modulating HK activity at the mitochondrial membrane where adenine exchange can allow passage for nucleotides directly to and from the HK active site. In neonatal rat ventricular myocytes, Akt can phosphorylate HK as a protective measure against oxidant- or Ca^{2+} -stimulated mitochondrial pore opening (Miyamoto et al., 2008). In yeast, the presence of easily fermentable carbon sources, such as glucose, can lead to dephosphorylation and dimerization, whereas lack of substrate results in the dominance of phosphorylated monomers (Randez-Gil et al., 1998). Substrate-induced autophosphorylation has also been suggested as a mechanism for HK control in yeast (Fernández et al., 1988). In hibernating ground squirrels, HK is dephosphorylated and substrate affinity is decreased, whereas higher phosphorylation state and affinity are seen in euthermic squirrels (Abnous and Storey, 2008). Control of HK by post-translational modification would be valuable in freeze-induced hypometabolism where all energy-expensive cell functions are suppressed (Storey and Storey, 2004a), and glucose phosphorylation must be tightly regulated as physiological needs and conditions change. Reversible phosphorylation of HK could be important for

balancing the need to maintain glucose in the cryoprotectant pool versus catabolising glucose for energetic and oxidative stress requirements by providing the mechanism to interconvert an existing pool of HK between low and high activity states.

The present data indicate that freezing converts muscle HK to a form that has lower activity and lower affinity for its substrates, probably arising from dephosphorylation of the enzyme. At least one major phosphatase, PP-1, is freeze-activated in wood frog skeletal muscle and has the potential to dephosphorylate HK. The percentage of PP-1 in the active state rose to 100% during the first 20 minutes of freezing in wood frog muscle, and then decreased somewhat with longer freezing, suggesting that it is an important regulator of events in frozen muscle (MacDonald and Storey, 2002). Although PKC and AMPK are both active after 24 hours of freezing (Holden and Storey, 1996; Rider et al., 2006), and can phosphorylate HK, the results of this study show that HK is more susceptible to dephosphorylation in the frozen state. High activity of both kinases and phosphatases over long-term freezing would seem to contrast one another, and the low phosphorylation state of HK argues that it is phosphatase activity, and dephosphorylation, that prevails over protein phosphorylation of HK. Protein targeting and subcellular organization may be a factor. As there are protein subunits that target phosphatases to glycogen in wood frogs (MacDonald and Storey, 2002; 2007), other subunits are known to target phosphatases to HK in yeast (Alms et al., 1999). Thus, depending on which signalling enzymes are being targeted to HK, it may be present in a high or low phosphate form *in vivo*. A future study may investigate which kinases or phosphatases are targeted to HK as whole-body freezing progresses.

This study has shown that HK in *Rana sylvatica* muscle is regulated during

freezing to be converted to a lower activity and lower substrate affinity form. Reversible phosphorylation appears to be the mechanism involved and allows an existing pool of HK to be interconverted between a high-activity, high-affinity form in control frogs and a low-activity, low-affinity form in frozen frogs. In addition, HK is influenced by intracellular pH, temperature, and intracellular glucose concentration. The results argue that HK is less active in frozen frogs, and that this aids the maintenance of high cryoprotectant pools of glucose.

CHAPTER 6

GENERAL DISCUSSION

For a variety of ectotherms that encounter subzero temperatures as part of their normal life cycle, freeze-tolerance is a key to their survival (Costanzo et al., 2000; Andersson and Johansson, 2001; Voituron et al., 2002; Voituron et al., 2003; Sinclair et al., 2004; Storey and Storey, 2004c; Sinclair and Chown, 2005; Holmstrup and Overgaard, 2007; Overgaard et al., 2007b; Bazin et al., 2007). For the wood frog, *Rana sylvatica*, subzero temperature causes animals to seek shelter on the forest floor, further insulated by falling leaves and snow (Schmid, 1982). Whole-body freezing then occurs, and as much as 65-70% of body water is converted to ice in extracellular and extra-organ spaces. All physiological processes are reduced as part of a total metabolic rate depression; heartbeat, respiration, and brain activity are all below detectable limits (Storey and Storey, 2004b; 2004c). Freezing may last for days or weeks, through the whole winter, as long as subzero temperatures persist, although freeze-thaw cycles may be observed in periods as winter sets in, as well as during the spring thaw (Storey, 1987b).

Numerous metabolic adaptations allow the wood frog to survive. These include the conversion of most of the body's glycogen into glucose in order to function as a cryoprotectant (Storey and Storey, 1985; 1986; Storey, 1987a, Crerar et al., 1988). This serves two functions: i) prevention of intracellular freezing (Storey and Storey, 1996) and, ii) colligative action to retain water inside cells and prevent a decrease in cell volume below a critical minimum (Churchill and Storey, 1993). In addition to changes in carbohydrate metabolism, other adaptations allow for freezing survival. Gene transcription and protein synthesis are mostly halted, as are most other anabolic processes (Storey and Storey, 2004c). ATP-driven ion-motive forces, responsible for the majority

of ATP consumption, are also depressed (Hemmings and Storey, 2001). However, some processes and biochemical reactions must continue, to ensure continued survival during the frozen period, as well as the further metabolic strain associated with arousal and recovery. To allow non-damaging ice formation in extracellular space, ice-nucleating proteins are synthesized, as are proteins that manage water/osmotic stresses imposed by freeze-thaw and dehydration-rehydration (Wolanczyk et al., 1990; Storey et al., 1992; 1997; Churchill and Storey, 1994). Transcription of several genes, such as ADP/ATP translocase and fibrinogen (Cai et al., 1997; Cai and Storey, 1997a), as well as the freeze-specific genes, *fr10*, *li16*, *fr47* are up-regulated (Cai and Storey, 1997b; McNally et al., 2002; 2003). Both transcription and translation of the acidic ribosomal phosphoprotein, and the mitochondrial phosphate carrier, are also upregulated (Wu and Storey, 2005; De Croos et al., 2004). As respiration has decreased below a detectable limit, no oxygen is available for oxygen-based catabolism of carbohydrates, lipids, and amino acids, nor can oxidative phosphorylation function. As such, energy production under anoxic and ischemic conditions is limited to anaerobic glycolysis (Storey and Storey, 1986; Storey, 1987b). At only two moles of ATP for each mole of glucose, anaerobic glycolysis may not be suited to keep pace with lowered, albeit still present energy demands. Further limitations are imposed by the need to maintain glucose as a cryoprotectant.

The energy strain encountered during the freezing process is further compounded by thawing and arousal. Oxygen reperfusion, after several weeks/months of no detectable respiration, leads to a sudden elevation of radical oxygen species, imposing an oxidative stress that must be met for survival (Joannis and Storey, 1996). Further demands are placed upon the re-establishment of ion gradients necessary for muscular movement

(Hemmings and Storey, 2001). A direct effect on the energy state of cells can be seen as a result of freezing and thawing. ATP levels and/or levels of the total adenylate pool may drop as a result of metabolic reorganization (Storey and Storey, 1984; Storey, 1987b). The Adenylate Energy Charge (AEC) $[ATP + 0.5ADP] / [ATP + ADP + AMP]$ is the important determinant in cellular energetics, and a high ratio of ATP to total adenylates must be present in order to preserve cell viability.

A number of previous studies have shown that metabolic regulation in freeze tolerance depends to a large extent on the regulation of an existing pool of enzymes or proteins and modifying their behaviour at the functional or post-translational level (Crerar et al., 1988; Vazquez Illanes and Storey, 1993; Holden and Storey, 1996; Greenway and Storey, 2000; Holden and Storey, 2000; MacDonald and Storey, 2002; Woods and Storey, 2006; Rider et al., 2006). Adjustment of enzyme activity by protein synthesis and degradation is energetically expensive, but regulation of an existing pool, without the alteration of total levels, is a strategy that conserves energy (Storey and Storey, 2004b; 2004c). Because of this, I undertook a series of studies of enzymes in this thesis, analyzing the properties of several enzymes of energy metabolism in control versus frozen states in wood frog tissues. Each enzyme was chosen because of its potentially key role in freeze-tolerance and its relation to energy metabolism, glucose utilization, or possibly the interrelationship between both.

AMPD is an enzyme involved in cellular energetics and the regulation of the relative concentration of adenylates. AMPD catalyzes the irreversible deamination of AMP to remove it from the adenylate pool. This helps to raise the AEC by decreasing the total adenylate pool, as well as drive the adenylate kinase reaction by removing its AMP

product (Chapman and Atkinson, 1973). Creatine kinase has a central role in cellular energetics, buffering ATP concentrations and mediating the PCr/Cr shuttle to move ATP equivalents around the cell from sites of ATP synthesis, in the mitochondria, to sites of consumption (Wallimann et al., 1992). G6PDH is the first enzyme in the pentose phosphate pathway, irreversibly oxidizing G6P and producing NADPH as a result (Kleitzen et al., 1994). NADPH is needed to provide an overall reducing environment for oxidative stresses encountered during the anoxia of freezing, and the oxygen reperfusion of thawing (Joanisse and Storey, 1996). HK phosphorylates intracellular glucose and commits to metabolic reactions; the cell membrane is impermeable to G6P and so it cannot leak out of the cell until dephosphorylated. G6P can then be catabolised in glycolysis (Wilson, 2003) or used by other pathways. Each of these enzymes is therefore likely to play its own important role in freeze tolerance.

As two enzymes that help to control adenylate metabolism, both AMPD and CK were found to be regulated in skeletal muscle of wood frogs. These two enzymes may each have a role in maintaining the AEC in the frozen state. AMPD, by catalyzing the degradation of AMP, removes that end product from the adenylate pool, and reduces the total adenylate pool relative to the highest-energy adenylate, ATP, thus raising the AEC. Removal of AMP from the adenylate pool also helps to drive the AK reaction toward the direction of ATP synthesis. Conversely, CK acts to regenerate ATP, without altering the size of the total adenylate pool. CK draws upon PCr reserves to convert ADP back into ATP. Depending on physiological needs, both of these enzymes can have a profound effect on cellular energetics and long-term survival. AMPD was found to be regulated by a combination of several mechanisms. The binding of AMPD to frog skeletal muscle

myofibrils is one mechanism that alters its behaviour, and caused a change from Hill to Michaelis-Menten kinetics. Additionally, only bound AMPD was differentially regulated in control versus frozen frogs; bound AMPD showed reduced substrate affinity in the transition to the frozen state, whereas the soluble form of AMPD showed no difference in kinetic parameters between control and frozen states. Furthermore, once bound, AMPD was subsequently regulated by reversible phosphorylation. This is a regulatory mechanism that was originally proposed for AMPD in exercising trout (Lushchak and Storey, 1994), yet it is a valid strategy for animals with depressed metabolism. Phosphorylation by endogenous protein kinases such as PKA, PKC, CaMK, and AMPK reduced substrate affinity (increased K_m AMP) for AMPD from control frogs, whereas dephosphorylation by endogenous protein phosphatases increased substrate affinity in AMPD from frozen frogs. Bound AMPD from frozen frogs was also more inhibited by low temperatures and high concentrations of glucose than the enzyme in control frogs. Bound AMPD in frozen frogs is the only form that is uniquely inhibited by IMP, the product of the reaction. With the addition of these factors together, this argues for an inhibition of AMPD activity in the frozen state.

Frog muscle CK was regulated by similar mechanisms to AMPD. CK also displayed different kinetic parameters between the control and frozen states. However, whereas AMPD affinity for its substrates decreased in the frozen state, CK activity and substrate affinity both increased. Additionally, CK, like AMPD, is regulated by reversible phosphorylation in frogs enduring freezing. PKA, PKC, PKG, AMPK, and CaMK were all capable of phosphorylating CK *in vitro*, and CaMK and AMPK had detectable effects on maximal activity. Phosphorylation of CK by these two kinases increased activity of

CK, as well as its affinity for one of its substrates, creatine. Conversely, dephosphorylation of CK decreased activity and its affinity for creatine. Overall, this argues for an increased and important role for CK in energy homeostasis as part of freezing survival.

It is interesting that regulation by reversible phosphorylation should have opposite effects on CK and AMPD, two enzymes that can regulate adenylate metabolism and directly affect the AEC. Two of the protein kinases tested were capable of directly affecting AMPD and CK activity and/or affinity by phosphorylation: CaMK and AMPK. Phosphorylation by these kinases increased CK function, by increasing both activity and substrate affinity, but decreased AMPD function, by decreasing its substrate affinity. This could imply a coordinated regulation of these two enzymes in such a way that will optimize the AEC, while still maintaining the total adenylate pool at a reasonable size. As ATP levels begin to drop, and the AEC declines, CK can mobilize stored energy reserves, in the form of PCr, to replenish ATP. Only when PCr reserves have been depleted, and AMP levels begin to accumulate to high concentrations, relative to ATP concentrations, will AMP finally begin to be deaminated. This strategy ensures that the adenylate pool will be drained only when no other fuel reserves are left to replenish ATP. AMPD and CK have been implicated to function together, and the activity of one of the two enzymes compensates for the loss of the other (Tullson et al., 1998; Steeghs et al., 1998). If the adenylate pool has been drained by the irreversible deamination of AMP, a high-energy phosphate bond must be expended to resynthesize AMP from IMP. When an energetic stress is encountered, expending energy to resynthesize AMP and replenish the adenylate pool may further antagonize energy homeostasis. It was suggested that AMPD activity

was suppressed in hibernators for this reason (English and Storey, 2000), and AMPD may increase in function only after the PCr pool has been sufficiently drained.

G6PDH is a unique enzyme in this study, as it was the only liver enzyme studied amongst several skeletal muscle enzymes. It was included in this study since it is an enzyme at a key point in wood frog metabolism. As the first enzyme of the PPP, G6PDH is likely to be regulated based on physiological needs for precursors of nucleotide synthesis, and reducing power in the form of NADPH. NADPH provides the necessary hydride ions for use in antioxidant defence, and since the frog encounters oxidative stress during anoxia and ischemia, followed by oxygen reperfusion during thawing, high antioxidant levels are needed to withstand the stress (Joanisse and Storey, 1996). However, just as there is a need for high antioxidant defences in freezing survival, so too is there a need for cryoprotection. Glucose, the cryoprotectant in wood frogs, is derived from glycogen stored in the liver. Glycogen is broken down into glucose-1-phosphate, which is then converted into glucose-6-phosphate. Glucose-6-phosphatase then hydrolyzes G6P into glucose, whereupon glucose can be exported from the liver to other organs and tissues, or potentially be directly used by G6PDH. Without regulation, and with enough NADP available, G6PDH can potentially use much of the G6P available in liver following massive glycogenolysis, leaving less glucose available for export and cryoprotection.

G6PDH was shown to be regulated in freeze tolerance. Affinity for both substrates of G6PDH, G6P and NADP, decreased in the frozen state. G6PDH was also less phosphorylated in frozen frogs than in control frogs, indicating that reversible protein phosphorylation is a mechanism that regulates G6PDH kinetic properties. It should be

noted, however, that the effect on kinetic properties of the enzyme is moderate. This may be a response to the need to balance high concentrations of cryoprotectant glucose, with the need for high antioxidant defence. Indeed, in estivating snails, which do not require the maintenance of high concentrations of glucose, G6PDH activity is raised despite an overall state of metabolic rate depression (Ramnanan and Storey, 2006). Thus, based on the moderate shift in kinetic parameters, G6PDH that is differentially phosphorylated in frogs may be subsequently regulated *in vivo* to further attenuate its activity.

HK showed decreased affinity and activity in skeletal muscle after 24 hours of freezing. As previously discussed, two additional reactions capable of maintaining a high AEC were included in this study: CK and AMPD, which increased ATP levels or decrease the total adenylate pool, respectively. HK, as the first enzyme in glycolysis, is capable of phosphorylating glucose, allowing it to be potentially carried through the entire pathway, and produce two moles of ATP per mole of glucose. In order for this to be energetically sound, however, metabolites of glycolysis must be pushed all the way through to the end of glycolysis, where a net increase of ATP is regenerated. This only occurs near the end of glycolysis and comes after two ATP-dependent steps: the phosphorylation of glucose by HK, and the phosphorylation of F6P by PFK. If any enzymes of glycolysis following PFK are inhibited and glycolytic metabolites are not allowed to regenerate ATP, then any phosphorylation of glucose and F6P is wasteful of ATP that is already in short supply. An additional argument in favour of the inhibition of HK is the need to retain high concentrations of cryoprotectant in all organs and tissues. In skeletal leg muscle this may be particularly important. When freezing begins, the extremities of the body such as legs, are the first to freeze and experience dehydration,

and since blood withdraws from these peripheral tissues early, they receive lower amounts of cryoprotectant glucose from the blood compared to other organs (Storey and Storey, 1984; Layne and Kennedy, 2002). This is why it is critical to maintain the available intracellular glucose for cryoprotection, in part by inhibiting glucose catabolism via HK.

HK was indeed regulated by reversible phosphorylation towards this end. A low-phosphate form of HK in frozen frogs showed decreased catalytic activity and affinity for substrates, glucose and ATP alike. Conversely, a higher phosphorylation state in control frogs led to increased HK activity and affinity. An interesting concept seen in the reversible phosphorylation of HK is that, although this enzyme could potentially cooperatively function with other energy-buffering enzymes in skeletal muscle like CK and AMPD, its regulation is somewhat contrary to that seen for the two aforementioned enzymes. For instance, *in vitro* stimulation of AMPK in skeletal muscle extracts can result in the phosphorylation of AMPD, CK, and HK, and alteration of their kinetic parameters. *In vivo*, AMPK is known to have higher activity in skeletal muscle of frozen frogs, than in control frogs (Rider et al., 2006). However, despite the high activity of AMPK, and that CK and AMPD are in a high phosphorylation state in frozen frogs, HK does not seem to be phosphorylated in the same way. The reasons underlying this would make an interesting direction for future study. In wood frog liver, PP-1 binding to glycogen is abolished during freezing, so that glycogen phosphorylase remains phosphorylated and can continue to function and hydrolyze massive amounts of glycogen, reaching the high concentrations of glucose that are necessary for freeze-tolerance (MacDonald and Storey, 1999). A similar mechanism may be at work in the

control of HK. Despite the ability of active AMPK, an energy-sensor kinase, to phosphorylate energy-buffering enzymes such as CK and AMPD in the frozen state, interactions between AMPK and HK may be inhibited, preventing phosphorylation. Alternatively, whereas phosphatases are prevented from binding to glycogen in liver, they may be specifically targeted to enzymes like HK in skeletal muscle; proteins that target phosphatases to HK have been shown in yeast (Alms et al., 1999). The targeting of specific kinases and phosphatases to metabolic enzymes may be a strategy for achieving specifically desired phosphorylation states, and resulting enzyme behaviour. Further studies would be needed to determine the factors influencing kinase and phosphatase action on enzymes.

Conclusions

The results presented here demonstrate that freezing does affect the behaviour of enzymes regulating adenylate metabolism and glucose utilization, by significantly changing their activities, kinetic properties, and subcellular localization. Changes in total protein levels did not appear to be as important a regulatory mechanism as post-translational modification. Reversible phosphorylation caused by freeze-induced signal transduction was the main mechanism regulating the function of all enzymes in this study. The enzymes presented in this study may also be regulated under a coordinated strategy. Adenylate metabolism enzymes were regulated so as to maintain a high AEC, without depleting the adenylate pool. Glucose-utilizing enzymes were also regulated so as to potentially allow low levels of glucose to be utilized by pathways for antioxidant

defence or energy production, but largely retain glucose in its free state for use as a cryoprotectant.

Future Directions

In vivo biochemistry: Several questions have been raised by this study regarding protein-protein interactions. CK and AMPD are known to complex together in muscle near sites of high-ATP turnover, such as ATP-driven ions pumps, or acto-myosin ATPases (Wallimann et al., 1992; Joannise, 2004). Signal transduction enzymes may form additional complexes with these enzymes, such as AMPK complexed to CK (Ponticos et al., 1998), in order to regulate their phosphorylation state in response to changing physiological conditions. Therefore, the question would be: if protein interactions are expected to happen, do they in fact occur during freeze/thaw? Is there indeed a close interaction between CK, AMPK, and AMPD? What allows CK and AMPD to be phosphorylated while AMPK is active, yet prevents HK phosphorylation? Are differentially phosphorylated forms of these enzymes differentially compartmented or organized subcellularly in order to further modulate their activity? To detect the organization of proteins among the cellular architecture, sections of tissue can be fixed to slides, and probed with primary antibodies directed against proteins of interest. Secondary antibodies would be conjugated to fluorophores and the technique of fluorescent microscopy could be used to analyze enzyme locations in the subcellular architecture (An et al., 2008). Layers of specificity could be added to this technique; proteins need not only be probed by pan-specific antibodies, but can also be probed by antibodies raised against phosphorylated forms of a protein of interest. This could allow

for separate analysis of the spatial organization of phosphorylated and dephosphorylated enzymes. Locations of different enzymes, or differentially phosphorylated forms of enzymes, would be compared to determine if enzymes of interest co-locate into potential protein complexes.

Time course studies: All enzymes in this study were assessed in control animals, which had not been frozen, as well as animals that had been frozen for 24 hours, to ensure complete whole-body freezing. The reasoning was that any metabolic changes should be in full effect after complete freezing. However, some enzymes may be differentially regulated at different time points over the course of ice accumulation over minutes or hours post-nucleation. For instance, in liver, at the onset of freezing, there is an initial burst of high protein kinase activity where second messenger concentrations (cAMP, cGMP and IP_3) are high, and the percentage of free PKA catalytic subunit correlates well with elevated cAMP concentrations (Holden and Storey, 1996). Over long-term freezing, PKA activity drops to control levels, and protein phosphatase activity is enduring, with significant increases in both the total levels of PP-1 and % active PP-1, as well as PP-2A and PP-2C (MacDonald and Storey, 2002). This implies that liver enzymes that are potentially regulated by PKA may be phosphorylated in the earliest stages of freezing, and then dephosphorylated later by protein phosphatases once PKA activity has decreased. A similar situation may arise in skeletal muscle with HK which is in a low-phosphate form in fully frozen animals. However, this might not be true in the early stages of freezing when selected protein kinase activities are known to be elevated. Thus, it would be interesting to determine if changes in protein kinase activities in the

early stages of freezing have an impact on the phosphorylation state of enzymes of interest, such as HK in this example, and what the physiological relevance of high enzyme activity in the earliest stages of freezing would be.

Other enzymes within the metabolon and metabolic pathways: Following studies to determine enzyme subcellular organization and possible interactions between enzymes, it would be useful to determine which additional enzymes may interact closely with those already assessed in this study and how any cooperative interaction between them would impact metabolism. AMPD and CK may complex together, and their close spatial proximity may be beneficial, since their combined catalyzed reaction allows for efficient maintenance of the AEC. It is possible that other enzymes may interact as well. Adenylate kinase has been widely implicated as having a cooperative nature with CK and AMPD (Wallimann et al., 1992; Savabi, 1994). CK can use PCr reserves to restore ATP levels during times of high ATP turnover. As PCr reserves decline and ADP begins to accumulate, the AK reaction can convert accumulating ADP into ATP and AMP. As AMP begins to accumulate, AMPD would then deaminate AMP, continuing to drive the AK reaction forward (towards ATP production), although at the expense of draining the adenylate pool. Hence, an analysis of frog muscle AK would also be instructive. Kinetic parameters and phosphorylation state could be assessed to determine if the enzyme is differentially regulated between control and frozen frogs.

In addition to enzymes of adenylate metabolism, there are enzymes in the glycolytic pathway which can also be analyzed. PFK and pyruvate kinase (PK) are two known control points within this pathway and it is possible that they are additionally

regulated to help control flux through glycolysis for energy production during freezing while at the same time helping to contribute to minimizing the catabolism of free glucose. The relative importance of HK, PFK and PK in this regard may vary depending on the tissue involved, so a complete organismal study could be conducted, analyzing each tissue systematically to assess the regulatory controls that help to balance cryoprotectant needs versus glucose catabolism in each tissue during freezing. For those tissues which are found to preserve as much free glucose as possible, and possess gluconeogenic capability, those enzymes unique to the gluconeogenic pathway should also be assessed to determine if their properties are modified to regenerate more free glucose in the frozen state, than in control frogs.

Signal transduction: Several of the main signal transduction enzymes have yet to be fully studied in *Rana sylvatica*. Although enzymes such as AMPK in muscle and liver, and PKA in liver have been analyzed, many more remain which could affect the phosphorylation state of the enzymes of energy metabolism studied to date in wood frogs. Levels of second messengers that activate PKA, PKC, and PKG have been analyzed in frog muscle and liver (Holden and Storey, 1996) but activities and kinetic analysis of PKC and PKG have yet to be determined. This would be important information because it would be useful to determine which of these kinases are actually active *in vivo* and compare these data with the *in vitro* findings about which kinases phosphorylate which enzymes.

Further studies could move even higher in the signal transduction pathway. AMPK is known to be more active in frozen frogs than in control animals. However,

what is its phosphorylation state, and which protein kinases or phosphatases contributed to this state? Previous studies in other models have suggested that LKB1 and CaMKK are AMPKKs which phosphorylate AMPK (Witters et al., 2006), but given the unique findings so far in the wood frog, studies should be done and their findings confirmed.

Additionally, the important role of protein phosphatases must not be forgotten. While there are many protein kinases, only a few protein phosphatases have been identified, and as such, their activities could be easily determined. Activities have been determined for PP-1, PP-2A, PP-2B, and PP-2C in wood frog liver, but only for PP-1 in muscle. Future studies should look to determine the activities and properties of protein phosphatases in muscle, how they regulate downstream enzymes, along with how they themselves are regulated.

LIST OF PUBLICATIONS

- Abnous, K., Dieni, C.A., Storey, K.B. (2008) Regulation of Akt during hibernation in Richardson's ground squirrels. *Biochim. Biophys. Acta* 1780, 185-193.
- Dieni, C.A., Storey, K.B. (2008) Regulation of 5'-adenosine monophosphate deaminase in the freeze-tolerant wood frog, *Rana sylvatica*. *BMC Biochem.* 9:12.
- Dieni, C.A., Storey, K.B. (2008) Creatine kinase regulation by reversible phosphorylation. Submitted to *Mol. Cell. Biochem.*
- Abnous, K., Dieni, C.A., Storey, K.B. Regulation of MAPKAP-K2 in Richardson's ground squirrels, *Spermophilus richardsonii*, during hibernation. In preparation.
- Dieni, C.A., Storey, K.B. Regulation of glucose-6-phosphate dehydrogenase in the freeze-tolerant wood frog, *Rana sylvatica*. In preparation.
- Dieni, C.A., Storey, K.B. Regulation of skeletal muscle hexokinase in the freeze-tolerant wood frog, *Rana sylvatica*. In preparation.
- Dieni, C.A., Storey, K.B. A unique liver glucokinase, and its regulation by reversible phosphorylation, in a freeze-tolerant wood frog. In preparation.

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APPENDIX

ENZYME ASSAY OPTIMIZATION

Machine settings and enzyme amount: A standard set of assay conditions is typically obtained from the literature; however enzymes must be individually optimized for each animal and tissue. The first step is to find the correct enzyme amount to provide a good intermediate rate of activity. An initial estimation must be made as to the possible activity and how much extract to use. For example, if high activity is expected, 5 μL of enzyme extract is used; if low activity is expected, 30 μL of extract should be used. An assay is run, and if activity is very high, another assay is run, this time cutting down the amount of enzyme. Conversely, if activity is very low, more enzyme is used. This is refined until a useful rate is obtained that produces a progress curve of about 45° over the time interval measured. In the end, as many assays as possible is desired from each enzyme preparation, so ultimately the amount of enzyme used per assay is kept low.

Reproducibility, Blanks, and Coupling Enzyme: With the correct scale settings and amount of enzyme to use, assays are repeated several times until the same slope can be generated repeatedly ($\pm 5\%$). Only when reproducibility is obtained can the rate of your enzyme be experimentally manipulated.

A blank must then be run; 2-3 wells are set up, leaving out the most specific substrate of the enzyme reaction. The activity observed within blanks should be zero or very nearly so; no extra non-specific activity that will interfere with measuring your enzyme is wanted. Some assay mixtures will always have substantial blank activity and this must be subtracted from enzyme rates. Ideally, blank activity is no higher than 10% of your total detectable activity.

For assays that use coupling enzyme(s), the activity of the coupling enzyme must be much higher (at least 10-fold) than the maximal rate of the enzyme of interest. This is done in two ways: 1) the product of the reaction of interest (ie. the substrate of the coupling enzyme) is added to a reaction microplate. A 10 mM solution of the coupling enzyme substrate should always be kept on hand for this purpose. The reaction should be so fast that a progress curve is no longer visible by the time the microplate is shaken and the reaction-monitoring software is started. This shows that no matter how the enzyme of interest makes substrate, the amount of coupling enzyme present is sufficient to instantly process it; 2) secondly, two reaction microplates are set up, one with the regular amount of coupling enzyme, and one with twice as much. Both are run, and should give the same rate of reaction. This shows that at the very, very low levels of product that the enzyme of interest is making, which are far below the substrate K_m levels for the coupling enzyme, the coupling enzyme is still using up the substrate instantly. If rate of the 2x microplate is faster, then the amount of coupling enzyme that is used must be increased.

Optimizing reaction conditions: With a sufficient enzyme rate, zero blank, and knowledge that the coupling enzyme is present in excess activity, the optimal reaction conditions that give the fastest rate of activity of your enzyme (ie. V_{max}) must be found. Buffer type and buffer pH both affect the reaction, and thus it is necessary to choose the buffer type and pH that produce the best activity. Enzymes are typically assayed over a range from pH 6.0-9.0 at increments of 0.2 pH units. Buffers used are MES for pH 6.0-6.6, MOPS for 6.8-7.8, and TRIS from 8.0-9.0. Once optimal pH is determined, it is important to alternate buffer types capable of buffering at that pH, and choose the best buffer type, ensuring that the pH optimum remains constant.

Substrate concentrations must also be optimized. In certain circumstances, standard concentrations are used. For example, 0.1 or 0.15 mM NADH is the standard for all dehydrogenases and higher levels cannot be used, as total absorbance at 340 nm becomes too high and outside of the linear range that the microplate reader can quantify. The most specific substrate is chosen first and assay wells are prepared with substrate levels of 0.5x, 1x and 2x, when x is the substrate concentration in the original assay conditions that were suggested. After assessing their relative rates, it must be determined whether the substrate concentration must be higher or lower to reach the maximal velocity. Assays are repeated with a new set of substrate concentrations (e.g. 0.1x, 0.2x, 0.5x or 2x, 4x, 10x), followed by refinements (e.g. 3x, 4x, 5x) until the optimum is pinpointed. If a second substrate must now be optimized, the concentration of the first substrate is set at its newly determined optimum, and the exercise is repeated, but this time varying the second substrate.

Finally, the concentrations of any ions that are needed for the enzyme reaction are optimized, holding the concentrations of all substrates constant at their newly determined levels. The resulting optimum substrate concentrations now determined give the measurable maximal velocity or V_{max} of the enzyme.

CURVE FITTING

Storey Lab custom kinetics software

Analysis of kinetic data is typically performed using a statistical program designed for the Storey lab, published in BioTechniques (Brooks, 1992). The program allows the input of X-Y scatter points, pertaining to activity versus the concentration of a

biomolecule which affects the enzyme (a substrate, inhibitor, or activator). The program is designed with appropriate equations for determining kinetic parameters.

Michaelis-Menten equation: The standard velocity versus substrate curve with hyperbolic kinetics. Will calculate V_{max} and K_m .

Hill equation with $h > 0$: This is used as a starting point for curve fitting. It fits a velocity versus substrate curve with sigmoidal, cooperative kinetics. This equation leaves open the possibility that the Hill coefficient is greater than 0 but less than one, indicating negatively cooperative kinetics. $S_{0.5}$ is used instead of K_m as a half-saturation constant.

Hill equation with $h > 1$: This is a velocity versus substrate curve with sigmoidal, positively cooperative kinetics. It should be used only with certainty that positively cooperative kinetics are present.

Activator equation with $h > 0$: This is a velocity versus an allosteric activator, when dealing with an activator that has a cooperative mechanism. This leaves open the possibility that activation may be negatively cooperative. Activator equations calculate the activation constant K_a , a V_{max} which is the maximum activated rate of reaction, and v_0 , the rate of reaction in the absence of inhibitor. In order to use any activation equations, the plotted activity in the absence of activator must not be zero.

Activator equation with $h \geq 1$: This is a velocity versus an allosteric activator, when dealing with an activator that has a positive cooperative mechanism.

Activator equation with $h = 1$: This is a velocity versus an allosteric activator, when dealing with an activator that has a Michaelis-Menten-like mechanism.

Linear inhibition equation: This is a velocity versus an inhibitor. As its name implies, the correlation between inhibition and inhibitor concentration is a linear one.

This equation calculates the concentration of inhibitor at which activity is 50% of the activity in the absence of inhibitor, I_{50} . In order to use any inhibitor equations, the plotted activity in the absence of inhibitor must not be zero.

Non-linear inhibition equation: This is velocity versus an inhibitor. As its name implies, the correlation between inhibition and inhibitor concentration is not linear one, and is based on the Hill equation. Depending on the equation used, one can calculate I_{50} or K_{50} , which is the apparent affinity constant for an inhibitor typically seen in competitive inhibition, and not necessarily equivalent to I_{50} .

Statistics with Excel

Having calculated multiple kinetic parameters, it is necessary to compare the two data sets from control versus stressed animals. Microsoft Excel software is used to calculate statistic significance based on a two-tailed distribution, unequal variance, T-Test.

Plotting with Origin

Origin is a graphing program that is more sophisticated than Excel. Its key feature is its ability to create curve-fitted kinetics graphs that are suitable for publication. Although the published Kinetics program is used to obtain kinetic parameters such as K_m and V_{max} , a printout of a V vs. [S] curve cannot be obtained. As a result figures have been redrawn in Microsoft Excel, but Excel can only “join the dots” and cannot curve fit properly onto an X-Y scatter plot. Sometimes selected curves are useful to show. In those cases, this new software can be used to produce proper curve-fitted graphs.

ENZYME STRUCTURES

AMP deaminase: AMPD regulatory and catalytic site information was obtained from Merkler and Schramm, 1990. A later paper from these authors (Merkler and Schramm, 1993) compared the yeast sequence to that of mouse AMPD and its crystal structure.

Creatine kinase: The crystal structure of CK was obtained from rabbit muscle CK (Rao et al., 1998). The information contained in this study presented both 3D and 2D structure within the enzyme, as well as important catalytic residues.

Glucose-6-phosphate dehydrogenase: Human G6PDH was used for structural studies (Au et al., 2000). Tetrameric G6PDH was obtained from the typical equilibrium between dimers and tetramers. This study revealed the dependence upon NADP^+ at the dimer interface for oligomerization.

Hexokinase: A high-resolution crystal of HK from yeast was used for structural studies (Kuser et al., 2000). This is an early crystal structure with the correct primary sequence of HK. The primary amino acid sequence is compared to rat, fungal, and plant species. Additional motifs, such as a catalytic sulfate ion and hydrophobic channel, are also presented.