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**REGULATION OF PROTEIN AND PHOSPHOLIPID
METABOLISM IN THE 13-LINED GROUND SQUIRREL,
SPERMOPHILUS TRIDECEMPLINEATUS,
AND THE WOOD FROG, RANA SYLVATICA**

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

Master of Science
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ABSTRACT

Mammalian hibernation and ectotherm freeze tolerance are unique forms of hypometabolism employed for animal winter survival. The present studies explore selected molecular mechanisms associated with hypometabolism in two model animals, the thirteen-lined ground squirrel, *Spermophilus tridecemlineatus*, and the wood frog, *Rana sylvatica*. Conservation of fuel reserves and of macromolecular integrity during hypometabolism depend on the coordinated suppression of pathways of macromolecular synthesis and degradation. Investigations of the regulation of protein degradation, protein synthesis, and phospholipid degradation pathways were undertaken comparing control and stressed conditions in skeletal muscle and liver from both animals. Several eukaryotic initiation factors were analyzed using western blotting and revealed animal specific changes in the regulation of protein synthesis. The activity of the proteasome, responsible for protein degradation, was assayed fluorometrically and was correlated with measurements of oxidatively damaged proteins (protein carbonyls) and immunoblot analysis of levels of ubiquitin-tagged damaged proteins. Proteasome activity was strongly suppressed in frozen wood frogs via changes in both the total amount and the phosphorylation state of the proteasome. However, proteasome activity remained constant in the hibernator model. Ubiquitination increased in the hibernator model, but decreased in the frog system highlighting a difference in the ubiquitin system upstream of the proteasome. Assays of the activity of cytoplasmic phospholipase A₂ (cPLA₂) provided estimates of the role of arachidonic acid signaling in hibernation and freezing survival. Phospholipase activity decreased significantly during hibernation, but was

enhanced during freezing. Control of the proteasome and cPLA₂ have implications for the repair mechanisms that deal with oxidative damage to cellular macromolecules.

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Onwards and upwards.

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LIST OF ABBREVIATIONS

AGE	advanced glycation endproducts
AMC	7-amido-4-methylcoumarin
ATP	adenosine triphosphate
BEL	bromoenol lactone
BMR	basal metabolic rate
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
DMSO	dimethyl sulfoxide
DNPH	2,4-dinitrophenylhydrazine
DTNB	5,5'-Dithiobis(2-nitrobenzoic acid)
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)- N,N,N',N'-tetraacetic acid
INA	ice nucleating agents
IP3	inositol triphosphate
kDa	kilo Dalton
MCP	multicatalytic proteinase
MW	molecular weight
PAF	platelet activating factor
PDH	pyruvate dehydrogenase
PDK4	pyruvate dehydrogenase kinase 4

PKC	protein kinase C
PLA ₁	phospholipase A ₁
PLA ₂	phospholipase A ₂
PLB	phospholipase B
PLC	phospholipase C
PLD	phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PMSF	phenylmethanesulfonyl fluoride
PVDF	polyvinylidene difluoride
RAGE	receptor advanced glycation end products
ROS	reactive oxygen species
stress	in this thesis can be interchangeable with “stressor”
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
TPA	12-0-tetradecanoylphorbol 13-acetate

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Chapter 1:
General Introduction

Animals in North America face many different environmental stresses. For example, seasonal fluctuations in temperature cause drastic variations in landscapes and bring problems of scarcity of food and water availability. Across most of Canada, the winter months are spent with a thick layer of snow on the ground and mean temperatures well below 0°C. Conversely, during the summer months temperatures can rise to over 30°C. Most Canadian animals, therefore, must have the ability to survive over a range of temperatures spanning at least 30° and often 40-60° Celsius.

Various strategies are employed by animals to survive the scarcity of food and frigid temperatures of the winter months. Some species, including many birds and insects, migrate to southern climates to avoid the winter cold. Others retreat to buffered sites under the snow, underground or underwater; some species enter dormancy whereas others remain active in these sheltered habitats. Some species remain active above the snow pack; the niches of some birds and some of the larger mammals are not as compromised in the winter and they can still function physiologically and ecologically. However, for those that cannot migrate or whose niche is compromised, one strategy remains: hibernation.

Hibernation is a form of hypometabolism (torpor, dormancy) that is typically a response to low environmental temperatures. Many species of amphibians and reptiles sink into a winter dormancy as a strategy for coping with the winter months (Storey and Storey, 2004c). Many types of insects and other invertebrates also display hypometabolism over the winter months, frequently including an obligatory period of dormancy, called diapause. Hibernation is best known as the winter survival strategy of

many small mammals where it has some specific features. Mammalian hibernators suppress their metabolic rate by over 90% and abandon homeothermy, one of the hallmarks of mammalian life, to allow their body temperature to drop to ambient. The animals remain in constant torpor for days or weeks but arouse intermittently, rewarming their bodies back to 37-38°C for a few hours at a time.

Common themes in hypometabolism:

Hypometabolism or metabolic rate depression is a strategy employed by animals to endure extreme environmental stresses that typically interfere with the animal's ability to maintain a normal active life. Environmental stresses can include high and low temperatures, oxygen limitation, desiccation, extremes of salinity, scarcity of food and various combinations thereof (Storey and Storey, 1990). Animals enter hypometabolic states as a survival strategy to reduce their energy consumption and greatly extend the time that a fixed reserve of endogenous fuels can be stretched to sustain viability until physiologically compatible environmental conditions resume.

At its core, a reduction in metabolic rate requires the coordinated net reduction of the rates of energy-producing processes and energy-consuming processes. Since in most cases the animal undergoing hibernation has stopped eating, no new energy sources are obtained and the animal must use stored fuel reserves over the hibernation period. With the absence of eating, energetic costs of digestion, nutrient absorption and peristalsis are eliminated (Storey and Storey, 2004b).

One of the major issues in studying hypometabolism is the prioritization of metabolic processes. When energy supply is low, which pathways and functions are

turned off, which are strongly suppressed, and which are deemed a priority and continue to function at normal levels? For example, processes of transcription and translation are energetically expensive. Transcription consumes up to 10% of the energy budget of cells (Rolfe and Brown, 1997) and translation (protein synthesis) can consume as much as 80% of ATP turnover in some organs (Fuery *et al.*, 1998). The synthesis of proteins is very energetically expensive, requiring ~5 ATP equivalents per peptide bond (Storey and Storey, 2004b). Although transcription and translation are obvious targets for ATP savings in a low energy scenario, there is growing evidence for the continued expression of selected stress-activated genes. These selected genes are expressed during hypometabolism and potentially aid the animal to endure and recover from the depressed metabolic state.

One of the main mechanisms that is frequently used to make major changes in the activity state of metabolic processes is reversible protein phosphorylation. For example, the phosphorylation of selected ribosomal proteins is a key mechanism in the inhibition of protein translation (Rhoads, 1993; Mikulits *et al.*, 2000). Suppression of the rates of various metabolic pathways is also brought about by the phosphorylation of key enzymes. Indeed, this is one of the main strategies used for re-organizing catabolic pathways of fuel consumption during hypometabolism. An individual enzyme/protein may be either activated or deactivated by a change in the amount of covalently bound phosphate attached to it. Changes in the phosphorylation state of an enzyme are governed by the action of cellular kinases and phosphatases.

Regulation of macromolecular catabolism is also of importance in periods of hypometabolism. Enzymes and proteins in cells are not only regulated by reversible

controls such as protein phosphorylation but also by degradation by cellular proteases. A decrease in temperature reduces the activities of all enzymes (Q_{10} is often about 2) in the cold, which prolongs the half-lives of cellular macromolecules. However, the overall rate of synthesis versus degradation of macromolecules such as DNA, mRNA, proteins and membrane phospholipids needs to remain balanced (Storey and Storey, 2004b). Given that the rates of transcription and translation are strongly suppressed by reversible phosphorylation controls in the hypometabolic state, it can be predicted that protein degradation would be similarly suppressed. This would preserve the levels of key macromolecular components throughout the dormant period, and facilitate the resumption of cellular processes upon rewarming.

Mammalian hibernation:

Many small mammals spend the winter months in hibernation. For some species hibernation is obligatory whereas others show a facultative response that is dependent upon the severity of the winter and the relative amount of food that has been hoarded (Humphries *et al.*, 2003). Small mammals gain the greatest advantage from hibernation. Allometric scaling states that the basal metabolic rate (BMR) of a euthermic mammal is inversely proportional to body mass with an equation of $3.4 M^{-0.28} \text{ ml O}_2 \text{ g}^{-1} \text{ h}^{-1}$ where M represents the body mass of the animal in grams (Heldmaier and Ruf, 1992; Geiser and Ruf, 1995; Buck and Barnes, 2000). Hibernating mammals, conversely, have a consistent basal metabolic rate of $\sim 0.03 \text{ ml O}_2 \text{ g}^{-1} \text{ h}^{-1}$ across a variety of sizes. Therefore, the difference between the euthermic BMR and hibernating BMR is greater in small mammals. Hence, hibernation is a more advantageous strategy for small mammals and

this suggests the reason why there are not many larger mammalian hibernators (Heldmaier *et al.*, 2004).

Mammalian hibernation is characterized by a rapid reduction in metabolic rate followed by a reduction in overall body temperature. Typically body temperature falls until it equilibrates with ambient temperature and a minimum metabolic rate is usually achieved at about 0-5°C. If ambient temperature drops below 0°C, the hibernating animal slightly elevates its metabolic rate to keep body temperature above freezing. Metabolic rate in hibernation is often reduced to just 2-4% of euthermic values (Carey *et al.*, 2003). Physiological functions are drastically reduced while hibernating. For example, in ground squirrels, heart rate is reduced from 200-300 to 3-5 beats/min, and respiration drops from 100-200 to 4-6 breaths/min (Zatzman, 1984; McArthur and Milsom, 1991).

Throughout the hibernation season, animals exhibit long bouts of torpor (2-3 weeks in ground squirrels) that are interrupted by brief periods of arousal back to high metabolic activity (~12 hours) (Figure 1.1). Body temperature and other physiological parameters are restored to euthermic values during these arousal periods. Although the process of entry into deep torpor can take up to one day, the arousal period is characterized by a very rapid increase of body temperature that can restore the animal to euthermic values within minutes to a few hours (Heldmaier *et al.*, 2004). The physiological reason(s) for these interbout arousals are not yet known (Heldmaier *et al.*, 2004). However, prolonged torpor can have several consequences including inhibition of lymphocyte proliferation resulting in reduced immunocompetence, dehydration that leads to compromised circulation and ionic balance, and the production of reactive oxygen species that results in the accumulation of oxidative damage products (Humphries *et al.*,

2003). Most of the negative consequences of torpor are thought to be lowered/reversed during the periodic arousals (Grigg and Beard, 2000). However, arousal is energetically costly and causes a significant depletion of energy reserves. For example, in the hibernating marmot, 72% of the winter reserves of metabolic fuels are spent during these arousal periods (Heldmaier *et al.*, 1993).

One of the main questions yet unanswered in the realm of hibernation studies is the type of biochemical or molecular cue(s) that govern entry into hibernation. Small mammals are able to enter hibernation independent of ambient temperatures. Metabolic rate decreases prior to the fall in body temperature indicating it is the reduced metabolic rate that causes the drop in body temperature and not vice versa (Heldmaier and Ruf, 1992). Metabolic rate depression also happens in a normoxic state indicating that neither oxygen limitation or surplus of carbon dioxide are suitable cues for entry into hibernation (Heldmaier *et al.*, 2004). Though carbon dioxide may not be a potential cue for the entry into torpor, all types of metabolic depression including hibernation, estivation, anaerobiosis, etc. are characterized carbon dioxide retention and acidosis.

Macromolecular fuel utilization in hibernation

The prehibernation period is characterized by a rapid weight gain. In preparation for the winter months, hibernators nearly double their body mass by the accumulation of body fat. This shifts the animals from a predominantly carbohydrate based metabolism in the summer to a lipid based metabolism in the winter. Indeed, the respiratory quotient (RQ) which represents the ratio of volume of carbon dioxide produced divided by the volume of oxygen consumed shifts from a value of ~ 1.0 which is indicative of

carbohydrate catabolism to an RQ of 0.7 during torpor which indicates lipid oxidation (South and House, 1967).

During hibernation, most organs are fueled by lipid oxidation and carbohydrates are preserved as fuels for selected organs such as the brain (Storey and Storey, 2004b). In hibernating ground squirrels glycolysis is suppressed chiefly by the use of reversible protein phosphorylation to turn off key enzyme loci (Brooks and Storey, 1992). One of these loci is pyruvate dehydrogenase (PDH), the enzyme responsible for carbohydrate entry into the citric acid cycle via the conversion of pyruvate into acetyl-CoA. Phosphorylation inhibits PDH and the enzyme that catalyzes PDH phosphorylation, pyruvate dehydrogenase kinase 4 (PDK4), is strongly up-regulated during hibernation. PDK4 transcripts and protein are strongly induced in the heart, skeletal muscle and white adipose tissue of ground squirrels during the entire hibernation period, including torpor states as well as interbout arousals (Buck *et al.*, 2002). While the enhanced expression of PDK4 acts to suppress carbohydrate catabolism during hibernation, other metabolic changes enhance lipid oxidation. For example, increased expression of pancreatic lipase increases fatty acid liberation at low body temperatures (Andrews *et al.*, 1998) and increased synthesis of the heart and adipose isoforms of fatty acid binding protein increase the capacity for transport of fatty acids to the mitochondria in hibernator organs (Hittel and Storey, 2001).

Stresses of hibernation:

Apart from the physiological stresses outlined above in hibernation, another serious problem is oxidative stress during hibernation. The peculiar circumstances of

hibernation and arousal greatly affect the production of reactive oxygen species (ROS) (Figure 1.2). During the arousal period, the dramatic increase in oxygen consumption that fuels the huge increase in mitochondrial oxidative metabolism that is needed to rewarm the animal may result in an associated huge increase in the production of ROS that exceeds the limits of the antioxidant capacity of the hibernator (Carey *et al.*, 2003). The potential for oxidative stress has been shown in both plasma ureate levels and ascorbate levels. Plasma ureate is produced when xanthine dehydrogenase is converted to xanthine oxidase due to attack by ROS. An increase in plasma ureate levels during the arousal period suggests an increase in the production of ROS at this point in the torpor cycle (Toien *et al.*, 2001). The same authors also found an increase in plasma ascorbate levels during the arousal period indicating that the synthesis of ascorbate may be part of an antioxidant defense.

Accumulated damage due to oxidative stress also appears to be part of long-term torpor. The concentrations of selected oxidative damage products of lipids (conjugated dienes, lipid peroxide metabolites) increased during the early phase of torpor. Conjugated dienes were generally found in tissues that had low perfusion and/or reperfusion rates during the torpor-arousal cycle (Carey *et al.*, 2000).

Animal model: Thirteen-lined ground squirrels

The animal model of mammalian hibernation used in this thesis is *Spermophilus tridecemlineatus*, the thirteen-lined ground squirrel. This species is one of several species of ground squirrels that are capable of hibernating. The habitat of *S. tridecemlineatus* spans the southern prairies of Canada down through the south-central United States

(Figure 1.3). The animals feed primarily on seeds, flowers and insects and prior to hibernation they roughly double their body mass from 130-180g to 220-240g. In this species the hibernation period begins in September to early October, and finishes between late March and early May. The enzyme studies undertaken in my project were conducted primarily on skeletal muscle and liver tissues. These organs were chosen for several reasons including: (a) their large size provided sufficient tissue for extensive studies, (b) they represent major functional organs of the squirrels – liver is a main anabolic organ, muscle is a major catabolic one, and (c) both have key roles in hibernation – liver maintaining/renewing metabolic homeostasis over the course of torpor/arousal cycles and muscle making a major contribution to arousal via shivering thermogenesis. Although there are surely minor tissue specific differences in other organs, I believe that the general patterns of response can be obtained from the studies on these two tissues.

Another look at hibernation: strategies for ectotherms

Surviving in a climate with drastic fluctuations in temperature provides specific stresses to both endotherms and ectotherms. For endotherms, a vast amount of energy is consumed to maintain internal body temperatures at 37°C, a temperature of maximal physiological and biochemical performance. Ectotherms on the other hand, have internal body temperatures comparable to the ambient temperature. Although the energetic costs of being an ectotherm are lower, they experience wide temperature fluctuations often outside the range that suits optimum physical and biochemical performance (Heldmaier *et al.*, 2004). Endotherms require on average eight times more energy than ectotherms at

the same body size and temperature (Else and Hulbert, 1981). This value can increase by up to two orders of magnitude when comparing the same animals at cold temperatures. The metabolic rate of ectotherms is much lower in the cold due the thermodynamic effects on their biochemistry, whereas the metabolic rate of the endotherms is raised due to thermoregulatory heat production (Heldmaier *et al.*, 2004).

Animal model: Wood frog

The wood frog, *Rana sylvatica*, is one of five known species of frogs that endure the freezing of extracellular body fluids as part of their winter hibernation strategy. The others are the gray tree frog: *Hyla versicolor*, the spring peeper *Pseudacris crucifer*, the western chorus frog, *Pseudacris triseriata*, and Cope's gray tree frog *Hyla chrysoscelis* (Schmid, 1982; Costanzo *et al.*, 1992; Storey and Storey 1986a). The range of *Rana sylvatica* spans most of Canada and reaches down into the eastern part of the United States (Figure 1.4). These frogs spend the winter months at or near soil surface where they are typically covered by layers of leaves and snow. Although their hibernation sites offer some thermal buffering, the temperature under the snow pack may reach as low as -6°C. Consequently, this is the approximate lower limit of survival for most freeze tolerant vertebrates (Storey and Storey, 2004c).

As mentioned earlier, ectotherm hibernation can be more extreme than endotherm hibernation, particularly for freeze tolerant animals. Whereas heartbeat and respiratory rate are slowed in endotherm hibernation, heartbeat and respiration stop completely during freezing, rendering the tissues of frogs anoxic throughout the freezing period. Where endotherms cope with a lowered body temperature, freeze-tolerant frogs actually

accumulate ice within their body cavities. Roughly 65% of total body water is frozen as extracellular ice that accumulates in the abdominal cavity, bladder, brain ventricles, eye lenses, plasma and between the muscle and skin layers (Storey and Storey, 2004c).

Entrance into freezing is very important for the subsequent survival of the frog throughout the period of the freeze. The freezing process is started by contact of the frog with environmental ice. Once this contact is made, the ice then penetrates the skin through pores and subsequently extracellular body fluids begin to freeze (Layne *et al.*, 1990). Optimal survival occurs when the freezing process is slowed to a rate of between -0.16°C to -0.18°C per hour, whereas frogs will not survive a higher cooling rate of -1.17°C per hour (Costanzo *et al.*, 1991). A slower cooling rate is more predominant in nature because temperatures change relatively slowly under the snow pack. After the onset of freezing, the survival rate depends on both the temperatures during the freeze and the duration of the freeze. For example, survival of wood frogs frozen at -4°C for two days was 100%, but this decreased to 50% when frogs were frozen at -6°C for eleven days (Storey and Storey, 1986a). At temperatures lower than -6°C , the survival rate plummets. At a milder freezing temperature of -2.5°C , frog survival can be extended to 13 days (Storey and Storey, 1986b).

Survival of whole body freezing is aided by glucose, a cryoprotectant synthesized in copious amounts during the early minutes/hours of freezing. The accumulation of high concentrations of glucose in cells provides colligative resistance to the loss of intracellular water into extracellular ice masses and maintains a critical minimum cell volume (Storey and Storey, 2004c). Small molecular osmolytes are also beneficial in the preservation of protein structure and help to stabilize enzyme kinetic properties over a

range of temperatures (Somero, 2003). Prior to freezing, plasma levels of glucose are 1-5 mM, similar to normal blood glucose levels in all vertebrates. At the onset of freezing, liver glycogen reserves that were built up over the summer and autumn are rapidly catabolized to form glucose that is exported to raise glucose levels in the plasma and core organs to 150-300 mM (Storey and Storey, 1984). The rate at which freezing progresses determines how adequately glucose is transported to the organs from the liver. Faster rates of freezing impair the transport of glucose around the body and increase the damage caused by freezing (Layne and Kennedy, 2002). Upon thawing, glucose is restored into liver glycogen over several days where it is available to be used again during the next freezing episode. During the several hours that it takes a frog to freeze, glucose is pumped out of a central reserve (liver) at the same time as the freezing front moves inwards from peripheral tissues towards the core. Hence, as shown in Figure 1.5, muscle tissues are the first to freeze and the liver the last and, consequently, cryoprotectant levels are lowest in peripheral organs (skin, muscle) and highest in the core (liver, heart). During thawing, this cryoprotectant distribution means that core organs have lower melting points than do peripheral ones and so liver is the first to thaw and muscles the last. Because liver and muscle represent two extremes of organ survival in the frozen frog, they were chosen as the model tissues for the studies presented in this thesis. The muscle tissue has a longer degree of dormancy and potentially receives more damage during the freeze-thaw cycle. Liver tissue is one of the best-preserved organs through the freezing and thawing process, but it is the key metabolic center supporting freezing survival by the whole animal, being not only the center of glucose production but also the site of synthesis of a number of freeze-responsive proteins that are secreted (Storey,

2004).

Macromolecular fuel utilization:

During the freezing period, blood plasma is frozen and so tissues receive no oxygen delivery. Cells use up their remaining oxygen and tissues become anoxic for much of the freeze. Therefore, to ensure survival, frogs switch to anaerobic metabolism. Glucose not only serves as a cryoprotectant, but a potential fuel during freezing conditions. An extensive study looking at key enzymes in glycolysis, gluconeogenesis, the citric acid cycle, fatty acid pathways, amino acid and adenylate metabolism showed that the activities of selected enzymes in several biosynthetic pathways (malic acid, NADP-isocitrate dehydrogenase, glyceraldehydes-3-phosphate dehydrogenase, glutamate dehydrogenase and glutamate-pyruvate dehydrogenase) were reduced during freezing which may indicate a decrease in ATP-dependent biosynthesis (Cowan and Storey, 2001). A significant reduction in selected enzymes of glycolysis was also consistent with the idea that glucose serves a primarily colligative function in the frozen frog rather than acting as a fuel source, and endogenous glycogen was hypothesized to be the main fermentative fuel throughout the frozen period (Cowan and Storey, 2001; Storey and Storey, 2004c). Although in this metabolic profile there were tissue specific changes, the general trend was a decrease in glycolytic enzymes and inhibition of biosynthetic pathways.

Stresses of Freezing:

In the process of freezing, the wood frog has to overcome a variety of stresses

involved in going from a physiologically active frog to one essentially frozen solid. Ice itself is a potential danger as during its growth it can rupture cell membranes and severely damage the viability of cells. To combat this, frogs limit ice growth to extracellular spaces using extracellular ice nucleating agents (INAs) to trigger ice formation in selected sites (Storey and Storey, 2004c). The amount of ice formed within the microvasculature of organs must also be limited so that ice expansion does not burst delicate capillaries. This is aided by a partial dehydration of organs with cellular water exiting into extra-organ ice masses (such as in the abdominal cavity) where ice crystals can do relatively little physical harm to tissues (Storey and Storey, 2004c).

Two other major stresses associated with freezing are anoxia and dehydration. These two stresses can be separated and looked at individually. This is useful in determining if a biochemical/molecular change between the control and frozen state is brought about by the anoxic or the dehydration component of freezing (Storey and Storey, 2004c). Anoxia arises from the freezing of blood plasma, and long-term ischemia is caused by the interruption of the delivery of oxygen and nutrients to tissue and cells. The anoxia experienced during the freezing period is followed by a rapid increase in oxygen uptake upon thawing when breathing and heartbeat resume. This could cause a surge in oxygen free radical formation leading to potential oxidative stress. Studies of antioxidant enzymes in wood frog organs (catalase, glutathione S-transferase, glutathione reductase, etc.) showed a consistently high level of antioxidant defense over the freeze-thaw cycle, which suggests that the frogs are well-prepared to deal with this source of oxidative stress (Joanisse and Storey, 1996). Lipid peroxide damage was also unchanged during freezing (Joanisse and Storey, 1996). Hence, these animals seem quite capable of

dealing with both the anoxia/ischemia stress of freezing and the oxidative stress during thawing.

Dehydration can happen both because of the water permeable skin characteristic of most amphibians, and within the cells and tissues of freeze tolerant frogs as an adaptive mechanism to channel the formation of ice in extra-organ spaces. Dehydration leads to an increase in blood viscosity and a decrease in volume leading to diminished cardiovascular function. During the thaw period, rapid water re-uptake from melting ice in abdominal masses and/or from water uptake across the skin restores blood volume and cardiovascular rhythms resume. Dehydration/rehydration patterns are analogous to ischemia/reperfusion stresses with respect to oxidative stress. Again the antioxidant capacity of the frog plays a role in the defense against oxidative stress during the recovery from dehydration (Hermes-Lima and Storey, 1998; Hermes-Lima and Zenteno-Savin, 2002).

Glucose, although an important molecule for the alleviation of the effects of cold/freezing and maintenance of protein function, may have detrimental effects at the high cellular concentrations achieved during freezing. High glucose present in diabetics causes damage to tissues via the nonenzymatic glycation of proteins and the formation of advanced glycation end products (AGEs). Furthermore, glucose, glycated proteins and AGEs can all stimulate the production of reactive oxygen species (Storey, 2004).

Proposed research and hypotheses:

Mammalian hibernation and freeze tolerance in frogs provide two very different strategies for hypometabolism and survival throughout the winter season. Although there

are marked differences in the metabolic adaptations needed for survival in these two models, a common theme of metabolic depression prevails and both model organisms must use regulatory mechanisms to suppress energy-expensive metabolic processes while hibernating. The regulatory mechanisms involved in metabolic suppression have been quite well examined with respect to some cellular functions (e.g. glycolysis, protein synthesis) whereas the control of other cell functions during hypometabolism have received little attention. If protein synthesis is suppressed during hypometabolism, it is reasonable to expect that protein degradation must also be suppressed to a comparable extent so that the macromolecular content of cells is sustained throughout the hypometabolic episode. However, little is known about the regulation of protein degradation in hypometabolic systems. There are multiple gaps in our understanding of which pathways are turned off and which are maintained during hypometabolism.

Hypothesis 1a: Activity of the multicatalytic proteinase, a major intracellular complex that degrades damaged and oxidized proteins, is suppressed during hypometabolism in mammalian hibernation and frog freezing.

Chapters 2 and 3 of this thesis focus on the characterization of the multicatalytic proteinase, also called the proteasome, in each animal model. Kinetic changes in the protein were examined between control and stress conditions to determine if the proteasome was regulated during hypometabolism. Levels of carbonyl proteins (a measure of oxidative damage to proteins) were also quantified to determine whether cumulative damage to cellular proteins occurs during hypometabolism, possibly as a result of an impaired ability to degrade damaged proteins in this state. Ubiquitin and ubiquitin conjugate levels were assessed using Western blotting to determine the effects

of hibernation and freezing on pathways upstream of proteasomal degradation.

Hypothesis 1b: Since protein synthesis is inhibited in squirrel hibernation, similar mechanisms of depression will be used to inhibit protein synthesis during freezing of the wood frog.

Chapters 2 and 3 also address aspects of protein synthesis through the determination of the levels and phosphorylation states of several key eukaryotic initiation factors that may be involved in the suppression of protein translation.

Phospholipid metabolism during hypometabolism is another area that has received minimal research interest to date. Membrane integrity and the maintenance of multiple membrane functions must be preserved in both mammalian hibernation and frog freeze tolerance, despite severe challenges to the physical properties of membranes caused by either (a) very low body temperatures of mammalian hibernators or (b) the extensive compression of membranes in cells that have lost a high percentage of their volume to extracellular ice. Several cellular signaling pathways are activated through the metabolism of phospholipids. These pathways could have key roles in the regulation of hypometabolism and/or the implementation of metabolic adaptations that aid survival. For example, protein kinase C is closely linked with phospholipid metabolism and the activity of PKC and levels of its second messenger inositol 1,4,5-trisphosphate (IP3) are elevated in both hibernation and freeze tolerance (Lee *et al.*, 2002; Holden and Storey, 1997). Arachidonic acid is a key precursor for the production of a number of signaling molecules in cells. It is released from phospholipids by the action of phospholipase A₂. Nothing is known, to date, about the involvement of this signaling system in

hypometabolism.

Hypothesis 2: Suppression of the activity of phospholipase A₂ will occur during hypometabolism as a means of reducing cell responsiveness to signals that mediate energy-expensive functions such as growth and differentiation.

Chapters 4 and 5 focus on the regulation of calcium dependant phospholipase A₂ in organs of hibernating squirrels and freeze tolerant frogs. Spectrophotometric assays are used to assess changes in enzyme activity in muscle and liver tissues under control and stressed conditions in ground squirrels and frogs. Incubation studies paired with Western blotting analyze the effects of phosphorylation on the activity of phospholipase A₂. The release of arachidonic acid would be predicted to decrease with the onset of stresses such as hibernation and freeze-tolerance.

Figure 1.1: Body temperature as a function of time in the golden-mantled ground squirrel. The inset shows the pattern of three torpor-arousal cycles highlighting multiple stages: the entrance into torpor (EN) which lasts up to 12 hours, the early torpor (ET) within 48 hours of entering torpor, the late torpor (LT), arousal (AR) with a duration of ~2 hours, and finally interbout arousal (IBA) with a duration of ~20 hours. (Carey *et al.*, 2003)

Figure 1.2: A model of stress tolerance during the torpor-arousal cycles in a hibernator, based on intestinal mucosa. Numbers in parenthesis indicate approximate durations in hibernation states (Carey *et al.*, 2003).

Figure 1.3: Range distribution of the 13-lined ground squirrel (Cleary and Craven, 1994).

Figure 1.4: Range distribution of the wood frog (Northern Prairie Wildlife Research Center Home Page, 1997).

Figure 1.5: Proton magnetic resonance imaging of the freezing and thawing process in the wood frog (Rubinsky *et al.*, 1994). Ice (i) is not visible in ^1H MRI and so it appears black as compared to unfrozen areas, which appear white. Left hand panels (A-F) show the progression from control to frozen state at time intervals A) 10min, B) 49min, C) 1h 59min, D) 3h 14min, E) 3h 43min, F) 7h 5min with the frog held at -7.0°C . Right hand panels show the thawing of the frog at 4.0°C for A) 0min, B) 34min, C) 52min, D) 1h 8min, E) 1h 27min, and F) 3h 8min. Tissues are labeled (m) leg skeletal muscle, (l) liver, (h) heart, and (g) gut.

Chapter 2:

Effects of Hibernation on Proteolysis and Protein Synthesis in the Hibernating Thirteen-Lined Ground Squirrels, *Spermophilus tridecemlineatus*

Introduction:

Conditions of cold temperature and/or low food supply stimulate a variety of mammalian species to enter a hypometabolic state for periods ranging from brief torpor bouts of a few hours to 6-9 months of winter hibernation (Wang and Lee, 1996; Storey and Storey, 2004a). Metabolic rate depression results from a strong reduction in net ATP turnover achieved by coordinated suppression of the rates of both energy-producing and energy-consuming processes while maintaining a relatively constant adenylate energy charge. Suppression of the rates of multiple cell functions occurs including enzyme activities, ion channels, protein synthesis, and gene expression often via the mechanism of reversible protein phosphorylation (Storey and Storey, 2004b).

One of the major energy costs to cells is protein turnover, the synthesis and degradation of cellular proteins. Estimates of the proportion of cellular ATP turnover devoted to protein synthesis vary from 12-80% depending on species, tissue and metabolic state (Fuery *et al.*, 1998). Not surprisingly, rigorous suppression of this energy expensive process is a key component of hypometabolic states, including mammalian hibernation (Frerichs *et al.*, 1998; Chen *et al.*, 2001). Inhibitory control over protein synthesis during hibernation has been traced to at least three ribosomal proteins: eukaryotic initiation factor 2 (eIF2), eukaryotic initiation factor 4 (eIF4) and the eukaryotic elongation factor 2 (eEF2) (Frerichs *et al.*, 1998; Chen *et al.*, 2001; Van Breukelen *et al.*, 2004). Phosphorylation of eIF2 on the α subunit inhibits the ability of this factor to bind and deliver the initiating methionine residue to the assembling translation complex. Studies with both brain and kidney of ground squirrels have shown a strong increase in the amount of phosphorylated eIF2 α in torpor as compared with

euthermic controls (Frerichs *et al.*, 1998; Hittel and Storey, 2002). Regulation of eIF4e is via control of its binding protein (4E-BP1); in its dephosphorylated form 4E-BP1 binds to and inhibits eIF4e. A study of ground squirrel liver showed hypophosphorylation of 4E-BP1 during torpor as compared with euthermia indicating that eIF4e is inhibited in torpor (Van Breukelen *et al.*, 2004). Protein synthesis during hibernation is also inhibited at the level of elongation with elevated amounts of phospho-eEF2 (the inactive form) found in brain and liver of torpid animals (Chen *et al.*, 2001). Predictably, a decrease in protein synthesis in the cells of hibernators should be accompanied by a comparable decrease in protein degradation so that cellular protein content remains largely unchanged during torpor and cells can return to normal euthermic functions as soon as the animal rewarms. Indeed, a strong reduction in protein turnover has been demonstrated in several hypometabolic systems (Storey and Storey, 2004b).

Under normal metabolic conditions, many cellular processes including protein metabolism, gene transcription and the cell cycle are governed by the activation and inactivation of proteins and their subsequent degradation. Cells have several strategies for protein degradation. The major proteases in mammalian cells are lysosomal cathepsins, calpains and the multicatalytic proteinase (MCP), also called the proteasome (Shringarpure *et al.*, 2001). Membrane bound proteins and cell surface proteins are mainly degraded by the lysosome (Doherty *et al.*, 2002), whereas the bulk of cytosolic proteins (80-90%) are tagged and sent to the proteasome for degradation (Orlowski and Wilk, 2000).

The proteasome is a large barrel-structured protein complex capable of degrading old, damaged or oxidized proteins into peptides averaging 7-9 amino acids in length that

can then be further broken down and recycled in the cell (Orlowski and Wilk, 2000). The basic form of the 20S proteasome is a cylindrical 700 kDa structure with four rings of seven subunits. The two outer rings consist of alpha subunits while the inner rings house the beta subunits that are responsible for the catalytic activity. The proteasome has within it three major types of protease activities: tryptic, chymotryptic and peptidylglutamyl-peptide hydrolytic activity. The 20S proteasome degrades many proteins and peptides but preferentially degrades oxidatively damaged proteins in an ubiquitin and ATP independent fashion (Orlowski and Wilk, 2003).

Alternative forms of the proteasome are present in the cell. The 26S proteasome (MW 2000 kDa) is composed of the native barrel of the 20S proteasome with two 19S regulatory caps on either end (Orlowski and Wilk, 2003). The 19S regulatory caps contain a ring of six ATPases that use energy from ATP hydrolysis to catalyze the unfolding of the incoming protein into the 20S barrel. Most of the substrates of the 26S proteasome undergo a tagging process of ubiquitination where polymers of ubiquitin are added in sequence to a damaged protein destined for proteolysis (Figure 2.1). There are three enzymes important in the ubiquitin cycle. E1 is the ubiquitin-activating enzyme, which activates ubiquitin in an ATP-dependent manner by forming a high-energy thiol-ester bond. Next the activated ubiquitin is passed to E2, the ubiquitin conjugating enzyme. Ubiquitin protein ligase (E3) associates with the target protein and then E2, E3 and target protein combine in a complex so that ubiquitin is dissociated from E2 and bound to the target protein. The E3 enzyme is implicated in much of the specificity of the pathway, as it has to recognize and bind to different target proteins (Doherty *et al.*, 2002, Coux, 2003). The final steps are repeated several times to give long chains of

Figure 2.1: The Ubiquitin-proteasome pathway showing the cycle of ubiquitin activation, ubiquitin conjugation, degradation by the 26S proteasome followed by deubiquitination and recycling of ubiquitin. Obtained from [www.hgu.mrc.ac.uk/ Research/Gordon/](http://www.hgu.mrc.ac.uk/Research/Gordon/)

ubiquitin tags on proteins and then these move to the proteasome. Recognition sites on the 19S regulatory cap of the proteasome help to remove the ubiquitin chains and recycle this valuable signal protein (Orlowski and Wilk, 2000). Although the 26S proteasome is capable of degrading proteins in a non-ubiquitinated fashion, the predominant targets are poly-ubiquitinated proteins (Orlowski and Wilk, 2000; Orlowski and Wilk, 2003). Van Breukelen and Carey (2002) showed that liver and gut levels of ubiquitin-conjugated protein rose by 2-3 fold during hibernation in ground squirrels (as compared with summer active levels) and suggested that this was best explained by an inhibition of the ATP-dependent 26S proteasome during torpor, a process that is generally highly sensitive to temperature reduction in mammalian cells. However, direct measurement of proteasome activity has not previously been undertaken for a hibernating species.

Finally, there is one alternate form of the proteasome called the immunoproteasome. Three beta subunits ($\beta 1$, $\beta 2$, $\beta 5$) are replaced by the γ -interferon inducible subunits LMP2, LMP7 and MECL1. The immunoproteasome can exist as a barrel, or attached to 19S regulatory subunits or to an alternate PA28 cap (Orlowski and Wilk, 2003). The immunoproteasome is responsible for breaking down foreign proteins and providing the peptides for MHC-Class I antigen presentation (Orlowski and Wilk, 2003).

The present study assessed multiple parameters of the control of protein degradation and protein synthesis in skeletal muscle and liver of thirteen-lined ground squirrels. This is the first assessment of any of these parameters in skeletal muscle of hibernators and the first instance where multiple parameters of both protein synthesis and degradation have been quantified in a single tissue of a single species. Activity of the

proteasome in skeletal muscle of euthermic versus hibernating thirteen-lined ground squirrels was measured using peptides that target the individual protease activities of the proteasome. The amount of proteasome, through western blotting, was also compared between euthermic and hibernating conditions to determine if more or less proteasomes were being synthesized during these stress conditions. Carbonyl content was measured in muscles to assess the amount of oxidative damage to cellular proteins during hibernation and ubiquitin and the amount of ubiquitin conjugates were determined in both muscle and liver to assess the effect of hibernation on events up-stream of the proteasome. Finally, the state of protein synthesis in muscle and liver during hibernation was assessed via analysis of the amount and the phosphorylation (activity) states of the ribosomal initiation factor eIF4e, as well as its modifying protein 4e-BP1, and the amount of initiation factor eIF4B to determine the effects of hibernation on protein synthesis.

Materials and methods:

Animals

Thirteen-lined ground squirrels, *Spermophilus tridecemlineatus*, were obtained from TLS Research (Bartlett, IL) in early autumn 2001 and 2002 and transported to the NIH (Bethesda, MD) where they were held in the animal hibernation facility in the laboratory of Dr. J.M. Hallenbeck (National Institute of Neurological Disorders and Stroke). The squirrels were housed individually in a room with an ambient temperature of 21°C and a 12 h: 12 h light: dark cycle, and were fed standard rodent diet and water ad libitum. Once the animals had completed the pre-hibernation phase characterized by maximizing their body lipid reserves and a rapid weight gain from 130g-180g up to 220-

240g, the animals were placed in a dark room with temperatures of 5-6°C to induce hibernation. Some animals were maintained at 21°C and sampled as euthermic controls. Animals that had been hibernating for 2-5 days, as well as controls, were sacrificed by decapitation and tissues were excised, immediately frozen in liquid nitrogen and transported to Ottawa where they were stored in a -80°C freezer until use. Samples of mouse (*Mus musculus*) skeletal muscle were provided from the Carleton University Animal Care facility and were stored at a -80°C until use.

Tissue homogenization and preparation

Samples of frozen hind leg skeletal muscle were ground under liquid nitrogen using a mortar and pestle and then 100 mg of tissue was mixed with 1 mL of homogenization buffer (50 mM Tris HCl, pH 7.8, 10 mM β -mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 50 mM NaF) and homogenized using a Polytron homogenizer. The addition of chelating agents (EDTA, EGTA) that inhibit protein kinases and NaF that inhibits protein phosphatases allows the native phosphorylation state of the proteasome to be maintained during tissue extraction. Samples were centrifuged at 9,000 x g for 15 min at 4°C and the supernatant was removed. In most cases, a measured volume of extract was then centrifuged through Centricon-500 (Amicon) concentrating devices (molecular weight cutoff 100 or 500 kDa, respectively) to remove small molecular weight proteins from the sample (large proteins are held back in the retentate). In selected cases (described in the Results), extracts were untreated or were centrifuged through Centricon-100 devices (100 kDa cutoff). Centricons were centrifuged at 5,000 x g at 4°C until sample volume was reduced by half. Retentate in the Centricon was then washed with the

addition of 2 volumes of homogenizing buffer followed by re-centrifugation, again until volume was reduced to about half of the original starting volume. The final retentate volume was then carefully measured and adjusted back to be equal to the original volume with homogenization buffer.

Assay conditions

Enzyme activity was assayed fluorometrically using the method described by Rogers and Dean (2003). Release of the fluorophore AMC from substrate-AMC conjugates was quantified using a Victor 1420 Multilabel counter (Perkin Elmer). All peptides were prepared in 3:1 v:v DMSO:water as higher amounts of DMSO could inhibit proteasome activity. The peptide substrates measured the three main proteinase activities of the proteasome: Suc-LEU-LEU-VAL-TYR-AMC (Sigma Chemical Co.) for chymotryptic activity, Cbz-ALA-ARG-ARG-AMC (Sigma Chemical Co.) for tryptic activity, and Cbz-LEU-LEU-GLU-AMC (Dalton Peptides) for the peptidyl-glutamyl-peptide-hydrolytic activity. Assays were conducted with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Preliminary studies optimized enzyme amount and the linearity of the reaction over time at both high and low assay temperatures (from this 45 min was chosen as the standard reaction time). Inhibitor experiments were carried out using a potent proteasome inhibitor, MG132 (Sigma Chemical Co.), at a final assay concentration of 50 μ M with samples incubated with the inhibitor for 20 min prior to assay. Assays were performed in 96 well microplates with a total volume of 200 μ L and standard reaction conditions: 50 mM Tris, pH 7.8, 20 mM KCl, 0.5 mM magnesium acetate and substrate varying from 0-200 μ M. To conduct low

temperature assays the fluorometer was placed in a cold incubator set at 8°C; the instrument was chilled overnight and before each assay, the microplate filled with assay mixture was pre-equilibrated in the incubator for ~15 min until the temperature in a blank well (filled with water) stabilized at 8°C, as determined by a thermister in the well. After the assay was completed, the temperature in blank well was measured again; temperature rose by 1-1.4°C due to heat generation by the machine during the run.

Protein determination and statistics

Protein concentration was determined using the Coomassie blue dye-binding method and the Bio-Rad prepared reagent. Enzyme activities were measured as AMC production over time (pmole/h/μg protein) based on a standard curve constructed with 0-15 μM of the AMC fluorophore. Data were analyzed using a two-tailed Student's t-test with statistical significance determined as $P < 0.05$.

Partial purification of multicatalytic proteinase

A Sephacryl-400 gel filtration column (90 cm x 2.5 cm) was equilibrated in 25 mM Tris-HCl, pH 7.5 buffer containing 2 mM EDTA, 2 mM β-mercaptoethanol, 10% v:v glycerol and 0.04% w:v sodium azide. The column was calibrated using high molecular weight standards: blue dextran (2000 kDa), thyroglobulin (670 kDa), Jack Bean urease (545 kDa), and *Bacillus pasteurii* urease (242 kDa). Tissues were homogenized 1:5 w:v in homogenization buffer, centrifuged at 9,000 x g and then the supernatant was loaded onto the column. A flow rate of 0.35 mL/min was obtained and 1 mL fractions were collected. Fractions were assayed with Z-LLE-AMC peptide.

Carbonyl assay for total protein oxidative damage

The protocol was modified from Reznick and Packer (1994). Tissue extracts were prepared 1:10 or 1:20 w:v in homogenization buffer and centrifuged for 15 min at 10,000 x g. An aliquot of 500 μ L of sample was added to 500 μ L of 10 mM 2,4-dinitrophenylhydrazine (DNPH) (dissolved in 2 M HCl). HCl both denatures the protein and allows the sample to be derivatized with DNPH. Blanks contained a 500 μ L sample aliquot added to 500 μ L of 2 M HCl. Both samples and blanks (run in duplicate in all cases) were then subjected to the following protocol. All were vortexed every 10 min for 1 h and then the reaction was stopped by the addition of 500 μ L of 30% TCA to precipitate protein. After a 10 min incubation on ice, samples were centrifuged at 11,000 x g for 15 min. Supernatants were decanted and pellets were washed in 1 mL of 1:1 v:v ethanol:ethylacetate. After a second centrifugation at 11,000 x g for 15 min, the supernatant was discarded and the pellets were washed three more times. Finally, pellets were solubilized in 6 M guanidine hydrochloride and incubated at 37°C for 30-60 min. Remaining insoluble material was removed by a final centrifugation at 11,000 x g for 10 min. Samples were measured on a Multiscan Spectrum spectrophotometer at 380 nm with an absorption coefficient of 22,000 $M^{-1}\cdot cm^{-1}$. Protein content was determined based on a standard curve of bovine serum albumin ranging from 0.25-2 mg/mL in guanidine hydrochloride read at 280 nm. Carbonyl content was measured as nmol/mg protein.

Native gel electrophoresis and silver stain

Native polyacrylamide gel electrophoresis was used to analyze peak column

fractions; 4% acrylamide gels were run for 2 h at 100 v using the Bio-Rad Mini Protean apparatus. Gels were silver stained by the method outlined by Blum *et al.* (1987).

Western Blotting of proteins in proteolysis and protein synthesis

Frozen tissue samples were homogenized (1:5 w/v) in homogenization buffer containing 20 mM Tris, pH 7.8, 150 mM NaCl, 10 mM β -glycerophosphate, 1 mM EDTA, 1% w/v Triton X-100, 1 mM NaF and 1 mM EGTA; a small aliquot of protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), was added just prior to homogenization. Samples were centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was collected and the protein concentration was determined using the BioRad protein assay with bovine serum albumin as a standard. Samples were then diluted to the desired concentration in sample buffer containing 100 mM Tris-HCl (pH 6.8), 4% SDS, 20% v/v glycerol, 5% v/v β -mercaptoethanol, and 0.2% w/v bromophenol blue. Samples were boiled for 5 min, cooled and then frozen at -20°C until use.

SDS-polyacrylamide gel electrophoresis was carried out using gels ranging from 4-20% acrylamide gel, depending on the molecular weight of the proteins to be separated. Protein amounts ranging from 10-200 μ g were loaded into each well, again depending on the protein being analyzed. Proteins were separated on gels using a BioRad Mini-PROTEAN 3 system. Gels were run at 180 V until the desired separation of colored molecular weight markers (Kaleidoscope marker, BIORAD) was achieved. Proteins were blotted onto polyvinylidene difluoride (PVDF) membranes (Pall) by wet transfer in cold 25 mM Tris pH8.5, 192 mM glycine and 20% v/v methanol. Transfer was carried out at 300 mA for ~90 min.

PVDF membranes were incubated in blocking solution composed of TBST buffer (10 mM Tris pH 7.5, 150 mM NaCl, 0.05% v/v Tween-20) and 5% non-fat dry milk for 30-60 min at room temperature. The blocking solution was then discarded and the membrane was incubated in fresh TBST containing primary antibody, diluted according to manufacturer's instructions (1:1000 v/v). Membranes were then incubated overnight at 4°C to allow primary antibody to bind. Rabbit polyclonal antibodies for the following proteins were used: anti-20S proteasome (Calbiochem, cat. No.539145- designed against the α 1-subunit), anti-ubiquitin and ubiquitin conjugates (Biomol, cat. No.UG9510), and eukaryotic initiation factors eIF4B (Cell Signaling, cat. No.3592), eIF4e (Cell Signaling, cat. No.9742), phospho-eIF4e against Ser209 (Cell Signaling, cat. No.9741) and 4e-BP1 (Cell Signaling, cat. No.9452). After incubation with primary antibody, membranes were washed with TBST in three short washes of five minutes each. Membranes were then incubated with secondary HRP-linked goat anti-rabbit IgG antibody (1:2000 v/v) in TBST for 1.5 h at 4°C. After incubation the membranes were washed again in TBST for 3 washes of 5 min. Final detection of the desired protein was carried out using an enhanced chemiluminescence assay with Super Signal West Pico Chemiluminescent Substrate (PIERCE). The results were scanned and evaluated by ChemiGenius Bio Imaging System (SynGene).

To confirm that a consistent amount of protein was added to each well, membranes were subsequently stained with Coomassie blue. Membranes were rescanned and selected strong bands that showed constant intensities between samples (and were not near the MW of the protein of interest) were chosen as control bands. They were quantified by densitometry and used to normalize the intensity of the immuno-reacted

bands between lanes. Quantification was performed using GeneTools software (SynGene).

A minimum of 4 independent isolations from pooled tissue was performed per treatment. Hibernating:euthermic ratios were calculated by first normalizing the euthermic and hibernating band intensities with Coomassie stained blots, and then calculating the ratio of hibernating values over euthermic values. The mean of these values was calculated $\pm$ SEM and the Student's t-test was employed to assess significant differences between euthermic and hibernating states.

Results:

Partial purification of the proteasome by Centricon concentrating devices

Figure 2.2 shows the effect of partial purification of *S. tridecemlineatus* skeletal muscle proteasome activity by use of Centricon concentrators. Activity of the proteasome in crude supernatant was relatively low but could be increased substantially by separating the proteasome from lower molecular weight metabolites and proteins using either Centricon-100 (MW cutoff 100 kDa) or Centricon-500 (MW cutoff 500 kDa) devices. Separated from lower molecular weight subcellular components using the Centricon-500, proteasome activity in muscle extracts was nearly doubled. Centricon-500 treatment was used subsequently for the standard preparation of enzyme extracts for kinetic studies. The effect of Centricon-500 treatment on activity suggested that possible endogenous inhibitors of the proteasome were being removed in the filtrate. Therefore, additional tests were performed on the filtrate in an effort to determine the nature of these potential low molecular weight inhibitors. Samples of the Centricon-500 filtrate were treated in

two ways: (a) passage through a small column of Sephadex G-50 column to separate protein versus metabolite fractions, or (b) boiled for 10 min to denature proteins. When added back to the partially purified proteasome in the Centricon-500 retentate, samples of untreated filtrate inhibited enzyme activity. However, neither the boiled filtrate nor the G-50 eluate affected proteasome activity when added back to the proteasome-containing retentate. The lack of inhibition by the boiled sample suggests that the inhibitor is a peptide or protein and the lack of inhibition by the G-50 eluate suggests that the peptide/protein inhibitor must have a molecular weight less than 50 kDa (and hence was retained on the column).

Centricon partial purification could also remove low molecular weight proteases that could cleave the substrate peptides and potentially yield false results for proteasome activity. As an internal check, several samples of Centricon retentate were incubated with a potent proteasome inhibitor MG132 prior to assay. In the presence of the inhibitor, activity was zero indicating that all activity in the uninhibited assay was due to the proteasome and not to other proteases. MG132 is also an inhibitor of lysosomal proteases and calcium dependent protease, but these proteases have low molecular weights and would be removed by the Centricon filtration.

Separation by Sephacryl-400 gel filtration

A 1:5 w:v muscle extract, prepared as stated above, was fractionated on a Sephacryl-400 column. After a void volume of 50 mL was eluted, fractions of 1 mL were collected and assayed using Z-LLE-AMC peptide to determine the native molecular mass of the proteasome. The proteasome complex eluted in a single peak corresponding to 900

± 12 kDa (Figure 2.3). Peak fractions from the column were pooled and concentrated and then separated on a native polyacrylamide gel. After silver staining a single strong band was seen that migrated with a higher molecular mass than the 670kDa thyroglobulin standard (data not shown). Hence, both size separation techniques are consistent with 20S form of the proteasome.

Assays of proteasome activity in euthermic and hibernating ground squirrels

K_m and V_{max} values for the proteasome were measured at 23°C in skeletal muscle extracts from euthermic and hibernating ground squirrels after partial purification by Centricon-500 filtration, which removes low molecular weight inhibitors. Table 2.1 shows values for each of the peptide substrates: Z-LLE-AMC, Z-ARR-AMC and Suc-LLVY-AMC. The proteasome showed distinctly different V_{max} and K_m values with each of the substrates, highlighting the different protease activities of the proteasome complex. However, for each individual substrate, no significant differences were seen for either substrate affinity or maximal activity between the enzyme from euthermic and hibernating animals.

Temperature effects on substrate K_m values

The effects of temperature on the K_m values for each of the three substrates of the proteasome were evaluated at 23°C and at 8°C, the cold temperature being similar to hibernating body temperature. Assays were performed on muscle extracts from euthermic and hibernating ground squirrels and additionally on mouse skeletal muscle extracts for comparison to a non-hibernating mammal. The effects of temperature were mixed among

the peptidase activities (Table 2.2). Assays at low temperature resulted in a significant increase in the K_m value (2.6-3 fold) for the peptide Z-LLE-AMC corresponding to the peptidyl-glutamyl peptide hydrolytic activity in all three cases. However, K_m values for the peptides measuring the tryptic and chymotryptic activities were either decreased or unaltered when assayed in the cold. The K_m values for Z-ARR-AMC of the proteasome from euthermic and hibernating muscle decreased to 19-24 % of the euthermic value whereas the corresponding K_m for the mouse proteasome was unaltered at low temperature. The K_m for SUC-LLVY-AMC decreased significantly by 34% for the euthermic enzyme at low assay temperature but neither the hibernator nor the mouse enzymes showed significantly altered K_m values for this substrate.

Enzyme maximal activity was also determined at two temperatures (Figure 2.4). The activity of the ground squirrel enzyme at 8°C was significantly reduced to ~30% of the value at 23°C; this corresponded to a Q_{10} value of 2.03. However, the mouse enzyme utilizing either Z-ARR-AMC or SUC-LLVY substrate was more strongly affected by low temperature with activity reduced to 13-19% of the value at 23°C.

Carbonyl content

The level of protein carbonyls is a measure of oxidative damage to cellular proteins. Carbonyl levels were assayed in muscle extracts from both euthermic and hibernating squirrels (n=6). Levels decreased significantly from 0.567 ± 0.118 nmole carbonyl/mg protein in euthermic squirrels to 0.125 ± 0.029 nmole carbonyl/mg protein in hibernating squirrels.

Western Blot analysis

The amount of proteasome present during euthermic and hibernating states was examined through Western blot analysis on squirrel muscle. Additionally ubiquitin and ubiquitin conjugates were assessed in muscle and liver tissue of euthermic and hibernating ground squirrel to determine if there was any regulation of the ubiquitination process upstream of protein degradation during stress conditions. Suppression of protein turnover during torpor could arise from a reduction in the amounts of protein biosynthetic and proteolytic enzymes. To evaluate the amount of proteasome present in muscle, Western blotting on SDS-PAGE gels was used with antibodies recognizing the α 1-subunit the proteasome, the central protein of the 20S core. The larger proteasomes (26S and the immunoproteasome) also contain the 20S core and so the antibody detected total α subunits in all forms of the proteasome. There was no significant difference in the amount of α -subunit detected in muscle from euthermic versus hibernating animals (data not shown), which indicates that the proteasome protein is retained during hibernation, even though the activity of the proteasome may be strongly reduced in the hypometabolic state.

Proteolysis during hibernation could also be suppressed by restricting the available pool of ubiquitin with which to tag proteins for degradation. To test this idea, the levels of ubiquitin were assessed by Western blotting. The results showed that pools of free ubiquitin, producing a single band at ~8 kDa, remained unchanged between euthermic and hibernating conditions in both muscle and liver (Figure 2.5). Ubiquitin conjugates of proteins show up as multiple bands because the tagged proteins are of many different molecular weights and the number of ubiquitin moieties bound on each protein

type also vary. Hence, ubiquitin conjugates were quantified by summing up the densities of multiple bands outlined in brackets in Figure 2.5. There was a significant increase in the amount of ubiquitin-protein conjugates, at a range of molecular weights, in both muscle and liver of hibernating animals indicating an accumulation of tagged proteins during hibernation. Ubiquitin conjugates were 1.70- and 1.23-fold higher in muscle and liver of hibernating animals, respectively, as compared with euthermic controls.

Western blotting was also used to assess the responses of the ribosomal initiation factor 4 during hibernation. Four parameters were tested – levels of subunits eIF4B and eIF4e, the relative amount of phosphorylated eIF4e, and levels of the binding protein 4e-BP1. There were no significant changes in muscle and liver of euthermic and hibernating animals in their expression of eIF4B, phospho-eIF4e and 4e-BP1. However, liver, but not muscle, of hibernating squirrels showed a significant increase in the total amount of eIF4e as compared with euthermic controls (Figure 2.6).

Figure 2.2: Effect of partial purification using Centricon-100 or Centricon-500 filters on ground squirrel skeletal muscle proteasome activity. Activity was assayed at 23°C with 100 μ M Z-LLE-AMC as the substrate and is expressed as pmole AMC produced per hour per mL, all retentate volumes having been adjusted back to equal the original volume added to the Centricon. In this way, activities are comparable between treated and untreated samples and not influenced by protein removed during the Centricon procedure. Values are means $\pm$ SEM, n=3.

Figure 2.3: Elution profile for the proteasome off Sephacryl-S400 and standard curve relating log molecular weight and elution volume for four protein standards. The elution position of the 20S proteasome is shown by an arrow on the standard curve. Activity in the fractions is expressed relative to activity in the peak fraction that is set to 100.

Table 2.1. Proteasome activity and K_m values with three substrates for the muscle enzyme from euthermic and hibernating ground squirrels. Assays were conducted at 23°C and values are means $\pm$ SEM, n=3.

	Peptide substrate		
	<u>Z-LLE-AMC</u>	<u>Z-ARR-AMC</u>	<u>Suc-LLVY-AMC</u>
K_m (μ M peptide)			
Euthermic	59.0 $\pm$ 4.9	20.7 $\pm$ 3.1	73.5 $\pm$ 4.5
Hibernating	60.4 $\pm$ 3.8	18.6 $\pm$ 1.9	70.6 $\pm$ 3.8
V_{max} (pmol/h/ μ g protein)			
Euthermic	16.3 $\pm$ 1.5	7.84 $\pm$ 0.6	5.30 $\pm$ 0.7
Hibernating	16.8 $\pm$ 1.9	9.52 $\pm$ 1.8	6.80 $\pm$ 0.9

Table 2.2. Effects of assay temperature on K_m values for the three substrates of the proteasome in skeletal muscle extracts from euthermic and hibernating ground squirrels and the nonhibernator mouse.

	Peptide substrate					
	<u>Z-LLE-AMC</u>		<u>Z-ARR-AMC</u>		<u>Suc-LLVY-AMC</u>	
	23°C	8-10°C	23°C	8-10°C	23°C	8-10°C
Euthermic	59.0 ± 4.9	176 ± 29.6*	20.7 ± 3.1	3.94 ± 1.9*	73.5 ± 4.6	48.9 ± 4.1*
Hibernator	60.4 ± 3.8	159 ± 25.9*	18.6 ± 1.9	4.49 ± 1.1*	70.6 ± 3.8	86.7 ± 19.6
Mouse	75.1 ± 3.1	195 ± 16.4*	15.7 ± 0.5	14.9 ± 2.3	87.1 ± 15.1	66.9 ± 11.6

Data are means ± SEM, n=6. The temperature range for the cold assay represents initial (8°C) versus highest final (10°C) temperature measured in microplate wells; the slight warming during the 45 min assay is due to the mechanical activity microplate reader. * - Significantly different from the K_m value at 23°C as assessed by the Student's t-test, $P < 0.05$.

Figure 2.4: Effect of temperature on the $V_{\max}$ activity of the proteasome in skeletal muscle extracts from euthermic and hibernating ground squirrels as well as the non-hibernator mouse. Shown are the relative activities at 8°C compared with 23°C; values are mean $\pm$ SEM, n=6.

Figure 2.5: Western blot analysis of the levels of free ubiquitin and ubiquitin conjugates in muscle and liver of euthermic and hibernating squirrels. The ratios of normalized band intensities of hibernation:euthermic are shown. Data are means $\pm$ SEM, n = 4 separate determinations on independent tissue extracts. Stars show a significant difference in normalized band intensities between the hibernating versus euthermic state, as determined by the Student's t-test $P < 0.05$.

Figure 2.6: Western blot analysis of eIF4e, phospho-eIF4e, 4e-BP1 and eIF4B levels in muscle and liver of euthermic and hibernating squirrels. The ratios of normalized band intensities of hibernation:euthermic are shown. Data are means $\pm$ SEM, n = 4 separate determinations on independent tissue extracts. Stars show a significant difference in normalized band intensities between the hibernating versus euthermic state, as determined by the Student's t-test $P < 0.05$.

Discussion:

The present study assessed proteasome activity in skeletal muscle from euthermic and hibernating ground squirrels at both warm and cold assay temperatures in an effort to elucidate the mechanism of regulation of the proteasome employed during hibernation. Related parameters of proteolysis and protein synthesis were also assessed in order to help evaluate the activity state of these processes in hibernation versus euthermia.

The use of a membrane filtration device for the partial purification of the proteasome was first described by Rogers and Dean (2003). In mirroring their purification step in the present study, we found that the activity of the proteasome increased after low molecular weight cellular constituents were removed by Centricon filtration. This suggested that low molecular weight proteins, metabolites or ions might be inhibiting the proteasome in crude extracts and subsequent attempts to characterize the inhibitor suggested that a low molecular weight protein was involved. Since there were no significant differences in the maximal activities of the proteasome in filtered extracts from euthermic versus hibernating conditions, it is possible that hibernation-related changes in the levels of small molecular weight inhibitors of the proteasome may be the means of suppressing proteasome activity during torpor. For example, proteasome inhibition has been shown to induce several classes of heat shock proteins (Ding and Keller, 2001; Kawazoe *et al.*, 1998).

Fractionation of the ground squirrel muscle proteasome by column gel filtration revealed one peak of activity and a native gel of the pooled peak fractions revealed a single band. Both were consistent with a molecular mass of ~900 kDa which is indicative of the 20S form of the proteasome. Subsequent assay of the peak column fractions in the

presence of ATP, to stimulate the 26S proteasome, or low concentrations of SDS, to stimulate the 20S proteasome also suggested that activity was the 20S proteasome (data not shown).

Kinetic analysis used peptide substrates to evaluate the three different proteolytic activities of the proteasome. Parameters of substrate affinity (K_m) and activity (V_{max}) gave unique results for each peptide. However, no significant differences between the kinetic parameters of the proteasome from euthermic versus hibernating animals were found (Table 2.1). All extracts and assays were conducted in the presence of additives that inhibited endogenous kinases and phosphatases so that any differences in the phosphorylation state of the proteasome between the two physiological states would be maintained. Some studies have shown that there are phosphorylation sites on the proteasome specifically on the C8 and C9 subunits (Castano *et al.*, 1995) that are phosphorylated by casein kinase II. Casein kinase II is also known to copurify with the proteasome under some conditions (Ludemann *et al.*, 1993) and, hence, phosphorylation could be a mechanism of proteasomal regulation. Since there were no significant changes in the kinetic parameters of the proteasome in euthermic versus hibernating conditions, this argues that changes in the phosphorylation state of the proteasome are not likely to be a mechanism of regulation of proteolytic suppression during entry into torpor.

Although there were no significant changes in K_m and V_{max} values for the proteasome from euthermic and hibernating samples when assayed at 23°C, assay temperature had substantial effects on K_m values for the different substrates. When assayed at 8-10°C, compared with 23°C, the K_m for Z-LLE-AMC increased significantly (ie. substrate affinity decreased) for both the euthermic and hibernating enzyme as well as

for the mouse proteasome. Conversely, the K_m for Z-ARR-AMC decreased significantly at low temperature for the euthermic and hibernating enzyme but not for the mouse proteasome. K_m for SUC-LLVY-AMC was temperature independent for the hibernator and mouse enzymes but decreased significantly for the euthermic enzyme. The differential effects of temperature on the K_m values for the different substrates undoubtedly arises due to temperature effects on the different types of weak bonds that contribute to binding each of the substrates (Hochachka and Somero, 1984) and may suggest that enzyme preference for different physiological protein substrates could change at the low body temperature of the hibernating state.

Maximal activity of the proteasome with each of the substrates was suppressed by low temperature. With a 15°C reduction in assay temperature, the activity of the proteasome in ground squirrel extracts decreased by ~70% (Figure 2.4); the calculated Q_{10} associated with this was 2.03, a value consistent with the general effect of temperature on the reaction rates of most enzymes. However, mouse proteasome activity was more strongly inhibited at low temperature (reduced to just 13-19% of the value at 23°C). This suggests that the protein from the hibernating species is better suited for function over a wide range of environmental temperatures. Retention of function of proteins over a wide temperature range can be achieved by making minor alterations in amino acid sequence (Somero, 2003).

Low tissue perfusion rates during torpor-arousal cycles can be a source of oxidative damage to tissues. Torpor is not continuous over the winter months but is interspersed with brief arousal periods where body temperature is rapidly returned to euthermic values before another torpor bout commences. Ischemia-reperfusion injury

may occur in areas where blood flow is not fully restored before elevations in body temperature (Carey *et al.*, 2003). Skeletal muscle may be susceptible to ischemic-reperfusion injury during hibernation as it is one of the first areas of the squirrel where blood flow is minimized. Quantification of carbonyl proteins is a very sensitive and widespread means of estimating oxidative damage to proteins and we predicted that carbonyl protein levels would rise in hibernation due to the stresses caused by torpor cycles. However, contrary to the expectations for a system that could show ischemia-induced damage, the level of oxidatively modified proteins actually decreased in hibernation. These results for protein carbonyls in skeletal muscle could be interpreted in two ways: (a) the proteasome remains active in degrading oxidatively damaged proteins during hibernation, and (b) oxidative damage to proteins is actually reduced in muscle during hibernation. In support of the former, it is been well known that the 20S proteasome selectively degrades oxidatively damaged proteins through recognition of hydrophobic surface patches (Grune *et al.*, 2003) and this function may continue during torpor to avoid the accumulation of damaged proteins. The latter possibility contrasts with the results of another study that showed elevated levels of oxidative damage products of lipids (conjugated dienes) in intestine of hibernating ground squirrels as compared with summer active animals sampled several weeks before hibernation (Carey *et al.*, 2000). It is also generally believed that hibernation and/or the arousal process (which is accompanied by a huge increase in oxygen consumption) must cause oxidative stress because several studies have documented enhanced levels of antioxidant defenses (both metabolites such as ascorbate and enzymes) during hibernation (summarized in Carey *et al.*, 2003). However, if antioxidant defenses are elevated prior to hibernation,

then the accumulation of oxidative damage products during torpor or arousal could be minimized or even suppressed below normal, an outcome that could be beneficial for maintaining the viability of cells/tissues over long weeks of continuous torpor. These issues of oxidative stress and antioxidant defense during hibernation are far from resolved and require more study to sort out a variety of issues including organ-specific responses, assessment of oxidative damage to different types of macromolecules (e.g. lipid, protein, DNA), and analysis of the patterns of changes in oxidative damage products and antioxidant defenses over the course of torpor-arousal cycles.

Van Breukelen and Carey (2002) found an increase in the level of protein-ubiquitin conjugates during hibernation and suggested a storage function for these conjugates so they may be broken down when euthermia resumes. Comparable results were found in the present study. Liver and muscle ubiquitin conjugates rose significantly during hibernation. However, the total pool of free ubiquitin did not decrease. Ubiquitin-dependent protein degradation via the 26S proteasome requires ATP input and, hence, it could be expected that this activity would be selectively suppressed as part of the overall metabolic rate depression of the hibernating state. The result would be an accumulation of ubiquitin conjugates. However, the ubiquitin conjugating enzymes E1-E3 would have to be kept active during hibernation to be able to tag and store damaged proteins. Protein degradation by the 20S proteasome is a more energetically favorable pathway and appears to continue to function during hibernation to serve a housekeeping purpose for elimination of oxidatively damaged proteins.

Western blot analysis of the responses of proteins of the eIF4 complex suggested relatively little regulation of this complex during torpor. Levels of the eIF4B subunit did

not change nor did the amount of 4E-BP1, an inhibitor of eIF4e. However, the total amount of eIF4e rose between euthermic and hibernating conditions in liver tissue. The eIF4e is responsible for recruiting mRNA to the ribosome for translation (Gingras *et al.*, 1999). This is a rate-limiting step in translation. Although an increase in eIF4e protein during hibernation seems contradictory to the expectation of protein synthesis suppression during hibernation that is predicted from the responses of other ribosomal factors (levels of phosphorylated eIF2 α and eEF2), van Bruekelen *et al* (2004) also reported elevated phospho-eIF4e in the liver of golden mantled ground squirrels *Spermophilus lateralis*. They suggested that perhaps this is a compensatory mechanism and that further inhibition of protein translation happens downstream of the eIF4e. However, although the present study detected increased amounts of eIF4e protein in liver of hibernating animals, the activity state of eIF4e was not enhanced in hibernation as shown by the lack of change in the relative level of phosphorylated eIF4e between euthermia and hibernation. Phosphorylation of eIF4e is required for its activation and the enhancement of translation. There was also no change in the level of the regulating protein 4e-BP1. In the absence of phosphorylation of 4e-BP1, it efficiently binds to eIF4e and inhibits translation. When 4e-BP1 is phosphorylated, it is no longer able to bind to eIF4e and translation occurs. Unlike the study conducted by Van Bruekelen *et al.* (2004), we were unable to determine the phosphorylation state of the binding protein. However, since there was no change in the phosphorylation state of eIF4e, and since there was a net increase in total eIF4e protein during hibernation in liver tissue, the control of translation during hibernation must lie in the phosphorylation state of 4e-BP1, or in the regulation of other components of the protein synthesis apparatus.

Chapter 3:
Effects of Hypometabolism on Proteolysis and Protein
Synthesis in the Freeze-tolerant Wood Frog, *Rana*
sylvatica

Introduction:

The wood frog, *Rana sylvatica*, is one of a small group of terrestrially-hibernating anurans that can survive whole body freezing during the winter months. Freezing survival requires a range of physiological and biochemical adjustments (Storey and Storey, 2004c). Breathing and heartbeat cease in the frozen animal and so all organs experience ischemia and anoxia for the duration of the freeze, which may last for days or weeks. A large fraction of intracellular water migrates out of cells to freeze in extracellular ice masses and, as a consequence, cell volume is greatly reduced. High concentrations of glucose are accumulated as a cryoprotectant (concentrations in core organs rise from 1-5 $\mu\text{mol/g}$ in controls to 150-300 $\mu\text{mol/g}$ in frozen frogs) to limit cell volume from decreasing below a critical minimum and to help protect/stabilize cellular macromolecules. A variety of genes are up-regulated during freezing to address specific needs in the frozen organism (Storey, 2004) and metabolic functions are reorganized to suppress those that are unnecessary in the frozen state and to conserve the energy generated from anaerobic metabolism for critical vital functions. By coordinating the suppression of energy-producing and energy-consuming processes, many organisms can readjust their metabolic rate to a new much lower rate of energy turnover (hypometabolism) and so greatly extend the time that they can survive using a fixed reserve of endogenous metabolic fuels (Storey and Storey, 2004b). Reversible protein phosphorylation is one of the key mechanisms that are employed for coordinated regulation of transitions to and from the hypometabolic state.

Protein synthesis can consume from 12-80% of cellular ATP depending on the tissue and metabolic state (Fuery *et al.*, 1998) and, not surprisingly, protein synthesis is

one of the energy-expensive cellular processes that is typically strongly suppressed in organisms entering energy-restricted states such as during freezing (Storey and Storey, 2004b). A primary mechanism of translation inhibition is the phosphorylation of various ribosomal initiation and elongation factors and/or proteins that regulate these factors. The phosphorylation of eukaryotic initiation factor 2 α , the effects of phosphorylation on eukaryotic initiation factor 4e and the effect of eukaryotic initiation factor 4B have been implicated in mammalian models as ways of suppressing protein synthesis during hypometabolism (DeGracia *et al*, 1996). If protein synthesis is suppressed in the hypometabolic state, it stands to reason that the opposing metabolic function, protein degradation, must also be suppressed in a coordinated fashion in order to maintain protein homeostasis in cells.

The proteasome, or multicatalytic proteinase (MCP) complex, is responsible for 80-90% of protein degradation in cells (Orlowski and Wilk, 2000) and, therefore, should be a primary target of regulation aimed at altering the rate of protein degradation in hypometabolic systems. The present study assessed the effects of freeze/thaw and related stresses (dehydration, anoxia) on the activity and properties of the proteasome in skeletal muscle of freeze tolerant wood frogs with the goal of determining how proteasome activity might be suppressed in these energy-restricted states. Assays used fluorescent peptides as substrates to target and measure the three protease activities of the proteasome. Western blotting quantified the relative amounts of proteasome protein over a freeze-thaw time course. Because the proteasome plays a major role in degrading proteins that have undergone oxidative damage, the content of protein carbonyls was also measured in muscle and liver in the different states, providing a direct measure of

oxidative damage to proteins and an indirect measure of proteasomal function. Additionally, ubiquitin and ubiquitin conjugate levels were measured using Western blot analysis to determine the effects of freezing on the ubiquitin tagging process upstream of protein degradation. Finally, the effect of freezing on protein synthesis was examined by analyzing the amounts of the initiation factors eIF2 α (in its phosphorylated form), eIF4B and eIF4e.

Materials and Methods:

Animals:

Male wood frogs (*Rana sylvatica*) were collected from breeding ponds in the Ottawa area during early April. Animals were rinsed in a tetracycline bath and housed at 5°C in plastic boxes containing damp sphagnum moss for 1-2 weeks before use. Control frogs were sampled from this condition, sacrificed by double pithing and tissues were rapidly dissected out, frozen in liquid nitrogen, and stored at -80°C until use. For freezing exposure, frogs were transferred to closed plastic boxes with moist paper toweling on the bottom and then placed in an incubator at -2.5°C. Under these conditions frogs cool and begin freezing after ~45 min due to nucleation by contact with ice forming on the paper toweling. Some frogs were sampled after 24 h frozen whereas others were returned to 5°C and sampled after 4 or 8 h of thawing.

Experimental dehydration studies were carried out as previously described (Churchill and Storey, 1994) with frogs held at 5°C in glass desiccators separated from silica gel by a 1 cm layer of sponge. Initial body water content of frogs prior to dehydration was 0.81 ± 0.02 g H₂O/g body mass (mean $\pm$ S.E.M., n=6). The rate of water

loss was 0.5-1% total body water lost per hour. Once animals had lost 40% of total body water, they were sacrificed as above. Experimental anoxia exposures were also conducted as previously described (Holden and Storey, 1996). Frogs were placed in 500 mL glass jars containing a 0.5 cm layer of 5°C water that had been previously bubbled for 20 min with 100% nitrogen gas. Bubbling with nitrogen gas was continued for 20 min and then the jars were tightly capped and sealed with Parafilm. Animals were held at 5°C and sacrificed after 8 h anoxia exposure.

Tissue homogenization and preparation:

Samples of frozen leg skeletal muscle were pulverized under liquid nitrogen using a mortar and pestle and then 100 mg of tissue was mixed with 1 mL of homogenization buffer (50 mM Tris HCl, pH 7.8, 10 mM β -mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 50 mM NaF) and homogenized and centrifuged as outlined in Chapter 2. The addition of chelating agents (EDTA, EGTA) inhibits endogenous protein kinases, whereas NaF inhibits protein phosphatases; these additions stabilize the native phosphorylation state of the proteasome during tissue extraction. After centrifugation, the supernatant was removed and applied to Microsep (Pall) concentrating devices with a molecular weight cutoff of 300 kDa to remove small molecular weight proteins. Concentrating devices were centrifuged spun at 7500 x g at 4°C until the sample volume had decreased to half. Homogenization buffer was then added to wash the sample, and the sample was recentrifuged. Final retentate was adjusted to the original volume with homogenization buffer.

Assay conditions

Enzyme activity was assayed fluorometrically using the method described by Rogers and Dean (2003). Enzyme assays were carried out as outlined in Chapter 2. Release of the fluorophore AMC from substrate-AMC conjugates was quantified using a Victor 1420 Multilabel counter (Perkin Elmer). The same three peptides (Suc-LLVY-AMC, Cbz-ARR-AMC, and Cbz-LLE-AMC) were prepared as stated in Chapter 2. Assays were conducted with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. Preliminary studies optimized enzyme amount and the linearity of the reaction over time at both high and low assay temperatures; from this, 45 min was chosen as the standard reaction time. Inhibitor experiments were carried out using a potent proteasome inhibitor, MG132 (Sigma Chemical Co.), at a final assay concentration of 50 μ M; samples were incubated with the inhibitor for 20 min prior to assay. Assays were performed as stated in Chapter 2. To conduct low temperature assays the fluorometer was placed in a cold incubator set at 8°C and chilled overnight. Before each assay, the microplate filled with assay mixture was pre-equilibrated in the incubator for ~15 min until the temperature in a blank well (filled with buffer) stabilized at 8°C, as determined by a thermister in the well. After the assay was completed, the temperature in blank well was measured again; temperature rose by 1-1.4°C due to heat generation by the machine during the run.

Incubation studies for the determination of the effects of endogenous kinases and phosphatases:

For these experiments only, tissue extracts were prepared in 50 mM Tris HCl, pH

7.8 containing 10 mM β -mercaptoethanol and were homogenized and centrifuged as stated above. Microsep treatment was not used for incubation studies as all endogenous kinases and phosphatases need to be retained in the extract. To test the effects on the proteasome of stimulating either endogenous protein kinases or protein phosphatases, aliquots of enzyme extract were incubated for 4 h at 4°C under the following conditions: (a) STOP conditions containing the additions 5 mM EDTA, 5 mM EGTA, and 30 mM NaF to the buffer to inhibit endogenous kinases and phosphatases in order to maintain the native phosphorylation state of the enzyme, (b) to stimulate protein kinases additions to the buffer were 30 mM NaF, 1 mM cyclic 3'5'-adenosine monophosphate (cAMP), 1 mM cyclic 3'5' guanosine monophosphate (cGMP), 5 mM ATP, 5 mM MgCl_2 , 1.3 mM CaCl_2 , and 1 $\mu\text{g/ml}$ phorbol 12-myristate-13-acetate (PMA), or (c) to stimulate protein phosphatases additions to the buffer were 5 mM MgCl_2 and 5 mM CaCl_2 .

Protein determination and statistics

Protein concentration was determined using the Coomassie blue dye-binding method and the Bio-Rad prepared reagent. Enzyme activities were measured as AMC production over time (pmole/h/ μg protein) based on a standard curve constructed with 0-15 μM of the AMC fluorophore. Data were analyzed using a two-tailed Student's t-test with statistical significance determined as $P < 0.05$.

Molecular weight determination

A Sephacryl-400 gel filtration column (90 cm x 2.5 cm) was equilibrated in the same buffer and calibrated as in Chapter 2. Tissues were homogenized 1:5 w:v in

homogenization buffer, centrifuged at 9,000 x g and then the supernatant was loaded onto the column. A flow rate of 0.35 mL/min was obtained and 1 mL fractions were collected. Fractions were assayed with Z-LLE-AMC peptide.

Carbonyl assay for total protein oxidative damage

The protocol was modified from Reznick and Packer (1994). Tissue extracts of liver and muscle were prepared 1:10 or 1:20 w:v in homogenization buffer and centrifuged for 15 min at 10,000 x g. The carbonyl protein assay protocol was followed as in Chapter 2. Carbonyl content was measured as nmol/mg protein.

Western Blotting of proteins in proteolysis and protein synthesis:

Frozen tissue samples of frog muscle and liver were prepared for Western blotting as outlined in Chapter 2. Homogenates were loaded onto SDS-gels of the appropriate concentration of acrylamide depending on the separation desired. Gels were run, proteins transferred onto PVDF membranes and membranes blocked with 2% milk solution as stated in Chapter 2. Rabbit polyclonal antibodies for the following proteins were used: anti-20S proteasome (Calbiochem, cat. No.539145 – designed against the α 1-subunit), anti-ubiquitin and ubiquitin conjugates (Biomol, cat. No.UG9510), and anti-eukaryotic initiation factors eIF4B (Cell Signaling, cat. No.3592), eIF4e (Cell Signaling, cat. No.9742), phospho-eIF2 α (Cell Signaling, cat. No. 9721). Membranes were incubated with antibodies (1:1000 v/v dilution) overnight in TBST and then washed and probed with secondary HRP-linked goat anti-rabbit IgG antibody (1:2000 v/v) in TBST for 1.5 h at 4°C. Bands were then detected, stained, normalized and quantified as outlined in

Chapter 2.

A minimum of 4 independent isolations from pooled tissue was performed per treatment. Ratios between control and frozen states were determined. The mean of these values was calculated $\pm$ SEM and the Student's t-test was employed to assess significant differences between control and frozen states.

Results:

Microsep concentrating devices are useful tools in the partial purification of the proteasome. Preliminary studies with ground squirrel tissue extracts showed an increase in proteasome activity in the retentate of the partially purified samples when compared to the activity of the crude protein extract (Figure 2.1). Removal of lower molecular weight proteins and metabolites by the use of concentrating devices increased the activity of the proteasome between 60-80% in squirrel extracts (see Chapter 2) and therefore this technique was also used in the preparation of the frog proteasome.

Microsep treatment could also remove low molecular weight proteases that could cleave the substrate peptides and potentially yield false results for proteasome activity. As an internal check, several frog muscle samples of Microsep retentate were incubated with the potent proteasome inhibitor MG132 prior to assay. In the presence of the inhibitor, proteasome activity in frog muscle extracts was zero indicating that all activity in the uninhibited assay was due to the proteasome and not to other proteases. MG132 is also an inhibitor of lysosomal proteases and calcium dependent protease, but these proteases have low molecular weights and would be removed by the Microsep filtration.

Sephacryl-400 gel filtration:

Fractionation of a frog muscle extract on Sephacryl-400 was used to determine the native molecular mass of the proteasome. The frog muscle proteasome complex eluted in a single peak corresponding to 830 kDa (Figure 3.1).

Effects of freezing, anoxia or dehydration exposures in vivo on skeletal muscle MCP

Microsep-treated extracts of skeletal muscle samples were prepared from wood frogs exposed to the following conditions *in vivo*: controls at 5°C, freezing at -2.5°C for 24 h, thawed at 5°C for 4 or 8 h after 24 h frozen at -2.5°C, 8 h anoxia exposure at 5°C, and dehydrated to 40% of total body water lost at 5°C. Figure 3.2 shows the K_m values for three substrates, Z-LLE-AMC, Z-ARR-AMC, and Suc-LLVY-AMC for the proteasome from skeletal muscle of control versus frozen frogs as well as K_m Z-LLE-AMC for muscle proteasome from the other experimental treatments. Significant changes were seen between control and frozen conditions with all peptide substrates; K_m Z-LLE-AMC was 42% higher for proteasome from frozen muscle, compared with controls, whereas K_m values for Z-ARR-AMC and Suc-LLVY-AMC decreased by 27 and 46%, respectively, during freezing. Thawing reversed the effect of freezing on the K_m Z-LLE-AMC, K_m values dropping again to control values. Experimental dehydration *in vivo* had no effect on the K_m Z-LLE-AMC but anoxia exposure increased the K_m value by 42%, mimicking the effects of freezing on the enzyme.

Table 3.1 shows the effects of experimental state on the maximal activity of the proteasome in frog muscle. Freezing strongly reduced the activity of the proteasome when assayed with either Z-LLE-AMC or Suc-LLVY-AMC; activity in frozen muscle

was just 60% and 7 %, respectively, of the corresponding control activity. However, freeze exposure did not alter the maximal activity with the peptide Z-ARR-AMC. Thawing for 4 h increased the $V_{\max}$ Z-LLE-AMC by 2.2-fold compared with the control value (3.6-fold over the value for frozen muscle) but the value declined back towards control levels after 8 h thawing. Both anoxia and dehydration significantly suppressed $V_{\max}$ to 65 and 49 % of the control value, respectively.

Temperature effects on substrate K_m value and $V_{\max}$ values:

Maximal activity and substrate affinity for Z-LLE-AMC were determined at warm (23°C) and cold (8°C) temperatures for the proteasome from skeletal muscle of control and frozen frogs (Table 3.2). In all cases, kinetic parameters were significantly reduced in assays at 8°C as compared with 23°C. The K_m value for Z-LLE-AMC from control muscle decreased by 37 % when assayed at 8°C whereas the decrease in K_m was 69% for muscle proteasome from frozen frogs; hence, substrate affinity increased at low temperature. Maximal activity also decreased with decreasing assay temperature; calculated Q_{10} values were 1.42 and 1.86 for the enzyme from control and frozen frog muscle, respectively.

Effects of stimulating endogenous kinases and phosphatases on proteasome activity:

With the significant difference in proteasome activity between control and frozen tissues, protein phosphorylation was considered as a mechanism of regulation. To assess whether frog muscle proteasome was susceptible to reversible protein phosphorylation, crude muscle extracts from control and 24 h frozen frogs were incubated *in vitro* under

conditions that would stimulate the activities of endogenous protein kinases or protein phosphatases. These incubations were compared to incubations in which both kinases and phosphatases were inhibited (STOP conditions), showing the enzyme in its native phosphorylation state. The lower $V_{\max}$ values for the untreated enzyme in Figure 3.3, as compared with Table 3.2, are attributable to the absence of the Microsep filtration step in the preparation of these extracts. Figure 3.3 shows that incubation under conditions that should stimulate the activities of endogenous kinases (addition of Mg.ATP and second messengers of protein kinases A, G and C) did not significantly affect the activity of the proteasome in extracts from either control or frozen muscle, as compared with the corresponding activities under the STOP condition. However, incubation under conditions that would stimulate protein phosphatase activities (addition of Mg^{2+} and Ca^{2+}) resulted in a marked increase in proteasome activity in extracts from both control and frozen muscle; activity increased by roughly 4- and 2-fold, respectively. Notably, proteasome activity in extracts from frozen muscle increased to the activity seen in untreated control extracts.

Carbonyl content:

Protein carbonyls form as a result of oxidative damage and, hence, the carbonyl content of tissues is a measure of oxidative stress under different experimental conditions. Table 3.3 shows the carbonyl protein content in skeletal muscle and liver from control, 24 h frozen and 4 h thawed frogs. The amount of damaged protein increased 1.4-fold in both muscle and liver from frozen frogs, as compared with controls. Carbonyl content returned to control values within 4 h of thawing in both tissues.

Western Blot Analysis:

Changes in the amount of the 20S proteasome in skeletal muscle were evaluated over a freeze-thaw cycle using Western blotting with an antibody recognizing the $\alpha 1$ -subunit of the 20S proteasome. Figure 3.4 shows a marked decrease in the amount of proteasomes present during freezing; the mean amount in frozen muscle was just 36% of the amount detected in controls. However, the amount of proteasome complex in muscle increased again after thawing.

An antibody detecting ubiquitin and ubiquitin-conjugates was used to examine the effects of freezing on the ubiquitin tagging system, which is upstream from protein degradation. Free ubiquitin was visualized as a prominent 8kDa band as indicated on Figure 3.5. Ubiquitin conjugates of proteins show up as multiple bands because the tagged proteins are of many different molecular weights and the number of ubiquitin moieties bound on each protein type can also vary. Hence, ubiquitin conjugates were quantified by summing up the densities of multiple bands outlined in brackets in Figure 3.5. The ratio control versus frozen for ubiquitin and ubiquitin conjugates band densities are shown in the histogram for both skeletal muscle and liver of wood frogs. In muscle, both free ubiquitin and the amount of ubiquitin conjugates remained constant during freezing. By contrast, however, liver pools of free ubiquitin were strongly elevated (by about 2.8-fold) during freezing whereas the levels of ubiquitin conjugate were reduced during freezing by about 30% as compared with control liver.

Western blotting was also used to assess the responses of the ribosomal initiation factors during freezing in both muscle and liver. Representative Western blots are shown

for eIF4B and eIF4E in liver from control and 24 h frozen frogs and the histogram shows relative band densities in both liver and muscle. The antibody detecting the phosphorylated form of eIF2 α showed no change in the amount of the phosphoprotein (inactive) form of this initiation factor during freezing in liver. Muscle extracts did not react with this particular antibody. However, the amount of eIF4B and eIF4e changed significantly in liver during freezing; eIF4B content decreased by about 60% in liver from frozen frogs whereas eIF4E content rose by 2.1-fold. There were no significant differences between control and frozen levels of eIF4B and eIF4e in muscle tissue.

Fig 3.1: Elution profile for the wood frog skeletal muscle proteasome off Sephacryl S400 and standard curve relating log molecular weight and elution volume for four protein standards. The elution volume of the frog 20S proteasome is shown by an arrow on the standard curve. Enzyme activity in the fractions is expressed relative to activity in the peak fraction that is set to 100.

Fig 3.2: K_m values for three peptide substrates for MCP from frog skeletal muscle under different conditions. The enzyme from control (5°C acclimated) and frozen (24 h at -2.5°C) frogs was assayed with all three peptide substrates: Z-LLE-AMC, Z-ARR-AMC, and Suc-LLVY-AMC. Data for the other conditions are for assays with Z-LLE-AMC only. Data are mean $\pm$ SEM, n=4 independent determinations on muscle from different animals. Stars show significant differences as compared with respective control values, as determined using the Student's t-test, $P < 0.05$.

Table 3.1: Skeletal muscle proteasome activity in wood frogs treated *in vivo* to experimental freeze/thaw, anoxia or dehydration. For muscle from control and frozen frogs, activities with three different substrates are shown. Freezing was at -2.5°C; all other conditions were at 5°C. Assays were conducted at 23°C and data are means $\pm$ SEM, n=4.

	Peptide substrates		
$V_{\max}$ (pmol/h/ μ g protein)	<u>Z-LLE-AMC</u>	<u>Z-ARR-AMC</u>	<u>Suc-LLVY-AMC</u>
Control	62.7 $\pm$ 7.8	20.6 $\pm$ 9.7	60.8 $\pm$ 14.3
Frozen	37.8 $\pm$ 6.9*	17.8 $\pm$ 0.7	4.5 $\pm$ 0.2*
4 h thaw	135.4 $\pm$ 17.1*		
8 h thaw	110.2 $\pm$ 22.3		
Anoxic	40.8 $\pm$ 4.1*		
40% Dehydrated	30.8 $\pm$ 3.8*		

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

Table 3.2: Effects of assay temperature on K_m values and proteasome activity in skeletal muscle extracts from control and frozen wood frogs.

	Assay at 23°C	Assay at 8°C
Control Muscle		
K_m Z-LLE-AMC (μ M)	135.5 $\pm$ 13.8	84.5 $\pm$ 5.3 *
V_{max} (pmol/h/ μ g protein)	62.7 $\pm$ 7.8	38.3 $\pm$ 2.0 *
Frozen Muscle		
K_m Z-LLE-AMC (μ M)	192.5 $\pm$ 19.5	59.3 $\pm$ 3.96 *
V_{max} (pmol/h/ μ g protein)	37.8 $\pm$ 6.9	19.0 $\pm$ 2.0 *

Data are means $\pm$ SEM, n=6. * - Significantly different from the corresponding values at 23°C as assessed by the Student's t-test, $P < 0.05$.

Fig 3.3: The effects of incubations that stimulated the activities of endogenous kinases and phosphatases on the activity of the 20s proteasome in skeletal muscle extracts from control and 24 h frozen wood frogs. Data are mean $\pm$ SEM, n=4 independent determinations on muscle samples from different animals. Stars indicate significant differences as compared to their respective control values (STOP), as assessed by Student's t-test, $P < 0.05$.

Table 3.3: Protein carbonyl content as a measure of oxidatively damaged proteins in skeletal muscle and liver of control, frozen (24 h at -2.5°C) and thawed (4 h at 5°C) frogs. Values are means $\pm$ SEM, n=6.

	Carbonyl content nmol/mg protein	
	Muscle	Liver
Control	0.422 $\pm$ 0.056	0.435 $\pm$ 0.04
Frozen, 24 h	0.601 $\pm$ 0.056*	0.616 $\pm$ 0.055*
Thawed, 4 h	0.298 $\pm$ 0.017	0.399 $\pm$ 0.029

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

Fig 3.4: Western blot analysis of the amount of proteasome protein in skeletal muscle sampled from control, 24 h frozen, 4 hour thawed and 8 hour thawed wood frogs. Representative blots are shown along with histograms showing normalized band intensities. Data in histograms are means $\pm$ SEM, n = 4 separate determinations on independent tissue extracts. Stars show a significant difference in normalized band intensities between the stress state and control state, as determined by the Student's t-test $P < 0.05$.

Fig 3.5: Western blot analysis of the levels of free ubiquitin and ubiquitin conjugates in muscle (upper blot) and liver (lower blot) of control and frozen frogs. The histogram shows ratios of normalized band intensities of frozen versus control; data are means $\pm$ SEM, $n = 4$ separate determinations on independent tissue extracts. Stars show a significant difference in normalized band intensities between the frozen versus control state, as determined by the Student's t-test $P < 0.05$.

Fig 3.6: Western blot analysis of phospho-eIF2 α , eIF4B and eIF4e levels in muscle and liver of control and frozen frogs. Representative blots for liver eIF4B and eIF4e are shown. Histograms show the ratios of normalized band intensities of frozen versus control; data are means $\pm$ SEM, n = 4 separate determinations on independent tissue extracts. Stars show a significant difference in normalized band intensities between the frozen versus control state, as determined by the Student's t-test $P < 0.05$.

EIF4B LIVER

EIF4E LIVER

Discussion:

The present study was aimed at analyzing the effects of freezing and related stresses on the activity and amount of the proteasome in skeletal muscle of the wood frog. Furthermore, *in vitro* incubations were used to evaluate the effects of endogenous kinases and phosphatases on the proteasome in order to determine if reversible protein phosphorylation might be a mechanism of proteasome regulation. Carbonyl content was measured to assess the level of oxidative damage to proteins during the freezing process and to correlate this level of damage to proteasome activity. The effects of natural freezing on the levels of ubiquitin and the accumulation of ubiquitin conjugates were also examined in skeletal muscle and liver of frogs tissue and the state of ribosomal initiation factors, reflecting the status of protein synthesis, were examined.

The proteasome from wood frog skeletal muscle was eluted from the gel filtration column in a single peak corresponding to a molecular mass of ~830 kDa indicative of the 20S proteasome. Assays in the presence of ATP and SDS, to stimulate the 26S and 20S proteasomes, respectively, also confirmed that this complex was the 20S core proteasome. Pooled peak fractions from the Sephacryl S400 column were run on a native polyacrylamide gel and a single protein band was seen at a molecular weight slightly greater than the 669 kDa band of the thyroglobulin marker, again consistent with the expected size of the 20S proteasome (data not shown).

Kinetic assessment of the frog proteasome showed expression of the three characteristic proteolytic activities of the proteasome: tryptic, chymotryptic and peptidylglutamyl peptide hydrolytic activities. Significant changes in substrate affinity, K_m , were found for all three peptides when the proteasome from skeletal muscle of

control versus frozen frogs was assessed. Substrate affinity for Z-LLE-AMC decreased (K_m increased) whereas affinity for Z-ARR-AMC and Suc-LLVY-AMC increased in muscle from frozen frogs (Figure 3.2). The significant increase in K_m Z-LLE-AMC under frozen conditions was also seen in frogs given anoxia exposure whereas dehydration had no effect. V_{max} of the MCP with Z-LLE-AMC also decreased in both frozen and anoxic muscle (and also during dehydration) (Table 3.1). These data suggest that the anoxia/ischemia caused by tissue freezing may be the underlying physiological trigger for the modification of proteasome kinetic properties in frozen frogs. Indeed, many of the metabolic and gene expression responses to freezing that are seen in wood frog tissues have been linked to either anoxia stress or cell volume reduction as the underlying triggers (Storey and Storey, 2004c).

The proteasome has been found to undergo covalent modification by phosphorylation at three sites: the alpha subunits 7 and 3 in the core 20S barrel and the ATPase subunit S4 in the 19S regulatory cap (Orlowski and Wilk, 2003). Phosphorylation is important in the assembly of the 19S regulatory cap(s) to form the 26S proteasome (Orlowski and Wilk, 2003). The data presented in Table 3.1 and Fig. 3.2 were derived from enzyme extracts that contained inhibitors of endogenous protein kinases and phosphatases in order to preserve the phosphorylation state of the proteasome during the process of isolation and assay. Stress-induced stable changes in the K_m and V_{max} values of enzymes are often the result of covalent modification via reversible phosphorylation and, hence, this provided presumptive evidence that the addition or removal of phosphate groups from the proteasome might be the cause of the freeze- and anoxia-responsive changes in proteasome properties. Therefore, incubation studies were

conducted using conditions that would stimulate the actions of endogenous protein kinases (incubation with Mg.ATP plus second messengers of protein kinases A, G or C) or phosphatases (incubation with Mg^{2+} and Ca^{2+}). A significant increase in proteasome activity was found when samples were incubated under conditions that stimulated endogenous phosphatases. This suggests that protein phosphorylation may be the mechanism responsible for suppressing proteasome activity and decreasing substrate affinity for Z-LLE-AMC under stress conditions (freezing, anoxia) whereas dephosphorylation elevates proteasome activity and could underlie the large increase in proteasome activity seen during thawing.

Casein kinase II has been shown to phosphorylate the proteasome subunits alpha 7 and 3 (Castano *et al.*, 1995). Additionally, a cGMP-dependent phosphorylation sequence has been found in the alpha 3 subunit (Mason *et al.*, 1996). Casein kinase II is also known to copurify with the proteasome (Ludemann *et al.*, 1993). This study and others (Mason *et al.*, 1996) indicate that reversible phosphorylation is a key mechanism for controlling the activity of the proteasome and, furthermore, the present research indicates that reversible phosphorylation is important in regulating proteasome activity with respect to environmental stresses (freezing, anoxia) that affect the energy status of frog organs.

Proteasome V_{max} activity was decreased significantly during freezing for the peptidylglutamyl-peptide hydrolytic and chymotryptic activities, whereas the tryptic activity was unaffected. This again shows the differential protease activities of the proteasome as measured by the three peptides. Two factors could affect this decrease in activity: phosphorylation, as shown by the incubation studies, and the amount of

proteasome expressed, as shown by the Western blot analysis. The relative activity state of the proteasome in control versus frozen states was not affected by incubations that promoted the activity of protein kinases (compare STOP with kinase conditions) (Figure 3.3). This suggests that the lower amount of proteasome activity present in the frozen state was not a result of freeze-induced phosphorylation of the enzyme. This was confirmed by the results of the western blotting which showed a significant decrease in the amount of 20S proteasome between control and frozen states. Proteasome activity also increased significantly at the 4 hour thaw time period as shown in Table 3.1. Again we look to phosphorylation studies and western blots. Western blot analysis does not show an increase in the amount of proteasome at the 4 hour thaw period. However, incubation studies showed that dephosphorylating the proteasome can be responsible for very large increases in activity. Thus, it can be inferred that the increase in activity seen after 4 hours of thawing could result from dephosphorylation of the enzyme, perhaps setting the stage for very active proteolysis in the newly thawed tissue to catabolize damaged proteins accumulated while the animal was frozen.

Temperature also had a significant effect on both K_m and V_{max} values for the proteasome from control and frozen wood frog muscle. The K_m values were significantly reduced in low temperature (8°C) assays as compared with 23°C. This shows a higher affinity of the proteasome for substrates at lower temperatures which is perhaps not unexpected since the animals used in the present study were collected just after they emerged from winter hibernation and they were maintained in the lab at 5°C. A proteasome optimized for function *in vivo* at near 0°C temperatures would be appropriate over the winter months. The temperature dependence of the proteasome substrate affinity

may change in the summer perhaps as a result of an altered subunit composition or the expression of a different mixture of subunit isoforms. This has not yet been tested. Maximal activity of the proteasome showed an expected decrease at 8°C as compared with 23°C with a calculated Q_{10} for this enzyme of 1.42 and 1.86 for the enzyme from control and frozen frog muscle, respectively.

During freezing, vital signs including breathing and heartbeat cease in wood frogs. Blood circulation is cut off as plasma freezes and tissue oxygen levels are depleted. An ischemic state is maintained until thawing allows the reperfusion of organs. In mammalian systems, reperfusion damage is common after ischemia and is caused by a burst of oxyradicals formed when blood flow is re-established. However, studies with wood frogs have shown that this species maintains high constitutive activities of antioxidant enzymes, apparently to deal with oxidative stress that occurs when oxygen is rapidly reintroduced to organs during thawing (Joannis and Storey, 1996). Furthermore, levels of glutathione and some enzymes increase during freezing; e.g. glutathione peroxidase rose in skeletal muscle (Joannis and Storey, 1996). As a result, when two indices of oxidative damage to lipids were assessed, no changes were found over the course of freeze/thaw (Joannis and Storey, 1996). However, the measurements of carbonyl content in the present study show that oxidatively damaged proteins do accumulate in muscle while frogs are frozen but are rapidly removed after thawing. This accumulation might be due to oxidative damage during freezing, but equally could be due to inhibition of the proteasome in the energy-limited frozen state. The proteasome catabolizes oxidatively damaged proteins through recognition of hydrophobic surface patches (Grune *et al.*, 2003). Hence, carbonyl proteins could accumulate due to a reduced

ability to catabolize them while frogs are frozen; this explanation also tallies with the observation that carbonyl protein levels in muscle were reduced to below control levels within 4 h of thawing.

In a mammalian form of hypometabolism (hibernation), Van Breukelen and Carey (2002), found an increase in ubiquitin conjugates during torpor in gut and liver of hibernating ground squirrels. The present data for frozen frogs showed a decrease in ubiquitin conjugates in liver during freezing to 30% of control values, but not in muscle. This highlights tissue specific differences between the frog organs during freezing. Skeletal muscle and skin, being peripheral tissues, are the first tissues to freeze and metabolism is likely arrested very quickly. Hence, unchanged levels of ubiquitin and ubiquitin conjugates in muscle are perhaps not unexpected if protein degradation processes are rapidly halted. The carbonyl protein data suggest that damaged proteins continue to accumulate throughout the freezing process so the lack of change in ubiquitin conjugate levels suggests a regulated suppression of the ubiquitin tagging process in frozen muscle. Ubiquitin tagging involves three enzymes (E1, E2, E3) and ATP is required for the activation of ubiquitin to bind to E1. This ATP requirement may mean that ubiquitin tagging is too energetically expensive under stress conditions and is shut down. The 20S proteasome is also much more efficient at degrading oxidatively damaged proteins than the ubiquitin tagged 26S proteasome pathway (Grune *et al.*, 1997), and therefore ubiquitin pathways may be turned off but the 20S proteasome activity retained to deal with oxidatively damaged proteins. The data from liver show elevated ubiquitin and decreased ubiquitin conjugates during freezing. Again, this could also be due to the regulation of ubiquitin tagging enzymes E1-E3. Liver is the last organ

to freeze in the frog and during the several hours that it takes a frog to freeze liver metabolism is highly active in both cryoprotectant synthesis and the production of freeze-responsive proteins. It is possible that during this time, existing ubiquitin conjugates continue to be degraded but ATP limitation and/or specific controls on E1-E3 halt the formation of new conjugates. The net result of this would be elevated free ubiquitin levels in liver of 24 h frozen frogs coupled with reduced conjugates. Future studies should look into the tissue specific regulation of ubiquitin enzymes E1-E3 to confirm these hypotheses.

Protein translational control in anuran hypometabolism is an area in which there has little research to date. Suppression of protein synthesis in mammalian systems of hypometabolism generally include phosphorylation the eIF2 α initiation factor as a prominent mechanism; this halts delivery of the initiating methionine residue. However, the present study showed that the amount of phospho-eIF2 α was unchanged in wood frog liver between control and frozen conditions. The initiation factor eIF4 is also involved in translational control (Redpath and Proud, 1994) and the present data suggest that it is a center of translational control in the frozen frog. Levels of eIF4B were significantly decreased in the frozen state in liver. This subunit of the eIF4 complex associates with the ATP-dependant RNA helicase eIF4A and accelerates the activity of the helicase in translation (DeGracia *et al.*, 1996). A decreased amount of available eIF4B may slow the translation process. As stated in chapter 2, eIF4e is responsible for binding the mRNA m⁷ GTP cap and initiating translation. As was seen in the mammalian model, there is an increase in the level of this subunit in frog liver under freezing conditions. Although contradictory to a theory of reduced protein synthesis in an energy depleted state, the

elevation of eIF4e content is a common response in these two very different hypometabolic states.

Chapter 4:

Cytoplasmic Phospholipase A2 Regulation in the Hibernating Thirteen-lined Ground Squirrel

Introduction:

Lipid membranes are comprised of a complex mixture of phospholipids and proteins which can be altered or changed under multiple circumstances to give these biological structures a dynamic function in cells. Phospholipids can vary with respect to their head group structure, hydrocarbon length, and their degree of saturation. Proteins that function in membranes can vary widely in type, amount, activity state, and translocation status (e.g. recruited to the membrane versus in a cytoplasmic pool). Lipid membranes are responsible for a host of cellular functions including membrane dynamics, protein regulation, signal transduction, import/export of nutrients, ions and other molecules, endocytosis and vesicular secretion (Asai *et al.*, 2003). The composition of lipid membranes relies on a balance between synthesis and catabolism as well as alterations of phospholipid composition to create unique membrane characteristics (Asai *et al.*, 2003). A unique class of enzymes called phospholipases brings about membrane catabolism and remodeling.

There are five classes of phospholipases: phospholipase A₁ (PLA₁), phospholipase A₂ (PLA₂), phospholipase B (PLB), phospholipase C (PLC) and finally phospholipase D (PLD). These classes differ in the location and type of bond that they can hydrolyze (Figure 4.1) (Brown *et al.*, 2003). PLA₁ and PLA₂ cleave the *sn*-1 and *sn*-2 sites, respectively, on the phospholipid generating a lysophospholipid and a free fatty acid. PLB enzymes also cleave at *sn*-1 and *sn*-2 positions. PLB has within it both hydrolase and lysophospholipase transacylase activity (Ghannoum, 2000). PLC cleaves the link between the glycerol and phosphate. This has lead to extensive studies of the role of PLC in generating second messengers and its role in membrane trafficking (Brown *et al.*,

Figure 4.1: Mechanism of action of phospholipases A1, A2, C and D (Roberts, 1996).

2003). Finally, PLD removes the polar phosphate group generating phosphatidic acid. PLD activity leads to calcium mobilization, adenylate cyclase inhibition and PLC activation (Roberts, 1996).

Arachidonic acid is a key molecule in the cell as it is a precursor to several metabolic pathways including the production of lipid messengers including the eicosanoids. Types of eicosanoids including prostaglandins, aromatic hormones, leukotrienes, thromboxanes, and platelet-activating factor are all derived from the metabolism of arachidonic acid. Eicosanoids play important cellular roles in regulating blood flow, migration, secretion, apoptosis and stimulation of the inflammatory response (Dessen, 2000). Arachidonic acid metabolites also play an important role in cell growth, cell differentiation, the regulation of ion transport, neurotransmitter uptake and release, and cytoskeleton and membrane remodeling (Han *et al.*, 2002; Brown *et al.*, 2003). In the resting state, the bulk of arachidonic acid is esterified in membrane phospholipids at the *sn*-2 position (Hirabayashi and Shimizu, 2000). The liberation of arachidonic acid from this position is brought about by the activation of the phospholipase A₂ family of enzymes.

There are three large subclasses of A₂ phospholipases that liberate phospholipids at the *sn*-2 position. The enzymes in these classes differ in their molecular size and their requirement for calcium. The best studied of this class are the secreted phospholipases (sPLA₂) which are classified as groups I, II, III, V and IX-XIV. All of these phospholipases have low molecular weights ranging from 12-19 kDa. They have been well-documented since they are the more prevalent phospholipases in secreted venoms. Their active sites all contain a conserved histidine residue that sits adjacent to an

aspartate residue. This allows the sPLA₂ to use the histidine residue, assisted by the aspartate, to polarize a bound H₂O molecule which then attacks the carbonyl group of the phospholipid (Balsinde *et al.*, 1999). At this point calcium is required to stabilize the intermediates of the reaction mechanism; millimolar amounts of calcium are required by this family of enzymes. Secreted phospholipase A₂ enzymes also have 5-8 disulfide bonds which help to stabilize the enzymes in the extracellular fluids where they reside (Balsinde *et al.*, 1999).

The second class of phospholipase A₂ is calcium independent (iPLA₂). These phospholipases are in group VI and are found predominantly in the cytoplasm. Calcium independent phospholipases are not regulated by post-translational covalent modifications (Balsinde *et al.*, 1999) and unlike other phospholipases in this family the iPLA₂s show no fatty acid preference in the *sn*-2 position (Roberts, 1996). Calcium independent iPLA₂s are stimulated by ATP and irreversibly inhibited by bromoenol lactone (BEL). Unlike other phospholipase members, iPLA₂ is not involved in arachidonic acid release, but instead in arachidonic acid metabolism. Calcium independent PLA₂ is involved in membrane remodeling by incorporating free arachidonic acid into membrane phospholipids. In this sense, iPLA₂ may be involved in the allocation of arachidonic acid and the relative amount of free arachidonic acid in cellular compartments (Balsinde *et al.*, 1999).

The third class of phospholipase A₂ enzymes encompasses cytoplasmic calcium dependent phospholipases (cPLA₂) belonging to Group IV. This group preferentially hydrolyzes arachidonic acid at the *sn*-2 position and requires micromolar amounts of calcium. However, unlike sPLA₂, the calcium is not required for the catalytic mechanism

of the enzyme but, instead, is needed for the translocation of the enzyme to cellular membranes where it attacks phospholipids. Three isoforms of cPLA₂ are known: α -cPLA₂, β -cPLA₂, and γ -cPLA₂. Although these isoforms have high sequence homology, they differ in their selectivity for arachidonic acid (Dessen, 2000). For example, the alpha form of cPLA₂ is highly selective for phospholipids with arachidonic acid in the *sn*-2 position, whereas the beta and gamma isoforms are not as selective. All isoforms have two catalytic domains and conserved serine residues in a lipid consensus sequence (Hirabayashi and Shimizu, 2000). This marks the major mechanistic difference between cPLA₂s and sPLA₂s. Cytosolic calcium-dependent PLA₂s are serine hydrolases. Both the alpha and beta isoforms contain an important C2 domain responsible for calcium-dependent binding of the enzyme to membranes (Hirabayashi and Shimizu, 2000). Calcium bound to Asp⁴³ and Asp⁹³ in the amino-terminal of the C2 domain is essential for the enzyme to bind to cellular membranes (Zhou *et al.*, 2003). Molecular weights are 85 kDa and 114 kDa for the alpha and beta isoforms, respectively, whereas the gamma isoform, lacking the C2 domain, and therefore being calcium-independent (but with sequence homology to the alpha and beta isoforms), has a size of 61 kDa (Jones *et al.*, 2004).

The only isoform that is known to be subject to post-translational modification is α -cPLA₂. This enzyme can be phosphorylated on up to four serine residues: S⁴³⁷, S⁴⁵⁴, S⁵⁰⁵, and S⁷²⁷. The effects of phosphorylation on S⁴³⁷ and S⁴⁵⁴ are not known as these residues are not well conserved between species. The source of S⁵⁰⁵ and S⁷²⁷ phosphorylation is also controversial but these two residues are conserved and have a direct impact on the activity of the phospholipase. In different systems, the source of S⁵⁰⁵

phosphorylation has been linked to the mitogen-activated protein kinase (MAPK) family but the identity of the MAPK isoform responsible is not yet known (Zhu *et al.*, 2001). Phosphorylation of S⁷²⁷ is brought about by protein kinases A or C in some systems but this phosphorylation is not necessarily correlated with an increase in activity (Hirabayashi and Shimizu, 2000).

Since arachidonic acid is such a valuable signal molecule in cells, the regulation of cPLA₂ is very complex. Not only does the activity of the enzyme potentially require the dual phosphorylation of S⁵⁰⁵ and S⁷²⁷, activity is also dependent on the amount of cytoplasmic calcium which the enzyme needs to associate with cellular membranes. There is evidence that the amount of calcium in the cytoplasm determines whether the phospholipase is sent to the Golgi, the endoplasmic reticulum, or the perinuclear membrane (Zhou *et al.*, 2003).

Since arachidonic acid is important in the production of eicosanoids and other downstream molecules and hormones, its regulation could be very important in the prioritization of cellular pathways during episodes of metabolic rate depression. Mammalian hibernation is a phenomenon where controlled metabolic rate depression is extremely important. Many enzymes and pathways are regulated during hibernation by means of reversible protein phosphorylation, and yet the responses of phospholipases during hibernation have received little study to date. In the late 1980s and early 1990s Brutovetsky and coworkers looked at the role of calcium dependant phospholipase A₂ in the regulation of oxidative activity in liver mitochondria in hibernating ground squirrels and gophers and showed that the enzyme was important in reversing the inhibition of the respiratory chain during torpor bouts (Brustovetsky *et al.*, 1989, 1992).

The present study focuses on cPLA₂ with the goal of determining whether the activity of the cytoplasmic phospholipase A₂ was regulated between euthermic and hibernating states in ground squirrel tissues and whether protein phosphorylation was the mechanism involved. Significant changes in activity of cPLA₂ were found between samples of skeletal muscle and liver from euthermic and hibernating animals. Subsequent incubation studies confirmed phosphorylation as a mechanism of regulation.

Materials and methods:

Animals and tissue preparation:

Squirrel tissues were obtained as outlined in Chapter 2. Samples of muscle and liver tissue was crushed under liquid nitrogen using a mortar and pestle and then 150 mg of tissue was mixed with 1 mL of homogenization buffer (50 mM Tris-HCl, pH 7.4 containing 1 mM EDTA, 30 mM NaF and PMSF added at the time of homogenization) and homogenized using a Polytron homogenizer. Samples were centrifuged at 10,000 x g for 15 minutes at 4°C and supernatants were held on ice until use. Assays followed the instructions of the cPLA₂ assay kit from Cayman Chemical Company.

Preparation of arachidonoyl thio-PC:

The arachidonoyl thio-PC substrate provided in the assay kit was dissolved in ethanol and solubilized as per manufacturer's instructions. Substrate in ethanol was bubbled under a gentle stream of nitrogen gas and then solubilized in assay buffer (80 mM HEPES, pH 7.4, 150 mM NaCl, 10 mM CaCl₂, 4 mM Triton X-100, 30% v/v glycerol, and 1 mg/mL bovine serum albumin) and vortexed until completely dissolved.

A solution in 12 mL of assay buffer contains arachidonoyl thio-PC at a final concentration of 1.5 mM.

Assay specificity:

Assay specificity for cPLA₂ was ensured by two methods: (1) using the inhibitor, bromoenol lactone, to irreversibly inhibit calcium-independent phospholipase (iPLA₂), and (2) partial purification of samples by gel filtration to separate out low molecular weight proteins including any sPLA₂ present. Enzyme extracts were layered on small columns of Sephadex G-50 (equilibrated in homogenization buffer) and then centrifuged for 1 minute at high speed in a IEC benchtop centrifuge to remove any thiol contamination that would interfere in the assay as well as low molecular weight (<30 kDa) metabolites and proteins. Samples of eluate were then incubated with the inhibitor bromoenol lactone at a final concentration of 5 μ M for 30 minutes prior to assay of cPLA₂ activity.

Assay:

cPLA₂ was assayed spectrophotometrically in a 96 well microplate with a total assay volume of 225 μ L. The reaction mixture contained assay buffer, thio-PC substrate and enzyme sample (after incubation with bromoenol lactone inhibitor). Each sample was tested at substrate concentrations varying from 0-1.3 mM for muscle tissue and 0-0.665mM in liver tissue in different wells. Reactions were started by the addition of enzyme samples to the microplate wells followed by incubation for one hour at 23°C. cPLA₂ cleaves the arachidonoyl thio-PC at the *sn*-2 position and the thiol is released.

Assays were stopped by addition of a mixture of 25 mM DTNB and 475 mM EGTA. The EGTA chelates the calcium required for phospholipase activity and the DTNB reacts with the thiol product to form a colored 5-thio-2-nitrobenzoic acid complex. Colour was developed for 5 minutes and then absorbance was measured on a Multiscan Spectrum spectrophotometer at 414 nm.

Incubations and western blotting:

Liver extracts were treated under incubation conditions that were designed to alter the phosphorylation state of cPLA₂. Tissue samples were homogenized in a simple buffer containing 50 mM Tris-HCl, pH 7.4 and PMSF added at the time of homogenization and centrifuged as described previously. Supernatants were centrifuged through a Sephadex G-50 column equilibrated in the same buffer and then extracts were divided into two aliquots. To the first aliquot, 1 mM EDTA and 30 mM NaF were added to inhibit phosphatases and kinases (referred to as STOP conditions). To the second aliquot, 5 mM MgCl₂ and 5 mM CaCl₂ were added to stimulate endogenous phosphatases. Enzyme extracts were incubated on ice for four hours (cold incubation provided better long term stability of the enzyme than incubation at room temperature) and then further processed. Aliquots were frozen until preparation for western blot analysis. Protein content of samples was determined by the BioRad assay and then samples were prepared and separated by one-dimensional SDS-PAGE and then subjected to Western blotting essentially as outlined in Chapter 2. Samples were loaded at a concentration of 60 µg per well onto 12% SDS-PAGE gels. After electrophoresis, the proteins were transferred to PVDF membranes and incubated with a peptide antibody against phosphorylated serine-

505 of cPLA₂ (Cell Signaling Cat. no. 2831), used at a dilution of 1:1000 v/v. Membranes were then incubated with secondary HRP-linked goat anti-rabbit IgG antibody (1:2000 v/v) in TBST for 1.5 h at 4°C. The blots were washed 3 X 5 minutes in 1X TBST. Blots were developed using an enhanced chemiluminescence assay using SuperSignal West Pico Chemiluminescent Substrate (PIERCE). Bands on the blots were scanned and evaluated using the Syngene.

To confirm that a consistent amount of protein was added to each well, membranes were subsequently stained with Coomassie blue. Membranes were rescanned and selected strong bands that showed constant intensities between samples (and were not close to the MW of cPLA₂) were chosen as control bands. They were quantified by densitometry and used to normalize the intensity of the immuno-reacted bands between lanes. Quantification was performed using GeneTools software (SynGene).

Results:

Assay and enzyme kinetics for squirrel muscle and liver cPLA₂:

cPLA₂ was assayed in ground squirrel muscle and liver sampled from both euthermic and hibernating animals. Figure 4.2 shows the maximum activity of cPLA₂ in both tissues (assayed at optimal substrate concentration of 1.33 mM for muscle extracts and 0.665 mM for liver). Activity was ~3-fold higher in muscle than in liver. Muscle did not show a significant change in $V_{\max}$ activity between euthermic and hibernating states whereas liver showed a significant decrease in $V_{\max}$ activity during hibernation (activity fell to 57% of control). K_m values for arachidonoyl thio-PC are shown in Figure 4.3.

Substrate concentrations of arachidonoyl thio-PC varying from 0-1.3 mM for muscle and 0-0.665 mM for liver were used to generate velocity versus substrate concentration curves. Liver cPLA₂ showed a markedly lower K_m than the muscle enzyme; in extracts from euthermic tissues, K_m was 0.70 ± 0.016 mM in muscle and 0.056 ± 0.005 mM in liver. In both tissues, the K_m value decreased significantly during hibernation, falling to just 28% in muscle and 61% in liver of the corresponding euthermic value.

Incubations and Western Blotting

Preliminary Western blot analysis showed strong cross-reactivity of the phospho-cPLA₂ antibody (detecting P-serine residue 505) with a protein of ~110 kDa (the expected molecular weight of cPLA₂) in ground squirrel liver extracts and, therefore, liver samples were used for incubation studies. Figure 4.4 shows that the phosphorylated form of the enzyme was readily detected in liver from euthermic animals but levels were very low under hibernating conditions, about one-third (38%) of the value in euthermic liver. To determine whether the change in phospho-cPLA₂ band intensity during hibernation was the result of dephosphorylation of the protein, incubation studies were carried out on liver samples from euthermic and hibernating squirrels. Liver samples were incubated under conditions that preserved the phosphorylation state of the enzyme (STOP conditions: EDTA to inhibit protein kinases, NaF to inhibit phosphatases) and under conditions that stimulated endogenous phosphatases (MgCl₂ and CaCl₂ added). Figure 4.5 shows that the phosphorylated form of cPLA₂ was present in substantial amounts in euthermic extracts incubated under STOP conditions, similar to the results seen in Figure 4.4. However, when incubated under conditions that stimulated

endogenous phosphatases, the intensity of the immunoreactive band was reduced 58% in extracts of euthermic liver. This indicates that the enzyme after incubation with phosphatases was in its dephosphorylated state. In incubated liver extracts from hibernating animals, phospho-cPLA₂ was present at very low amounts under both the STOP and phosphatase incubations. This implies that the enzyme in liver of hibernating animals was in the dephosphorylated form.

Figure 4.2: Vmax values for phospholipase activity in squirrel muscle and liver tissues at both euthermic and hibernating samples. Assays were carried out at 23°C. Values are mean $\pm$ SEM, n = 3.

Figure 4.3 : Km values for phospholipase activity in squirrel muscle and liver tissues at both euthermic and hibernating samples. Assays were carried out at 23°C. Values are mean $\pm$ SEM, n = 3. Stars indicate a significant difference between hibernating and euthermic samples as determined by the Student's t-test $P < 0.05$.

Figure 4.4: Western blot analysis of the levels phosphorylated cPLA₂ in muscle and liver of euthermic and hibernating squirrels. The ratio of normalized band intensities of euthermic and hibernating samples is shown. Data are means $\pm$ SEM, n = 4 separate determinations on independent tissue extracts. Stars show a significant differences in normalized band intensities between the hibernating versus euthermic state, as determined by the Student's t-test $P < 0.05$.

Figure 4.5: Western blot analysis of the levels phosphorylated cPLA₂ in muscle and liver of euthermic and hibernating squirrels after incubation periods. Bands labeled STOP indicate samples that were incubated to preserve the natural phosphorylation state of cPLA₂. Bands labeled phosphatase were incubated in conditions to stimulate endogenous phosphatases. The ratio of normalized band intensities of euthermic and hibernating samples is shown. Data are means $\pm$ SEM, n = 4 separate determinations on independent tissue extracts. Stars show a significant differences in normalized band intensities between the hibernating versus euthermic state, as determined by the Student's t-test $P < 0.05$.

Discussion:

Calcium dependent cytosolic phospholipase A₂ was measured in skeletal muscle and liver of euthermic and hibernating ground squirrels in an effort to determine whether this important enzyme contributes to the regulation of hypometabolism. There is currently great interest in PLA₂ due to its action in arachidonic acid release which initiates signal transduction cascades that have important roles in biochemistry, physiology and, more recently, pharmacology (Balsinde *et al.*, 1999). Phospholipids were initially considered to have functions simply in the structure of cells, but they are now known to have important roles in cellular signaling, the cleavage of phospholipids by several different enzymes providing a variety of second messenger molecules including arachidonic acid. Arachidonic acid release triggers the production of eicosanoids which include cyclooxygenase-derived prostaglandins and lipoxygenase-derived leukotrienes (Smith, 1992). Additionally, after the cleavage of arachidonic acid, 2-lysophospholipid is produced and in some systems is used to create the platelet-activating factor (PAF) that is also involved in inflammation (Balsinde *et al.*, 1999). More recently, the activity of cPLA₂ has been under investigation because of its direct ability to alter membrane curvature, and thus affect the function of the membrane. The release of arachidonic acid from the membrane and the mechanism of action of cPLA₂ leaves a inverted cone shaped lysophospholipid, and a cluster of such inverted cones produces a fold in the membrane as shown in Figure 4.6. This positive curvature folding inwards in the cell may initiate the formation of membrane tubules (Brown *et al.*, 2003). Therefore, the diversity of reactions and cascades that follow from the action of cPLA₂ are still in the process of being discovered.

Figure 4.6: Membrane curvatures after the reaction of phospholipase A2 (Brown *et al* 2003)

In this study, the properties and phosphorylation state of cPLA₂ were investigated in skeletal muscle and liver of euthermic versus hibernating ground squirrels. There were no significant changes in maximal activity of cPLA₂ measured in muscle tissue under euthermic versus hibernating conditions, but there was a significant decrease in cPLA₂ activity during hibernation in liver. The K_m for arachidonoyl thio-PC decreased significantly in both tissues during hibernation. A change in the substrate affinity of the enzyme during hibernation may be due to a change in phosphorylation state of the enzyme.

The precise activation of cPLA₂ and its regulation is currently not fully understood. Initially, it was thought that the activation of cPLA₂ was determined by its phosphorylation state and the amount of intracellular calcium. The interaction of calcium with the calcium lipid binding domain to allow enzyme binding to membranes and an activation of the enzyme by phosphorylation were thought to give the enzyme access to its substrate. However, recent studies indicate the need for cPLA₂ interaction with other membrane lipids such as cholesterol, phosphatidyl inositol biphosphate, and ceramides, as well as interaction with proteins like annexins, p11 and vimentin, for the complete activity of cPLA₂ (Bailleux *et al*, 2004; Chakraborti, 2003).

cPLA₂ can be phosphorylated by several serine/threonine kinases and tyrosine kinases, phosphorylating the enzyme on different serine and/or tyrosine residues. Phosphorylation of the serine residue 505 is known to cause an increase cPLA₂ activity (Clark *et al.*, 1995). The antibody used in this study was specifically designed to recognize the peptide sequence containing the phospho-Ser⁵⁰⁵ residue. Phosphorylation of this residue is brought about by MAPKs, but the specific isoform responsible is not

known. In ground squirrel liver we found high levels phospho-Ser⁵⁰⁵ cPLA₂ in the euthermic state and very low levels of the phosphoenzyme during hibernation. This is consistent with an active cPLA₂ during euthermia and a suppression of enzyme activity during hibernation, probably mediated by a dephosphorylation of the Ser⁵⁰⁵ site (and possibly changes in the phosphorylation states of other residues on cPLA₂). Because of the difficulty of stimulating all MAPKs in vitro, we decided to examine the effects of dephosphorylating the enzyme via endogenous phosphatases. The data in Figure 4.5 show that incubation of liver extracts under conditions that were designed to stimulate enzyme dephosphorylation resulted in a decrease in the amount of phospho-Ser⁵⁰⁵ cPLA₂ detectable. This suggests that the changes in the amounts of phospho-Ser⁵⁰⁵ cPLA₂ detected between euthermic and hibernating states in ground squirrel liver were due to dephosphorylation of the enzyme during torpor. Hence, dephosphorylation of this residue (or perhaps changes in the phosphorylation state of other residues as well) during hibernation could be responsible for the significant decrease in the K_m for substrate of the liver enzyme under hibernating conditions and the reduced maximal activity. Alkaline phosphatases have been shown to reverse agonist-induced increases in cPLA₂ activity in numerous cell types (Clark *et al.*, 1995).

The activity of cPLA₂ was previously examined in hibernators by Brustovetsky *et al* (1989). They found that the activity of cPLA₂ decreased in liver mitochondria from the European ground squirrel (*Citellus undulates*) during hibernation. They postulated that the suppression of cytosolic calcium dependant PLA₂ in hibernation was linked to the decrease in mitochondrial respiration in hibernation, though the mechanism was unknown.

Phospholipase A₂ is also implicated in the control of the activity of many membrane bound enzymes. Of particular importance is Na⁺/K⁺ ATPase, a membrane bound ATPase which has important roles in regulating cell volume, cytoplasmic pH and calcium levels, and secondary transport processes such as sodium dependent glucose and amino acid transport. Na⁺/K⁺ ATPase is one of the major consumers of ATP in cells and, as such, the enzyme is a target for regulation with respect to many cellular requirements and is a major target of suppression for organisms entering hypometabolic states (Storey and Storey, 2004a). During hibernation in ground squirrels, Na⁺/K⁺ ATPase is inhibited in multiple organs with studies of the skeletal muscle enzyme demonstrating that the mechanism is phosphorylation-dependent (MacDonald and Storey, 1999). Interestingly, this ATPase is sensitive to changes in membrane fluidity (Therien and Blostein, 2000). The action of cPLA₂ in producing free fatty acids in the membrane tends to inhibit Na⁺/K⁺ ATPase by decreasing the thickness and fluidity of the surrounding membrane (Oishi *et al.* 1990). Arachidonic acid and its subsequent metabolites tend to also have an inhibitory effect on Na⁺/K⁺ ATPase and thus could contribute to the decrease in metabolic rate in tissue of hibernators.

Chapter 5:
Cytoplasmic Phospholipase A2 Regulation in the
Freeze Tolerant Wood Frog

Introduction:

The role of arachidonic acid release has not been studied with respect to animal freeze tolerance, in particular in relation to wood frog freezing survival. A number of studies have addressed arachidonic acid metabolism in relation to glucose and insulin secretion in diabetics (Dunlop and Clark, 1995; Jones *et al.*, 2004). Although elevated intracellular glucose levels may be one of the only conditions that diabetics share with the frozen wood frog, links between these two conditions may provide insight into the regulation and role of cPLA₂ during freezing.

As outlined in Chapter 1, during freezing the wood frog switches to a reliance on anaerobic metabolism and turns down/off the catabolism of lipids as well as proteins (see Chapter 3). The metabolism of phospholipids has not been analyzed before in the wood frog although some indication of phospholipid involvement in signaling during natural freeze/thaw came from a study of the regulation of protein kinase C (PKC), a downstream kinase of phospholipase C (PLC). The action of PLC cleaves the link between the glycerol and phosphate in the phospholipid, releasing diacylglycerol and inositol trisphosphate (IP3) that subsequently activates PKC and calcium mobilization (Brown *et al.*, 2003). In wood frogs, levels of IP3 rose dramatically during freezing. Holden and Storey (1996) postulated that IP3 and PKC may be involved in metabolic responses that deal with the consequences of long-term freezing, such as ischemia resistance or cell volume control. Although this study is perhaps peripheral to my current interest in cPLA₂, it does indicate a role for phospholipid signaling during freezing. The release of calcium brought about by PKC may also be linked to calcium dependent binding and consequently activation of cPLA₂.

The present study focuses on cPLA₂ with the goal of determining whether the activity of the cytoplasmic phospholipase A₂ was regulated between control and frozen states in wood frog tissues and whether protein phosphorylation was the mechanism involved. Significant changes in the activity of cPLA₂ were found between samples of skeletal muscle and liver from control and frozen animals. Subsequent incubation studies confirmed phosphorylation as a mechanism of regulation.

Materials and methods:

Animals and tissue preparation:

Frog tissues were obtained as outlined in Chapter 3. Tissue extracts of muscle and liver were prepared as outlined in Chapter 4 using the same homogenization buffer (50 mM Tris-HCl, pH 7.4 containing 1 mM EDTA, 30 mM NaF and PMSF added at the time of homogenization) and centrifugation treatments (10,000 x g for 15 minutes at 4°C). Assays followed the instructions of the cPLA₂ assay kit from Cayman Chemical Company. The arachidonoyl thio-PC substrate was prepared as in Chapter 4. The assay specificity for cPLA₂ was ensured by using bromoenol lactone to inhibit iPLA₂ and gel filtration (Sephadex G-50) to remove low molecular weight proteins including any sPLA₂ present, as explained in Chapter 4.

Assay:

cPLA₂ was assayed spectrophotometrically using the method described in Chapter 4. The reaction mixture contained assay buffer, thio-PC substrate and enzyme sample (after incubation with bromoenol lactone inhibitor). Each muscle sample was tested at

substrate concentrations varying from 0-1.3 mM in different wells while liver tissues were tested in a range from 0-0.665 mM. Reactions were started by the addition of enzyme samples to the microplate wells followed by incubation for one hour at 23°C. Assays were stopped by addition of a mixture of 25 mM DTNB and 475 mM EGTA. After colour development, the absorbances of microplate wells were measured at 414 nm.

Incubations and western blotting:

Liver extracts were treated under incubation conditions that were designed to alter the phosphorylation state of cPLA₂ as outlined in Chapter 4. Tissue samples were homogenized in a simple buffer containing 50 mM Tris-HCl, pH 7.4 with PMSF added at the time of homogenization and centrifuged as described previously. Supernatants were centrifuged through a Sephadex G-50 column. Samples were divided into two aliquots and treated as described in Chapter 4. Enzyme extracts were incubated on ice for 4 hours (cold incubation provided better long term stability of the enzyme than incubation at room temperature) and then further processed. Protein content of samples was determined by the BioRad assay and then samples were prepared and separated by one-dimensional SDS-PAGE and subjected to Western blotting essentially as outlined in Chapter 2. Samples were loaded at a concentration of 60 µg per well onto 12% SDS-PAGE gels. After electrophoresis, the proteins were transferred to PVDF membranes, probed with anti-cPLA₂ antibody as outlined in Chapter 4, and band densities were quantified using the Syngene.

To confirm that a consistent amount of protein was added to each well, membranes were subsequently stained with Coomassie blue. Membranes were rescanned

and selected strong bands that showed constant intensities between samples (and were not close to the MW of cPLA₂) were chosen as control bands. They were quantified by densitometry and used to normalize the intensity of the immuno-reacted bands between lanes. Quantification was performed using GeneTools software (SynGene).

Results:

Assay and enzyme kinetics for frog muscle and liver cPLA₂:

cPLA₂ was assayed in muscle and liver sampled from both control and frozen wood frogs. Figure 5.1 shows the maximum activity of cPLA₂ in both tissues (assayed at optimal substrate concentration of 1.33 mM for muscle extracts and 0.665 mM for liver). In control samples the phospholipase activity was comparable between tissues. The $V_{\max}$ activity of cPLA₂ did not change in muscle between control and frozen states but in liver $V_{\max}$ activity increased significantly during freezing (activity rose 2.3 fold). K_m values for arachidonoyl thio-PC are shown in Figure 5.2. Substrate concentrations of arachidonoyl thio-PC varying from 0-1.3 mM for muscle and 0-0.665 mM for liver were used to generate velocity versus substrate concentration curves. Liver cPLA₂ showed a consistently lower K_m value than the muscle enzyme; in extracts from control tissues, K_m was 0.071 ± 0.0046 mM in muscle and 0.03 ± 0.0018 mM in liver. In both tissues, the K_m value increased significantly during freezing rising 1.3-fold for muscle and 2.1-fold for liver tissue.

Incubations and Western Blotting

Preliminary Western blot analysis showed strong cross-reactivity of the phospho-

cPLA₂ antibody (detecting P-serine residue 505) with a protein of ~110 kDa (the expected molecular weight of cPLA₂) in wood frog liver extracts. Therefore, liver samples were used for incubation studies. Figure 5.3 shows that the phosphorylated form of the enzyme was readily detected in liver extracts from control animals but levels were much lower in liver from 24 h frozen frogs, just 42% of the value in control liver. Incubation studies were carried out on samples of liver extracts from control and frozen frogs to determine whether protein phosphorylation could be responsible for the change in Western blot band densities detected between control and frozen samples. Liver samples were incubated under conditions that preserved the phosphorylation state of the enzyme (STOP conditions: EDTA to inhibit protein kinases, NaF to inhibit phosphatases) and under conditions that stimulated endogenous phosphatases (MgCl₂ and CaCl₂ added). Figure 5.4 shows that the phosphorylated form of cPLA₂ was present in substantial amounts in control extracts incubated under STOP conditions, similar to the results seen in Figure 5.3. However, when incubated under conditions that stimulated endogenous phosphatases, the intensity of the immunoreactive band was reduced to 32% of control liver values. This indicates that the enzyme after incubation with phosphatases was in its dephosphorylated state. In incubated liver extracts from frozen animals phospho-cPLA₂ was present in very low levels under both the STOP and phosphatase incubations. This implies that the enzyme in liver of frozen animals was in the dephosphorylated form and therefore not susceptible to further dephosphorylation by phosphatase action.

Figure 5.1: Vmax values for cPLA₂ activity in wood frog skeletal muscle and liver sampled from control (5°C acclimated) and 24 frozen (at -2.5°C) animals. Assays were carried out at 23°C. Values are mean ± SEM, n = 3. Star indicates a significant difference between frozen and control samples as determined by the Student's t-test P<0.05.

Figure 5.2: K_m values for arachidonoyl thio-PC for cPLA₂ from wood frog muscle and liver comparing samples from control and frozen frogs. Assays were carried out at 23°C. Values are mean $\pm$ SEM, n = 3. Stars indicate significant differences between frozen and control samples as determined by the Student's t-test $P < 0.05$.

Figure 5.3: Western blot analysis of the levels of phosphorylated cPLA₂ (Ser505) in muscle and liver sampled from control and frozen frogs. Representative blots are shown along with a histogram showing normalized band intensities for control and frozen samples. Data are means $\pm$ SEM, n = 4 separate determinations on independent tissue extracts. Star shows a significant difference in normalized band intensities between the control and frozen state, as determined by the Student's t-test $P < 0.05$.

Figure 5.4: Western blot analysis of the effect of stimulating phosphatase activity on the amount of phosphorylated cPLA₂ detected in muscle and liver extracts from control and frozen frogs. Bands labeled STOP indicate samples that were incubated under conditions that preserve the native phosphorylation state of cPLA₂. Bands labeled phosphatase were incubated under conditions that stimulated endogenous phosphatase activity. Representative Western blots are shown along with a histogram showing normalized band intensities of control and frozen samples. Data are means $\pm$ SEM, n = 4 separate determinations on independent tissue extracts. Star shows a significant difference in normalized band intensities between the control and frozen states, as determined by the Student's t-test $P < 0.05$.

DISCUSSION:

The goal of this study was to examine the potential role of cPLA₂ in the response of wood frogs to natural freezing and determine whether this enzyme is activated or inactivated during hypometabolism. Previous studies showed that some of the downstream effects of phospholipase PLC action (via stimulation of PKC) are activated as a response to freezing (Holden and Storey 1996), but the pathways involving cPLA₂ and signaling by arachidonic acid metabolites have not yet been characterized. In this study, the activity of cPLA₂ was examined in muscle and liver from both control and frozen frogs. These two tissues are important model tissues in the frog. As a peripheral tissue, skeletal muscle is one of the first tissues to freeze and does so with minimum levels of cryoprotectant whereas liver is the metabolic center of adaptive responses during the freeze. Phosphorylation was also considered as a means of cPLA₂ regulation during the freezing period. cPLA₂ in muscle showed no significant changes in activity between control and frozen conditions, but in liver activity increased substantially in the frozen animal. The K_m for arachidonoyl thio-PC increased in both tissues in the frozen state, indicating a reduced substrate affinity of the enzyme. Stable changes in the kinetic properties of an enzyme between two physiological states are often the result of covalent modification, frequently by reversible phosphorylation of the enzyme. Hence, phosphorylation was considered a means of altering the kinetics of cPLA₂.

Incubation studies were carried out on the liver extracts to assess whether phosphorylation could be the mechanism of cPLA₂ regulation during freezing. The antibody used in Western blotting detected the presence of phosphorylation on serine residue 505. This residue is considered to be the main player in the regulation of enzyme

activity by the MAPK family of protein kinases. When this residue is phosphorylated, the enzyme is active, and when it is in its dephosphorylated form, the enzyme is less active (Clark *et al.*, 1995).

The results of the incubation studies on frog liver cPLA₂ seem somewhat contradictory to the previous findings. The western blot analysis showed that the content of phospho-serine 505 protein decreased during freezing and the incubation studies were consistent with a high phosphate enzyme form in control frogs and a low phosphate form in the frozen animals. Yet despite this, the measured enzyme activity actually increased in liver of frozen frogs. However, the strong increase in substrate K_m (ie. reduced substrate affinity) during freezing is consistent with a less active form of the enzyme being present in the liver of frozen frogs. Hence, phosphorylation of serine 505 may be only part of the mechanism of cPLA₂ regulation in response to freezing. There are four known sites of cPLA₂ phosphorylation (S⁴³⁷, S⁴⁵⁴, S⁵⁰⁵, S⁷²⁷) and one of the other three may contribute to cPLA₂ control during freezing. For example, protein kinase C, which has been shown to be activated under freezing conditions (Holden and Storey, 1996), has a recognition site for phosphorylation at S⁷²⁷ (Hirabayashi and Shimizu, 2000). Hence, PKC-mediated phosphorylation may occur during freezing and although the function of phosphorylation on S⁷²⁷ is still speculative, it might contribute to a change in the activity state of cPLA₂ during freezing. Phosphorylation of this site may lead to the association of cPLA₂ with the membrane in a calcium independent manner (Gigon *et al.*, 1999).

PKC may have a pronounced regulatory role on cPLA₂ in frogs. In a study conducted by Dunlop and Clark (1995), using labeled phosphate, they were able to determine that PKC caused the activation of cPLA₂ under conditions of high glucose in

neonatal rat pancreatic islet cells. This demonstrates that a vertebrate cPLA₂ was phosphorylated under conditions of high glucose in a PKC-dependent manner. This study was the first to describe the phosphorylation stimulated by a non-receptor agonist resulting from exposure to a nutrient stimulus (Dunlop and Clark, 1995). The phosphorylation by PKC does not seem to require MAP kinase activation (Dunlop and Clark, 1995). Although this study was unclear as to which residue was phosphorylated by PKC, it was important in showing that MAP kinases may not be the only kinase family capable of regulating cPLA₂. During freezing, the core organs of wood frogs have very high concentrations of glucose, and PKC activity has already been shown to be increased during freezing. This might lead to glucose-mediated, PKC-stimulated phosphorylation of cPLA₂ in addition to the apparent freezing-stimulated dephosphorylation of Ser⁵⁰⁵ and achieve a net adjustment of enzyme activity and substrate affinity that best serves the metabolic needs in the frozen state. Separate incubation studies and assays to stimulate PKC in the presence of high glucose would help to confirm this hypothesis.

Other indications of the potential for increased cPLA₂ activity are demonstrated in a study by Wong *et al.* (2003). High glucose levels, as shown using diabetics as models, cause damage to tissues via the nonenzymatic glycation of proteins and the formation of advanced glycation end products (AGEs). This is also a potential stress for freezing frogs as the cellular levels of glucose in core organs are vastly elevated during the freeze. Wong *et al.* proposed a pathway in which AGEs bind to their membrane receptor and generate intracellular superoxide (O₂⁻). The generation of superoxide stimulates a redox-sensitive pathway in which protein kinases (not specifically named) induce the

phosphorylation and activation of cPLA₂. This causes an increase in arachidonic acid release followed by the stimulation of NADPH oxidase and increased ROS generation (Figure 5.5; Wong *et al.*, 2003). In this manner, AGEs promote oxidative stress via the activation of cPLA₂ and generation of arachidonic acid. Interestingly, the receptor for advanced glycation end products (RAGE) was one of several genes that were identified as freeze responsive in wood frog heart (Storey, 2004). Hence, this study suggests that increased cPLA₂ activity in liver of frozen frogs may be associated with elevated levels of AGEs.

This present work indicates that an increase in cPLA₂ activity occurs in liver tissue during freezing. Coupled with the potential for oxidative damage caused by AGEs, one would expect the damage caused by ROS to be prevalent in the frozen liver. Indeed, protein carbonyl content in liver increased during freezing (refer to Table 3.3). AGEs, as mentioned previously, can lead to increased ROS generation through the activation and action of cPLA₂. This ROS production, in turn, causes an increase in the level of damaged proteins during freezing.

Figure 5.5: Proposed pathway leading from the formation of advanced glycation endproducts through the intracellular generation of superoxide to the stimulation of intracellular kinases to activate cPLA₂. The activation of cPLA₂ leads to the generation of arachidonic acid and the subsequent stimulation of NADPH oxidase and the generation of more ROS (Wong *et al.*, 2003).

Chapter 6:

General Discussion

One of the main themes of this study was to look at two types of hypometabolic states and determine if there were any commonalities in the prioritization and regulation of pathways involving protein degradation, protein synthesis and phospholipid metabolism. Global characteristics of depressed metabolism are often useful in both comparing systems, and hypothesizing about other hypometabolic systems that may be discovered. Unfortunately, no such global strategies of hypometabolism were seen in this study. Conversely, this comparative study rules against hypotheses of a global nature in highlighting that, in almost all aspects studied in this thesis, the hypometabolic states occurring in hibernating ground squirrels and frozen wood frogs were in fact, total opposites.

At the physiological level, both of these hypometabolic states have some common features. In both cases there is a decrease in heart rate and breathing, and a lowering of metabolic rate and body temperature. Although the degree to which these physiological parameters change is different between the models, the concepts are uniform. A depressed metabolic rate carries with it the ability to suppress both the energy-consuming and energy-producing pathways in a coordinated fashion in order to conserve energy reserves for duration of the hypometabolic period.

After this point, the models diverge. During mammalian hibernation, squirrels switch to a lipid-burning metabolism, whereas frozen frogs depend on anaerobic glycogen catabolism for fuel. Circulation is maintained, albeit at a strongly reduced level during ground squirrel hibernation, whereas frog blood plasma is frozen and tissues are left anoxic throughout the freeze. The duration of mammalian hibernation can last up to

several months, with a series of interbout arousals every few weeks. No such hibernation pattern exists for the frozen frog.

Protein metabolism:

During hibernation, the data suggest that the proteasome from ground squirrel muscle remains active. This was also reflected in the lower level of protein damage caused by ROS generation in hibernation as seen from the strongly reduced levels of carbonyl proteins in muscle of hibernating versus euthermic ground squirrels. The 20S proteasome remained active and apparently served to remove damaged proteins throughout the hibernation bout. The wood frog, however, does not share this priority. The activity of the proteasome was strongly suppressed in the frozen state either by protein phosphorylation or the rate of protein translation or both. As a result, carbonyl content increased strongly during freezing, but fell upon thawing and the resumption of normal cellular function.

Indications of upstream regulation of proteolysis were considered by the examination of ubiquitin pools and ubiquitin conjugates. In general, ubiquitin pools remained unchanged between control and stress conditions, with the frog liver as the only exception showing a significant increase. Ubiquitin conjugates rose in both muscle and liver in the hibernating squirrel, but fell in frog liver and remained unchanged in frog muscle during freezing. The data from the squirrel proteasome activity and carbonyl content, paired with ubiquitin conjugates suggests that the 20S proteasome remains active during hibernation, perhaps because its energy requirement for protein degradation is low, whereas the 26S proteasome which degrades ubiquitin tagged proteins in an ATP

dependent manner is suppressed leading to the rise in tagged proteins during torpor. This tagging of damaged proteins by the enzymes E1-E3 in the ubiquitin pathway may serve as a mechanism to store these proteins to be degraded upon the return to euthermia. In the frog scenario, both the activity of the proteasome and the levels of ubiquitin conjugates were unaltered or lowered in the frozen state. This suggests perhaps a global suppression of proteolysis during freezing from the ubiquitin chain pathway downwards to the proteasome. In both cases, the pathways leading to ubiquitin conjugation would be key targets for future research.

Analysis of the regulation of the enzymes E1-E3 would help to elucidate more about the preparation for proteolysis upstream of the proteasome. Although an energetically expensive process, is maintaining of these enzymes a priority in mammalian hibernation? In the frog scenario, are the activities of E1-E3 suppressed due to their energetically expensive nature? Alternatively, is the level of protein damage during freezing decreased due to the actions of high concentrations of glucose that can stabilize protein structure, therefore facilitating a reduction in the number of proteins that are tagged by ubiquitination in the frozen state? These are important questions that need to be addressed.

The suppression of protein synthesis is also an area in which the mechanisms seem to differ between the two model species. In the hibernator model, evidence was presented for regulation brought about by the phosphorylation of eIF2 α . In the frog model, this mode of regulation did not seem to occur; the phosphorylation state of eIF2 α was the same in control and frozen states. Alternative mechanisms for protein suppression may lie with the regulation of other initiation factors such as eIF4B. Another

question that remains unanswered in both contexts is the activity of eIF4e. Both squirrel and frog liver show an increase in the levels of this initiation factor despite cellular conditions that should suppress overall protein synthesis.

Phospholipid metabolism:

As was the case with protein metabolism, phospholipid metabolism also differed between the mammalian and anuran models. The activity of calcium dependant phospholipase A₂ significantly decreased in squirrel liver during hibernation, whereas the activity in liver of the wood frog increased during freezing. Although protein phosphorylation was a potential means of regulation for both contexts, the specific mechanisms of regulation may not be entirely understood. The squirrel model of phosphorylation and activation follows the literature for the known means of regulation. The activity and regulation of cPLA₂ in the frog model initially seemed contradictory until studies were found in a context of high glucose. These studies were comparable to the frog model and showed similar patterns of phospholipase regulation as seen in diabetic models.

Possible links between the 20S proteasome and cPLA₂:

One of the stresses in common between mammalian hibernators and freeze-tolerant frogs is the threat of ischemia/reperfusion injury and the potential for damage caused by ROS. Ischemia can induce a proinflammatory response that may increase tissue vulnerability to further injury upon reperfusion (Eltzschig and Collard, 2004). Reperfusion can lead to the production of superoxide ions, hydroxyl radicals,

hypochlorous acid, hydrogen peroxide and peroxynitrite derived from nitric oxide. In combination these ROSs can damage cell membranes through lipid peroxidation and damage proteins via the production of carbonyl groups. In considering these two areas of macromolecular damage by ROS, interesting comparisons can be made between the squirrel and the frog model.

In the squirrel, concentrations of conjugated dienes increased in tissues of hibernating ground squirrels, providing evidence of lipid peroxidation during torpor (Carey *et al.*, 2003; Carey *et al.*, 2000). Conversely, protein carbonyl content, representing oxidative damage to proteins, was reduced during hibernation in the present study. In the frog model, the opposite was found. Joannis and Storey (1996) found no significant increase in lipid peroxidation products during freezing, whereas this study found a significant increase in protein damage during freezing as evidenced by increased carbonyl content. When placed side by side, these trends follow with the data presented in this thesis.

In the squirrel model, protein damage was apparently kept low due to the continued activity of the 20S proteasome throughout the hibernation bout. The activity of phospholipase A₂ significantly decreased during hibernation, and lipid peroxidation was shown to increase. Polyunsaturated fatty acids are generally present in the *sn*-2 position of phospholipids. This site is especially prone to lipid peroxidation. As the phospholipase A₂ family of enzymes preferentially cleaves at the *sn*-2 position, this family has been implicated in the repair of damaged phospholipids (Chakraborti, 2003). The entire phospholipase A₂ family is capable of hydrolyzing peroxidized fatty acids from the membranes and the oxidized fatty acid is repaired by the action of glutathione peroxidase

(GSH-Px) as shown in Figure 6.1 (VanKuijk *et al.*, 1987; Chakraborti, 2003). Therefore, in the mammalian hibernation model, by decreasing the activity of cPLA₂, damage due to lipid peroxidation could increase as was reported here.

In the frog scenario, the protein carbonyl content was significantly increased, apparently due to the inhibition of the 20S proteasome. Lipid peroxidation did not increase significantly over the freeze-thaw cycle (Joannisse and Storey, 1996), whereas the activity of cPLA₂ was significantly increased in freezing as shown in this study. This phenomenon could be explained in part due to the repair function of cPLA₂ in reducing damage caused by lipid peroxidation.

The differential damage to proteins and lipids throughout the two stresses of hibernation and whole body freezing can be reflected in the choice to regulate proteasomal or phospholipase functions. The question then follows, why can't a system retain both activities during hypometabolic states to defend against both protein and lipid damage?

Why the proteasome and cPLA₂ cannot act in concert

Although energetics plays an important role in the prioritization of metabolic pathways in hypometabolism, the activation of both of these enzymes may go beyond energetics. In the past few years, the proteasome has been a target for cancer treatment. Via the inhibition of proteolysis, arachidonic acid release and metabolism has been shown to increase in cells treated with the receptor agonist 12-0-tetradecanoylphorbol 13-acetate (TPA) (Levine, 2000). Lactacystin, a proteasome inhibitor, was used to inhibit the degradation of short-lived proteins, while PMSF was used to inhibit the degradation

of long-lived proteins (Levine, 1998). Combined with the requirement of calcium, amplification of arachidonic acid release metabolism was found. Levine (1998) proposed that by inhibiting the degradation of short lived proteins such as kinases and phosphatases, an increased half-life may help to give these proteins more of a chance in phosphorylating and activating cPLA₂ to induce arachidonic acid metabolism. These studies using proteasome inhibitors show that the proteasome may have an effect in regulating the activation of cPLA₂. Downstream effects of arachidonic acid metabolism have implications in cancer therapy with the induction of apoptosis, prevention of tumor growth and stimulation of bone formation (Levine, 2004).

Conclusion

The aim of this study was to determine if there were some common themes in two distinct hypometabolic states by examining the regulation of two major macromolecular pathways: protein synthesis and degradation, and phospholipid metabolism. However, through examining enzyme kinetics, protein amounts, and phosphorylation states of the proteasome and phospholipase, no regulatory phenomena were found to be common between the hibernating squirrel and the freeze-tolerant wood frog. Through this study, more insight was gained into the mechanisms of regulation of two important pathways of macromolecular catabolism, therefore increasing our knowledge base of two very different hypometabolic states.

Figure 6.1: Model of repair of oxidized phospholipid by cPLA₂. Ovals represent polar head groups. (a) shows the native structure of the lipid bilayer with incoming reactive oxygen species. (b) shows the reaction of these ROS with the most polyunsaturated fatty acid of the bilayer causing a disruption in structure. The site for cPLA₂ hydrolysis is indicated by an arrow. (c) shows the release of the oxidized fatty acid to be detoxified with GSH-Px and the resulting phospholipid after the removal of the damaged fatty acid. Finally, (d) shows the reacylation with long-chain fatty acyl coenzyme A (FA-CoA) and the completion of the repair.

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