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**ENZYME THERMAL ADAPTATIONS
AND SIGNAL TRANSDUCTION INVOLVEMENT IN
GROUND SQUIRREL HIBERNATION**

by

Justin Anthony MacDonald

B.Sc. (hons.) Mount Allison University, 1993

**A thesis submitted to the faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of**

DOCTOR OF PHILOSOPHY

**Ottawa-Carleton Institute of Biology
Carleton University
Ottawa, Ontario
May, 1998**

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

**Enzyme Thermal Adaptation and Signal Transduction
Involvement in Ground Squirrel Hibernation**

Submitted by

Justin A. MacDonald, B.Sc. (hons.)

In partial fulfillment of the requirements
for the degree of Doctor of Philosophy

Chair, Department of Biology

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1998

ABSTRACT

Regulatory enzymes are integral to the regulation of metabolism, and reversible protein phosphorylation by kinases and phosphatases controls most aspects of cell life. Brown adipose (BAT) PKA, liver PP-1 and muscle phosphofructokinase (PFK) from the ground squirrel were purified to homogeneity; physical and kinetic properties of these were assessed with respect to function at low temperature. An assessment of PKA catalytic subunit function via *in vitro* incubations of ground squirrel BAT extracts with ^{32}P -ATP revealed differences in the patterns of phosphorylated proteins between euthermic and hibernating animals and between 37 and 5°C. The addition of 10 % (w/v) polyethylene glycol reversed all negative effects of cold temperature, low pH and urea on PFK stability. A reduction in assay temperature from 37° to 5°C had numerous effects on ground squirrel enzymes including changes in pH optima, decreases in K_m values for substrates, and reduced inhibition by salts. The percentage of membrane associated PKC increased during hibernation in liver, but the % active PKA was unaffected by hibernation in any tissue. Changes in PP-1 and -2 activities in tissues of euthermic and hibernating animals showed that phosphatases are regulated in hibernation. The subcellular localization of PP-1 in liver and muscle was affected by hibernation. The mitogen-activated protein kinase (MAPKs) MAPK-activated protein kinase (MAPKAPK-1 and -2) activities were affected by hibernation. p70^{S6K} kinase activity decreased in kidney during hibernation. c-jun kinase activity increased in 4 hibernating tissues but decreased slightly in brain. p38 was activated in hibernating muscle and heart. The responses of immediate-early gene products c-jun, egr-1, and c-myc were tissue specific with no apparent overall pattern. Maintenance of muscle energy status at the

expense of the total adenylate pool was accompanied by a 60% decrease in the Na^+K^+ -ATPase activity. Enzyme activity was reduced *in vitro* when incubated with protein kinase activators and was relieved by addition of alkaline phosphatase. A change in the ATP dependency of the enzyme also occurred in hibernation. Overall, protein phosphorylation is key to the regulation of hibernating metabolism in ground squirrels, and hibernator enzymes demonstrate functional adaptations to cold temperature.

ACKNOWLEDGEMENTS

I rarely spend five hours composing one paper's acknowledgements, but this thesis demanded it. Merely drawing up the planetary charts and meditating on the astrological gestalt were not sufficient. I also cast the runes, consulted the *I Ching*, and pored over my recent dreams. But nothing worked. In desperation, I danced in circles till I got dizzy, fell down, and banged my head on the moon. At last a vision arrived. I saw that I was being asked to explore the spiritual possibilities of exhaustion. Only when I had worn myself out failing at every technique that's always worked before was I graced with a cockeyed deliverance.

Thanks to T. for her emotional balancing act. Kudos to Janet Storey for her subliminal editing suggestions and to Ken Storey for his daily harangues and lectures on life, and to both for their devotion to science and everything good in the world. Gratitude is extended to Drs. Steve Perry and John Vierula for attending committee meetings and offering their comments and criticisms. Thanks to my father, without whom there would be no wonder in my world. And to all those who shared the Storey lab experience - wow, that was some crazy ride man! I must rest now . . . . .

Sometimes a scream is better than a thesis. Ralph Waldo Emerson

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LIST OF ABBREVIATIONS

ACC - Acetyl CoA carboxylase

ACL - ATP-citrate lyase

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

AMPK - AMP activated protein kinase

BAT - brown adipose tissue

BMR - basal metabolic rate

BSA - bovine serum albumin

cAMP - 3',5'-cyclic adenosine monophosphate

cGMP - 3',5'-cyclic guanosine monophosphate

CRE - cAMP response element

CREB - cAMP response element binding protein

DAG - diacylglycerol

DE-52 - diethylamino cellulose

E_a - energy of activation

EDTA - ethylenediamine tetraacetate

EGTA - ethyleneglycol bis(β-aminoethyl ether) N, N'-tetraacetic acid

ERK - extracellularly regulated kinase

F16Pase - fructose 1,6,-bisphosphatase

F26Pase - fructose 2,6,-bisphosphatase

F6P - fructose 6-phosphate

GWW - gram weight weight

H-7 - 1-(5-isoquinolinesulfonyl)-2-methyl piperazine

HEPES - (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid])

HPLC - high performance liquid chromatography

I₅₀ - inhibitor concentration reducing enzyme velocity by 50%

IBMX - isobutylmethylxanthine

IEF - isoelectric focusing

IMP - inosine monophosphate

JNK - c-jun N-terminal protein kinase

K_m - Michaelis-Menten constant

MAPK - mitogen activated protein kinase

MOPS - 3-(N-morpholino)propanesulphonic acid

NAD⁺, NADH - oxidized and reduced forms of nicotinamide adenine dinucleotide

Na₃VO₄ - sodium vanadate

NE - norepinephrine

n_H - Hill constant

PCA - perchloric acid

PDH - pyruvate dehydrogenase

PEG - polyethylene glycol

PFK/PFK-I - 6-phosphofructo-1-kinase

PFK-2 - 6-phosphofructo-2-kinase

pI - isoelectric point

PK - pyruvate kinase

PKA - cAMP-dependent protein kinase

PKAc - PKA catalytic subunit

PKA-I - PKA inhibitor peptide (5-24)

PKC - Ca^{2+} and phospholipid dependent protein kinase

PKG - cyclic GMP-dependent protein kinase

PMSF - phenylmethanesulfonyl fluoride

PP-1 - protein phosphatase type-1

PP-2 - protein phosphatase type-2

PS - phosphatidylserine

PTK - protein tyrosine kinase

PTP - protein tyrosine phosphatase

Q₁₀ - factor by which a 10°C temperature change reduces the rate of a chemical reaction

RPTK - receptor protein tyrosine kinase

RPTP - receptor protein tyrosine phosphatase

RSK - ribosomal S6 kinase protein

SAPK - stress-activated protein kinase

SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis

SRE - serum response element

T_b - body temperature

TCA - trichloroacetic acid

TPCK - tosylphenylalanylchloromethyl ketone

TRE - tetradecanoyl-phorbol ester response element

TRIS - (Tris[hydroxymethyl]aminomethane)

V_{max} - maximal enzyme velocity

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CHAPTER 1

General Introduction

Mammalian Hibernation

The majority of the food eaten by mammalian endotherms is required as fuel for generating heat to maintain their relatively high and constant body temperatures.

However, seasonal environments presents strong thermoregulatory challenges to small mammals. Energy demands increase during cold winters at the same time as food supplies decline. Whereas many birds may migrate to areas of the world where temperature is warm and food is abundant, terrestrial animals generally lack this option. Some small mammals store food for survival over the harsh winter months; this is used to supply sufficient energy to their metabolism. Other mammals, primarily some members of the orders Rodentia, Insectivora, and Chiroptera, accumulate large reserves of metabolic fuels within their bodies and enter hibernation, a period of deep torpor, or winter dormancy, which lasts weeks or even months in colder climates.

High metabolic rates and limited capacities for fuel storage are two compounding factors linked to the size of small mammals. These metabolic problems are amplified most when food availability is restricted during the cold winter months because a large surface area to volume ratio results in high rates of heat loss. For most small mammals, thermoneutral conditions mean an environmental temperature above 20°C, or for the smallest hibernating mammals, above 30°C (Cannon and Nedergaard, 1990). On a winter's day, small mammals are exposed to a cold stress well below the thermoneutral state, and would have to increase their metabolic rate by several-fold to counteract heat loss to the surroundings. Instead of using this expensive option, the hibernator allows its T_b to drop to that of its surroundings. Hibernation represents an elegant biochemical adaptation that via metabolic depression readily circumvents overwintering energy

problems of small animals. Indeed, Wang (1978) has estimated that, under natural winter conditions, the Richardson's ground squirrel saves approximately 90 percent of the energy that would otherwise be utilized to maintain normal metabolic rates.

In mammals which exhibit hibernation, two clear physiological states exist annually: a non-hibernating phase and a hibernating phase. There are distinct physiological and biochemical manifestations associated with each phase of the annual hibernation cycle and the alternation of the two phases is apparently regulated by an endogenous circannual rhythm (Mrosovsky, 1978). During the non-hibernating phase, the body weight of an adult animal is relatively constant and exposure to cold results in increased heat production and maintenance of euthermia. As the animal approaches its hibernating phase, its body weight increases dramatically, often doubling in 4 to 6 weeks due to hyperphagia and fat deposition. Lipids are stored in two forms, as white adipose tissue (the depot reserves that fuel basal metabolism) and as brown adipose tissue, BAT, (the essential energy source for the non-shivering thermogenesis that rewarms the hibernator during arousal) (Dark and Ruby, 1993). The animals select hibernacula that are dry and relatively sheltered from the winter environment; sheltered hibernacula provide sites where the temperature remains above 0°C so that the animal can let T_b fall to the ambient temperature of the den even though the air temperature may be far below zero. Once a weight maximum is reached, the animal becomes anorexic and is likely to be in its hibernating phase. Exposure to cold at this time typically elicits torpor. The cooling of T_b lasts roughly 1-3 days or until the hypothalamic set point for T_b during torpor has been reached (Davenport, 1992). Shivering occurs periodically to stabilize the body temperature at a level that is a few degrees above ambient temperature (4-10°C).

Torpor may last from a few hours to several weeks during which time all physiological functions are at a minimum. Following complete arousal from torpor, a period of euthermia lasting from a few hours to a few days ensues before the animal reenters another bout of torpor.

Body functions are greatly slowed with the lowered body temperature characteristic of hibernation (Wang, 1989). Total blood flow is typically reduced to about 10% of normal, although the head and brown fat tissue receive a much higher blood flow than other tissues. Cardiac output slows considerably and is characterized by a marked decrease in heart rate, the occurrence of arrhythmia due to enhanced parasympathetic activity, and a progressive increase in vasoconstriction to maintain blood pressure with decreased heart rate. A decrease in respiratory rate with irregular periods of apnea along with a significant decrease in oxygen consumption occurs during hibernation. There is an apparent loss of the ventilatory response to hypoxia challenge during hibernation. Breathing either 100% nitrogen or 1-3% oxygen does not result in stimulation of ventilation, but in contrast, breathing of 5-7% carbon dioxide results in elevated ventilation indicating the retention of respiratory sensitivity to increased PCO_2 during hibernation as also occurs in euthermia. The trigger for the onset of hibernation torpor has yet to be determined. It was thought that BAT possessed endocrine functions that triggered the onset of hibernation; however, BAT is now known to be a purely thermogenic tissue responsible for the arousal and rewarming of the animal from torpor (Horwitz, 1989). Research has attempted to implicate a brain peptide hormone as the "torpor trigger," but the putative hibernating promoting factors (e.g., antabalone, hibernation induction trigger, and alarmone) are still physiologically undefined (for

reviews see Nurnberger, 1995; Wang, 1986). The role of serotonin (5-HT) in the induction and maintenance of hibernation has attracted attention because of its regulation of sleep. There is no current biochemical evidence to suggest that sleep and hibernation are promoted by the same set of chemical messengers. Sleep promoting factors have been chemically defined and they include several peptides and lipid soluble substances, but whether these sleep-promoting factors promote hibernation remains unknown (Krueger and Shoham, 1986). In the Richardson's ground squirrel, intravenous administration of 5-HT increases heat loss only in the animal's non-hibernating phase but exerts an additional heat suppressing effect in its hibernating phase. The implication is that an enhanced brain serotonergic activity during the hibernating phase may facilitate the transition from euthermia to hibernation through both inhibition of heat production and enhancement of heat loss (Glass and Wang, 1979). Cai et al. (1989) suggest that the ratio of noradrenaline to serotonin is the most important factor for the induction of and maintenance of hibernation. More studies are needed to confirm the roles of neurochemicals on thermoregulation in hibernating mammals.

Geiser (1988) has observed that overall metabolic rate depression may be regulated by different mechanisms, depending on the size of the hibernating animal and its body temperature during torpor. This was revealed by a comparison of Q_{10} values for oxygen consumption during hibernation which decreased from approximately 4.5 to 2.5 as body size increased and as the difference between euthermic and hibernating body temperature decreased. Since biological processes typically have Q_{10} values between 2 and 3, Q_{10} values above 3 (observed in smaller animals and in animals with higher hibernating body temperatures) imply that these animals actively depress metabolic processes when

entering torpor. However, more recent work (Brooks and Storey, 1992) has shown that this relationship may not apply to mechanisms of glycolytic control in hibernating mammals. They reported phosphorylation/dephosphorylation enzymatic control mechanisms both in the small hibernator *Zapus hudsonius* (meadow jumping mouse) and in the larger hibernating golden mantled ground squirrel *Spermophilus lateralis*, though phosphorylation control seemed more rigorous and widespread in the smaller animal. In these animals, changes in the degree of enzyme covalent modification and in the concentration of allosteric enzyme activators are an important part of an overall glycolytic rate depression strategy in hibernation. This work suggests that glycolytic rate is actively depressed during hibernation and is not a consequence of a change in overall body temperature.

Specifically, the percentages of active glycogen phosphorylase and PDH were observed to decrease in both species during hibernation. Decreased glycogen phosphorylase activity is consistent with a switch to fatty acid oxidation during hibernation; decreased liver glycogen phosphorylase will limit glucose export to the blood and endogenous oxidation. The importance of fatty acid oxidation during hibernation has been demonstrated by measurements of respiratory quotients that indicate a switch to a primary dependence on lipid oxidation during hibernation, possibly supplemented by gluconeogenesis from amino acids (Lyman et al., 1982; Hochachka and Guppy, 1987). Furthermore, during hibernation, glycogen phosphorylase activity is suppressed to spare carbohydrate reserves as opposed to starvation where glycogen is rapidly depleted (Hochachka and Somero, 1984).

PDH activity in heart and kidney of *S. lateralis* decreases during hibernation (Brooks and Storey, 1992); it is surmised that this effect also promotes a metabolism based largely on lipid oxidation during hibernation. PDH is responsible for the production of acetyl-CoA from pyruvate in the mitochondrion and represents the major route for the entry of carbohydrates into the tricarboxylic acid cycle. The enzyme is controlled by phosphorylation to produce an inactive enzyme with activation caused by dephosphorylation via a soluble phosphatase (Denton et al., 1987; Reed and Yeaman, 1987). Two conditions may act to stimulate the membrane bound PDH kinase to phosphorylate the enzyme: 1) a decrease in pyruvate availability due to the regulation of glycolytic activity, and 2) an increase in acetyl-CoA due to increased fatty acid oxidation. Although the changes in glycolytic flux and fatty acid oxidation have been proposed to result in the suppression of PDH activity, the exact time-course of the changes in PDH and glycogen phosphorylase as well as the switch to fatty acid oxidation, has not been ascertained (Brooks and Storey, 1992).

Hibernators do not exploit the energy savings associated with metabolic depression to the maximum; all hibernating mammals studied to date are known to rouse periodically from the torpid state. These arousals are energetically very costly and represent approximately 90% of the total energy utilization over the hibernation season (Wang, 1978; Thomas et al, 1990). Thus, arousals are the single most important factor determining hibernation energy budgets and possibly causing over-winter mortality due to energy short-falls. Arousal from torpor is an explosive event; full arousal from a 5°C body temperature to euthermia can be completed in 20 min in small rodents (Wang and Hudson, 1970) and bats (Hayward, 1968), whereas a few hours are necessary for

complete arousal in the larger rodents like ground squirrels (Wang, 1979). Arousal begins with an increase in the breathing rate (hyperventilation) to promote the inhalation of O₂ and exhalation of CO₂. Non-shivering thermogenesis in the BAT occurs for initial heat production followed after partial rewarming, by an eventual mobilization of glycogen reserves to initiate shivering thermogenesis in skeletal muscle of the outer extremities (Davenport, 1992). Thermogenesis in brown fat is activated by the sympathetic nervous system through the release of norepinephrine, which binds to the receptors of brown adipose cells. Through a protein kinase associated second messenger mechanism, thermogenesis is initiated (Nedergaard and Cannon, 1990; Cannon and Nedergaard, 1985; Klaus et al., 1991). Normal ATP utilization for cellular processes rises in these fat cells in response to the sympathetic signals, accounting for a small part of the increased heat production. Brown fat mitochondria generate the majority of heat by short-circuiting normal respiratory control and dissociating (uncoupling) substrate oxidation and respiration from oxidative phosphorylation (ATP synthesis); the resulting electrochemical energy that would otherwise be used to produce ATP is dissipated as heat. Heat generated in the brown fat pads is then rapidly dispersed to other parts of the body by blood flowing through the extensive vascularization of BAT.

The occurrence of these spontaneous arousals is perplexing, especially when one considers that up to 90% of the energy used during the winter may be associated with this process (Thomas et al, 1990). Presumably the arousals and subsequent euthermic intervals have some physiological value, but the reasons for them remain the least understood part of the hibernation phenomenon. It was believed that periodic arousals were controlled by a circadian rhythm (Pengelley and Kelley, 1966; Mrosovsky, 1978).

As well, it became apparent that the temporal patterns of arousal events were influenced by environmental temperature (French, 1982). As the ambient temperature rose, both the frequency and the duration of homeothermal periods in midwinter increased. The strength of this temperature dependency suggested that the arousals were not timed by a circadian clock. The arousal frequency from deep torpor was unlike any circadian oscillation and mirrored fluctuations in body temperature and changes in the torpid metabolic rate. This link between arousal frequency and metabolic rate has led many investigators to suggest that arousals might be both initiated in response to, and necessary for the elimination of, metabolic wastes that build up and/or for the replenishment of fuel in species that eat between torpor bouts (Fisher, 1967; Fisher and Manery, 1967). However, this hypothesis has been displaced by an attractive theory that proposes that the animal needs to re-establish cellular ionic balance which is disrupted during a hibernation bout (Willis, 1982).

Experimental Animals

Ground squirrels are small, diurnal mammals that can avoid the winter climate and its associated hardships by retreating into subterranean burrows and entering hibernation. The majority are found in western North America. Species of the genus *Spermophilus* live in a variety of habitats including hot dry deserts, grassland, alpine meadows, and arctic tundra. The Richardson's ground squirrel, *Spermophilus richardsonii*, and Golden-mantled ground squirrel, *Spermophilus lateralis*, are two representative hibernating ground squirrels which will be used in this thesis. The natural abundance of these animals and the ease with which they can be maintained in captivity due to their small

size make these animals good subjects on which to base biochemical studies of hibernation.

During the autumn months, these rodents (approximately 0.1-0.4 kg in mass) enter their underground burrows and enter an extended period of torpor (lasting weeks to months) during which body temperature drops from the normal euthermic temperature of 37°C to approximately 5°C. Body temperature approximates the ambient burrow temperature although if the burrow cools to less than 5°C, the animal will thermoregulate to stay at 5°C. A review by Davis (1976) summarized field studies on the timing of seasonal torpor and the duration of the torpor season in ground squirrels. This information was usually obtained through regular census and/or systematic trapping. With the advancement of biotelemetry, Wang (1978) was able to more thoroughly characterize patterns of torpidity under natural conditions in a population of Richardson's ground squirrels located in northwestern Alberta (Figure 1.1). The duration of torpor in early autumn was typically short, averaging around 3 days. The duration of torpor gradually lengthened as the season progressed and when the burrow temperature decreased to near 0°C in January peak torpor duration reached 19 days. Subsequently, the duration of torpor shortened until the animals emerged from hibernation in late March.

Enzyme Regulation and Protein Phosphorylation

One of the keys to survival in the hypometabolic state is the ability to control metabolic pathways. In hibernation, some key control enzymes are capable of regulating metabolism; for example, a strong inhibition of carbohydrate metabolism and a

Figure 1.1. Hibernation bout duration and body temperature changes in hibernating ground squirrels. The top figure illustrates the body temperature changes over a complete torpor season in a juvenile male *Spermophilus richardsonii* under field conditions (taken from Wang, 1989). The bottom figure illustrates the pattern of body temperature within a single torpor bout in *Spermophilus richardsonii* under field conditions (taken from Wang, 1989).

reliance on lipid oxidation occurs during the transition from euthermia to hibernation.

Enzymes can be controlled by a number of mechanisms. Allosteric effectors, including activators and inhibitors, influence enzyme activity by binding to enzyme sites in an area other than the active site, thus changing the enzyme's conformation and its catalytic efficiency. Allosteric modification may vary at different temperatures since temperature induced changes in enzyme conformation may alter the affinities for substrates and may affect the efficiency of activators and inhibitors (Storey, 1992).

Reversible covalent modification of proteins by phosphorylation and dephosphorylation plays a dominant role in controlling complex cellular functions including contractility, membrane transport, cell growth and differentiation (Hashimoto and Yamamura, 1991), the transcription and translation of genes (Meek and Street, 1992), and the regulation of metabolic pathways during environmental stress (Storey, 1989). In mammalian cells, about one in every three proteins is now known to contain covalently bound phosphate, and protein kinases and phosphatases may account for as many as 5% of human genes. The phosphorylation (or dephosphorylation) of serine, threonine, or tyrosine residues triggers conformational changes in regulated proteins that alter their biological properties, the level of phosphorylation at any instant reflecting the relative activities of the protein kinases and phosphatases that catalyze the interconversion processes. Protein kinases and phosphatases may have many different enzyme proteins as substrates, and hence reversible protein phosphorylation may coordinate control over multiple enzymes and/or pathways to allow for an integrated response to metabolic need. Different metabolic pathways may also be controlled by identical protein kinases and protein phosphatases, or by identical effector molecules that regulate different protein

kinases and phosphatases. The role of reversible phosphorylation in controlling both individual enzyme changes and the overall metabolic rate depression in hibernation is explored in this thesis.

Protein kinases

A protein kinase cascade can begin with the binding of a hormone, such as adrenaline, to a receptor located on the plasma membrane of a cell (Shenolikar, 1988). A receptor-mediated increase in the intracellular concentration of a second messenger ensues and the second messenger then binds to the regulatory subunit of the protein kinase to cause an activation of the catalytic subunit. Active protein kinases are then free to phosphorylate proteins which control and regulate cellular processes. The signal amplification exhibited by the protein kinase cascade system is important to cellular metabolic control because a coordinating signal to many target enzymes ensures that many metabolic pathways can be controlled simultaneously with the release of low levels of hormone. Many examples are known in which "second messengers" relay hormonal signals directly to appropriate protein kinases or phosphatases, leading to an altered phosphorylation state of one or more key protein substrates. The prototypes for this kind of signal transmission are the activation of cAMP-dependent protein kinase (PKA) by cAMP and the activation of protein kinase C (PKC) by calcium. (Figure 1.2).

PKA is one of the most widely distributed kinase enzymes in the cells of mammalian tissues. PKA activity is controlled by a dissociation reaction in which the holoenzyme is catalytically inactive, but when cAMP binds to the regulatory subunit dimer, the catalytic subunits dissociate and are then able to phosphorylate substrates

(Taylor and Radzio-Andzelm, 1994). This method of activation also allows the free catalytic subunits to migrate between cellular compartments otherwise inaccessible to the larger holoenzyme. The covalent modification of regulatory glycolytic enzymes is partially achieved through the actions of protein kinases like PKA (Cohen, 1993). The classic example of PKA action is the phosphorylation of phosphorylase kinase (which then phosphorylates glycogen phosphorylase b to convert it to the active a form). The end result of this cascade is the activation of glycogenolysis. PKA also mediates the phosphorylation of multiple protein substrates that are important regulators of cellular growth, metabolism, gene transcription and translation, and homeostasis (Scott, 1993).

PKC is another important protein kinase involved in overall cell function (Nishizuka, 1995). The activation of PKC is a very complex cascade of molecular events that begins with the binding of a hormone to the plasma membrane of a cell. A membrane bound enzyme then catalyzes the breakdown of phosphatidylinositol 4,5-bisphosphate to yield diacylglycerol (DAG) and freely soluble inositol-1,4,5-triphosphate (IP₃). IP₃ causes the release of calcium ions from calcisomes and thereby increases the intracellular concentration of calcium. PKC translocates from the cytosol to the plasma membrane where it binds to DAG and becomes activated. PKC activity at the membrane also depends on the presence of the membrane phospholipid, phosphatidylserine.

Figure 1.2. An overview of signal transduction and the mechanisms of cAMP-dependent protein kinase and protein kinase C action. Key to symbols: growth modulators (GF, PDGF, EGF), receptor (R), phosphatidylinositol 4,5-bisphosphate (PIP₂), inositol-1,4,5-triphosphate (IP₃), diacylglycerol (DAG), 12-O-tetradecanoyl phorbol-13-acetate (TPA), protein kinase C, phosphoinositide specific phospholipase C (PLC), cAMP-dependent protein kinase (PKA), cytosol (CYT), plasma membrane (PM), and endoplasmic reticulum (ER). Adapted from Malviya et al., (1988).

Cells also respond to extracellular stresses by activating signal transduction pathways which culminate in changes in gene expression (Cahill et al., 1996). A critical component of the signal transduction is the activation of protein kinases which phosphorylate nuclear transcription factors which in turn control the induction of various genes (reviewed in: Kyriakis and Avruch, 1994; Seger and Krebs, 1995; Cobbs and Goldsmith, 1995). These kinases transduce signals to the nucleus in response to growth factors and other cellular stresses, such as inflammatory cytokines, ischemia, reversible ATP depletion, heat shock, osmotic shock, endotoxin and genotoxic stresses. The dominant mechanism by which cells respond to extracellular signals can be summed up simplistically as follows (Figure 1.3). Signaling commences on binding of an effector molecule to its receptor on the cell surface. The binding activates a protein tyrosine kinase. The tyrosine kinase activity can either be an intrinsic property of the receptor itself (e.g. growth factor receptors) or may reside in a distinct and separate kinase molecule associated with the receptor (e.g. cytokine receptors); the former has come to be known as a receptor tyrosine kinase (RTK). Kinase activation leads to autophosphorylation of the receptor or other substrates, and downstream activation of a number of common signaling molecules, either directly or through a cascade of biochemical reactions. Frequently activated proteins include GTPase activating protein, p34cdc/cyclin, Raf and MAP-kinases, phospholipase C- γ , and phosphatidylinositol 3-kinase. MAPK signalling pathways are highly conserved transducers of receptor and post-receptor events to the nucleus and are activated by a wide spectrum of extracellular

Figure 1.3. The mitogen-activated protein kinase cascade (adapted from Seger and Krebs, 1995)

stimuli (reviewed in: Seger and Krebs, 1995; Kyriakis and Avruch, 1994). These kinase cascades are characterized by sequential activation of a MAP kinase kinase kinase (MAPKKK) and a MEK (a MAPKK). Three distinct families of MAPKs have been identified to date: the extracellular signal regulated kinases (ERKs), the stress activated protein kinases or c-jun N-terminal kinases (SAPKs/JNKs), and p38 kinase. A number of other pathways, such as those originating from G proteins, merge into this scheme at various points. Eventually this chain of events result in the activation of transcription factors through phosphorylation, in turn affecting the transcription of specific genes, and/or other kinases including MAPK activated protein kinases (MAPKAPKs) and ribosomal protein S6 kinase (RSK). Once the signalling is complete, a given phosphoprotein must revert back to its resting state, a reaction catalyzed by the hydrolysis of the phosphate groups by protein phosphatases.

Protein Phosphatases

Although protein kinases have received paramount emphasis in studies of signal transduction, it is gradually being recognized that protein phosphatases must be equally important. In order for phosphorylation to play a regulatory role, it needs to be a reversible process for otherwise a phosphoprotein would not revert back to its dephosphorylated, inactive state. A direct role of protein phosphatases in a number of signal transduction pathways has been revealed (Cohen, 1989). Like their kinase counterparts, protein phosphatases are generally specific for either phosphotyrosine (protein tyrosine phosphatase or PTP) or phospho-serine or threonine (protein Ser/Thr phosphatase or PP). However, a few phosphatases with dual specificity have also been

reported, e.g. MAP kinase phosphatase-1, (Denu and Dixon, 1995). Unlike the kinases, the majority of the phosphatases are not regulated by second messengers and seem to be more promiscuous in their choice of substrates, giving rise to speculation that they may not be as numerous as the kinases.

The four major Ser/Thr phosphatases have been classified as either Type 1 (PP1), or Type 2 (PP2A, PP2B or PP2C) based on their ability to dephosphorylate the α - or β -subunit of phosphorylase kinase, and their sensitivity to two small heat-stable proteins, termed inhibitor -1 and inhibitor-2 (reviewed in: Cohen, 1989; Shenolikar, 1994; Wera and Hemmings, 1995). PP2B and PP2C have absolute requirements for Ca^{2+} and Mg^{2+} , respectively, while PP2A and PP1 are active toward most substrates in the absence of divalent cations. Three of these enzymes, PP1, PP2A, and PP2B are members of the same gene family, while PP2C appears to be distinct. PP1, PP2A and PP2B are complexed to other subunits in vivo, whereas PP2C has only been isolated as a monomeric protein. In addition, these phosphatases exhibit differential sensitivity to the marine sponge toxin, okadaic acid (MacKintosh and MacKintosh, 1994). Okadaic acid concentrations of 1 nM are required to completely inhibit PP2A, whereas much higher concentrations are required to inhibit PP1 and PP2B; PP2C activity is not affected at all by okadaic acid. These four Ser/Thr phosphatases have broad and overlapping specificities in vitro and account for virtually all measureable phosphatase activities in tissue extracts toward a variety of phosphoproteins. But new species of phosphatases are still being discovered (Cohen, 1997); phosphatases PPV, PPY, PPZ1, PPZ2 are examples

of newly discovered phosphatase family members which exhibit intermediate properties and are generating novel branch points in the classification scheme.

The active forms of PP1 are largely particulate, and their association with subcellular structures is mediated by targeting subunits that direct the PP1 catalytic subunit to particular locations, enhance its activity toward certain substrates, and confer important regulatory properties upon it (Hubbard and Cohen, 1993). This concept has been best established for the glycogen bound phosphatase in skeletal muscle and liver (PP1-G) and the myofibrillar bound form in skeletal muscle (PP1-M) (Cohen, 1989). The activity of PP1 is controlled indirectly by the levels of second messengers, cAMP and calcium, though this control is dependent on the form of the enzyme and the tissue. PP1 can be inhibited by cAMP in a variety of cells through the PKA-catalyzed phosphorylation of inhibitor-1. In skeletal muscle, phosphorylation of the glycogen binding subunit of PP1-G by PKA promotes translocation of the catalytic subunit from glycogen particles to cytosol, inhibiting the dephosphorylation of glycogen metabolizing enzymes. PP1 can also be activated indirectly by calcium through the ability of PP2B to dephosphorylate inhibitor-1. The inactive cytosolic form of PP1 (PP1-I) can be activated *in vitro* through the glycogen synthase kinase-3 (GSK-3) catalyzed phosphorylation of its inhibitory subunit (inhibitor-2).

The catalytic subunit of PP2A is found complexed with a variety of regulatory subunits, generating a number of different holoenzymes (Wera and Hemmings, 1995). Although the physiological significance of so many holoenzymes remains unknown, they may represent a mechanism of regulation of PP2A. Substrates include RSK, the small growth suppressor protein (p53), and members of the MAP kinase family. In general,

protein kinases involved in cell growth and transcription factor activation appear to be frequently regulated by PP2A. PP2A exhibits high activity *in vitro* toward the regulatory enzymes of glycolysis/gluconeogenesis, fatty acid synthesis and amino acid breakdown, and its cytosolic location suggests that it may be the major phosphatase acting on these substrates *in vivo*. The finding that addition of okadaic acid to intact cells rapidly stimulates gluconeogenesis and lipolysis, inhibits fatty acid synthesis and stimulates phosphorylation of the regulatory enzymes of these pathways (PFK-II/F26Pase, PK, PFK-I, F16Pase, ACC, ACL) supports the view that PP2A is the dominant phosphatase *in vivo* (Cohen, 1989).

The specific functions of PP2B remain undetermined; however, it shows a much narrower substrate specificity *in vitro*, and unlike members of the other three classes, PP2B shows an absolute requirement of Ca^{2+} -calmodulin (Wera and Hemmings, 1995; Cohen, 1989). In contrast to phosphatase types 1 and 2A, PP2B does not regulate the rate-limiting enzymes of metabolic pathways; the most effective substrates for PP2B thus far identified are proteins that regulate the activities of other kinases and phosphatases, namely the regulatory RII subunit of PKA, inhibitor-1, and the calmodulin dependent cAMP phosphodiesterase. Since these proteins are all substrates for PKA, PP2B may act via Ca^{2+} to attenuate the actions of cyclic AMP. PP2B may also serve to regulate intermediates in signal transduction pathways that are yet unknown.

At present, only limited information is available on the function of the Mg^{2+} -dependent protein phosphatase, PP2C (reviewed in Cohen, 1989). The enzyme is monomeric and two major isoforms have been cloned. PP2C was suggested to play a role in the Ca^{2+} -dependent signal transduction in the brain. Recent analysis of yeast strains

has indicated that the PP2C isoform is important in the heat shock response (Shiozaki et al., 1994). PP2C is implicated in signal transduction processes involved in osmoregulation (Shiozaki and Russel, 1995) where it appears to regulate the osmosensing MAP kinase cascade (e.g. HOG1 kinases). However, whether PP2C directly regulates the kinases or their downstream targets still remains to be established.

Na⁺K⁺-ATPase

There are three classes of ATPase enzymes that directly couple ion transport against electrochemical gradients with the hydrolysis of ATP. Included in the P class of ATPases is the Na⁺K⁺-ATPase pump, a membrane protein that couples the hydrolysis of ATP to the transport of Na⁺ and K⁺ ions against their respective electrochemical gradients (for review: Lingrel and Kuntzweiler, 1994). Structurally, the protein consists of two subunits: a large polypeptide (α , or catalytic) and a smaller glycoprotein (β , of unknown function). The transporter plays an important role in the regulation of cell volume, heat production, and free Ca²⁺ and Na⁺ concentrations, as well as membrane potential.

Ion gradients have been shown to vary with physiological state in mammals; for example, starvation is associated with a decrease in the activity of Na⁺K⁺-ATPase and a reduction in tissue ion gradients (Milligan and Summers, 1986; Clausen, 1986). Na⁺/K⁺ gradients, or tissue K⁺ concentration gradients, have been measured in three instances of metabolic depression: in the anoxic turtle (Lutz et al., 1985; Chih et al., 1989; Buck and Hochachka, 1993), in the tissues of hibernators (Willis, 1987), and in the tissues of the

estivating Australian goldfields frog (Flanigan et al., 1993). These studies provided evidence that ion gradients were maintained, or at least were dissipated slowly, in successful metabolic depression accompanying anoxia tolerance or hibernation. This is in contrast to the rapid and damaging dissipation of ion gradients in animals that are not stress tolerant and do not show stress-induced metabolic rate depression (Willis, 1987; Kamm 1979). The regulatory mechanisms that control the activity of Na^+K^+ -ATPase during cold stress have yet to be determined. Control of Na^+K^+ -ATPase by reversible protein phosphorylation (via protein kinases and protein phosphatases) has been observed previously in mammalian tissues (Bertorello et al., 1988; 1991; Ewart and Clip, 1995) and may hence regulate this important metabolic process.

Summary of Aims and Objectives

The control of cellular function, including metabolic rate, must be governed by fast and readily reversible mechanisms. Studies of glycolytic regulation during environmental stress in a wide variety of animals have served to illustrate the molecular mechanisms which mediate transitions to and from the hypometabolic state (Storey and Storey, 1990). These mechanisms can include reversible enzyme phosphorylation, changes in the percentage of enzymes associated with subcellular structures to directly alter enzyme activity, and changes in enzyme activity via allosteric regulation. Since the hibernator's body temperature ranges from about 37°C in euthermia to near freezing temperatures in deep torpor, the functional ability of the mechanism(s) involved in controlling the hypometabolic state must be maintained over a wide range of temperatures. The first aim of this thesis was to assess the kinetic parameters of the key

regulatory enzyme of glycolysis, PFK-I, from hibernating ground squirrels and determine how changing temperature affects enzymatic function and glycolytic regulation.

Reversible protein phosphorylation has emerged as the pre-eminent control mechanism of cellular metabolism. The role of key phosphorylation/dephosphorylation systems has not been examined in the hibernation process, although preliminary investigations have determined that various enzymes of carbohydrate metabolism in these animals are modified by phosphorylation during hibernation (Storey, 1987a; 1987b; Brooks and Storey, 1992). The second aim of this thesis was to investigate whether phosphorylation/dephosphorylation has a wider role. Thus, I have chosen to assess the activity of the primary cellular ATP-consumer, Na^+K^+ -ATPase, to determine if it is modulated during hibernation by reversible phosphorylation. Studies also attempted to determine the kinase(s) involved in regulating Na^+K^+ -ATPase activity.

The third aim of this thesis will be to determine if signal transduction cascades, particularly protein kinases and phosphatases, are involved in the metabolic adjustments that occur during mammalian hibernation. The activities of kinases (PKA, PKC, and MAPKs) and phosphatases (PP1, PP2A, and PP2C) in the hibernating state were compared with euthermic activities.

The fourth aim of this study was to determine the effects of temperature on the kinetic and thermodynamic parameters of a representative protein kinase (PKA) and phosphatase (PP1). This was accomplished by purifying these enzymes and characterizing them at temperatures approximating those seen during a hibernation bout. Data collected yielded a clearer overall picture of how mammalian signal transduction

enzymes function over wide ranges in temperature to regulate cell function in the cold, hypometabolic state.

Hypotheses

The following hypotheses were tested:

- A. The activity of Na^+K^+ -ATPase is modulated by reversible protein phosphorylation in the hibernating state.
- B. The regulation of phosphofructokinase activity in the hypometabolic state occurs by temperature-induced changes in the enzyme conformation that affect the efficiency of its allosteric activators and inhibitors.
- C. Changes in tissue protein kinase and/or protein phosphatase activities are responsible for initiating the molecular adjustments seen in mammalian hibernation.
- D. PKA from the hibernating mammal is adapted to maintain catalytic function over a wider range of temperatures than the non-hibernating mammalian enzyme isolated from rabbit.
- E. PP1 from the hibernating mammal is adapted to maintain catalytic function over a wider range of temperatures.

CHAPTER 2

**Temperature, pH, and phosphate effects on allosteric phenomena of
muscle phosphofructokinase from hibernating ground squirrel**

INTRODUCTION

Hibernation requires a variety of metabolic adaptations including those that regulate and coordinate a profound metabolic depression as well as those that reorganize metabolism for long term survival at a reduced body temperature. Changes in fuel use are well known: lipid oxidation becomes primary, ketosis occurs, and gluconeogenesis from amino acids is increased (Hochachka and Somero, 1984). Carbohydrate reserves are spared as opposed to starvation where glycogen is rapidly depleted, and measurements of respiratory quotients indicate the oxidation of fatty acids provides the primary fuel for metabolism (Hochachka and Guppy, 1987). Glycolytic rate in most or all tissues should be reduced and 6-phosphofructo-1-kinase (PFK) is likely one of the key enzymatic sites where control over glycolysis is exerted. PFK gates the commitment of sugar phosphates (derived from glycogen or glucose) into the triose phosphate portion of the pathway. Since PFK is considered to be the first rate-controlling enzyme unique to glycolysis, it appeared to be the enzyme most likely to be affected in temperature adaptation. PFK is subject to a wide variety of regulatory mechanisms including allosteric controls by powerful activators and inhibitors (Kemp and Foe, 1983), acid-base changes, post-translational modification by reversible protein phosphorylation (Benoit et al., 1994), enzyme aggregation and enzyme binding to subcellular macromolecules (Brooks and Storey, 1988; 1991). PFK is an enzyme of central importance to the regulation of carbohydrate metabolism and arguments for inhibitory control of PFK in the hibernator have been presented (Brooks and Storey, 1992; Storey, 1987; Hand and Somero, 1982; 1983).

The mechanisms that control the decrease in body temperature during entry into hibernation are still not fully understood; however, two theories have been proposed. For some species (and authors) it has been concluded that metabolic rate reductions in hibernation are simply a consequence of the low T_b (Geiser, 1988). This interpretation is supported by calculations of Q_{10} values for oxygen consumption that are often found to be 2 -3 in many hibernator species. The reduction in the rate of most chemical processes with temperature is two- to three-fold for a 10°C interval (a Q_{10} of 2 to 3) and is similar to that found for most isolated enzymes or tissue metabolic rates. So, when a hibernator's body temperature falls from 37°C to about $2-7^{\circ}\text{C}$, the animal can count on a 8-27 fold reduction in metabolism as the simple effect of reduced temperature and hence, a corresponding 8-27 fold savings in the metabolic fuel consumption needed to survive the winter.

In reality, the reduction in metabolic rate for many species (particularly those with small body mass) is often more profound than would be expected from the typical effect of temperature on reaction rates alone. Based on these observations, authors have suggested that Q_{10} effects are enhanced during hibernation by specific mechanisms of metabolic inhibition (Geiser, 1988; Malan, 1982), and that an active suppression of metabolism consequently results in higher Q_{10} values (Heldmaier and Ruf, 1992a; 1992b; Storey and Storey, 1990). Although hypothermia appears to be the consequence and not the cause of reduced metabolic rates in hibernators, the cause of the active suppression has yet to be determined. The active suppression of metabolic rate during hibernation has been proposed as an effect of the lowered temperature, since the pH of neutrality is increased, and there is a tendency for an 'acid inhibition' of metabolism (Malan, 1982).

It has been well established that the pH of muscle in hibernation is similar to that in euthermia (Bickler, 1984; Malan, 1982; Malan, et al., 1985). Tissue pH is held constant even though the pH of neutrality of water increases as temperature decreases. The result is a relative acidosis of the tissue as body temperature decreases toward hibernation levels. Several authors maintain that this acidosis may be the factor that inhibits metabolism during hibernation (Malan et al., 1988). Specifically, acidotic inhibition of PFK has been implicated as the method by which glycolytic flux is decreased during hibernation (Hand and Somero, 1983).

The mechanism proposed for the pH induced cold inactivation of PFK favours an initial protonation of specific amino acid residues that is followed by an isomerization of the native tetramer to an inactive form and subsequent dissociation into dimers (Bock and Frieden, 1976a; 1976b). Enzyme reactivation is proposed to occur by a kinetically different pathway involving the deprotonation of the inactive subunit, followed by dimerization to the native state. The temperature and pH interactions on the activity and structure of skeletal muscle PFK has been suggested as a framework for interpreting the changes in glycolytic flux in hibernators; the acidotic conditions found in muscle tissue during hibernation leads to an increase in imidazole protonation and these effects may be translated into alterations in PFK kinetic and structural properties (Bock and Frieden, 1976b).

In this study we have re-evaluated experiments that examined low temperature and pH effect on subunit assembly of purified PFK, with a view to the compounding effects of dilute solutions on protein structure and function. An initial study of enzyme interaction with regulatory metabolites at low temperature revealed that PFK from the

skeletal muscle of the golden mantled ground squirrel (*Spermophilus lateralis*) exhibited altered kinetic properties that were consistent with temperature induced changes in allosteric phenomena. We then completed a detailed analysis of low temperature effects on the kinetic properties of the glycolytic control enzyme, PFK, from a hibernating mammal.

MATERIALS AND METHODS

Animals and chemicals

Adult golden mantled ground squirrels, *Spermophilus lateralis*, were obtained in the Crooked Creek area of the White Mountains of California during June 1992. All animals were individually housed in rat cages at the animal care facility of the University of California at Irvine, and maintained at 22°C on a fall (10L:14D) photoperiod. The squirrels were maintained on a diet high in polyunsaturated fats (15% sunflower oil providing linoleic acid, 18:2) which results in a depot fat composition very close to that seen in the wild and leads to a normal pattern of hibernation by the animals (Frank and Storey, 1995). The animals were allowed to feed and fatten for the next 7 weeks. At the end of this time (Sept.), several squirrels were sacrificed by decapitation while in a euthermic condition. Several remaining ground squirrels were induced to hibernate at an ambient temp of 3.5°C. After 30 days (Oct.) of hibernation, these were sacrificed while torpid. Samples of hind leg skeletal muscle were quickly excised and frozen in liquid nitrogen. Tissues were transported frozen on dry ice by a commercial air cargo service to Carleton University, and were then stored at -80°C until use. All biochemicals and

coupling enzymes were obtained from Boehringer Mannheim, Montreal, PQ, or Sigma Chemical Co., St Louis, MO.

Purification and standard assay of phosphofructokinase

Frozen leg muscle from euthermic ground squirrels was homogenized in 1:5 w/v in ice-cold buffer A (25 mM KH_2PO_4 , 0.1 mM EDTA, 0.1 mM EGTA, 15 mM KF, 10 mM 2-mercaptoethanol, 10% v/v glycerol, pH 8.0) with a few added crystals of phenylmethylsulfonyl fluoride using 3 x 20 s bursts of a Tekmar tissuemizer. The homogenate was centrifuged in a Sorvall RC5B refrigerated centrifuge at 23,400 g for 20 min at 4°C. The supernatant was saved and PFK was purified following a procedure modified from Ramadoss et al. (1976). The remainder of the purification procedure was performed at room temperature unless stated otherwise. The supernatant was applied onto a Sephadex DE-52 column (1.5 x 10 cm) equilibrated in buffer A. The column was washed with buffer A until the A_{280} of the effluent dropped to near zero; PFK activity was then eluted by 50 mL of a linear gradient of 0-1 M KCl in buffer A. Fractions (1 ml) were collected and assayed and those fractions with highest activity were pooled and dialyzed for 1 h at 4°C against 1 L of buffer B (25 mM imidazole-HCl, 0.1 mM EDTA, 0.1 mM EGTA, 15 mM KF, 10 mM 2-mercaptoethanol, 10% v/v glycerol, pH 8.0). The dialyzed enzyme was applied to an agarose-hexane-adenosine-5'-triphosphate affinity column (1.5 x 2 cm) equilibrated in buffer B. The column was washed with buffer B until the A_{280} of the effluent dropped to near zero, and then washed sequentially with 10 ml of 2 mM F6P in buffer B, 30 ml of buffer B, and 10 ml of 2 mM ADP in buffer B. PFK activity was then eluted with 10 ml of 2 mM ADP + 2 mM F6P in buffer B. Peak

fractions containing purified PFK were pooled, dialyzed against 1 L of buffer A for 1 h at 4°C, and then concentrated to 1 mL by dialysis against solid polyethylene glycol 8000. Purified PFK was used immediately or stored for up to 1 week in 30% v/v glycerol at -20°C.

Phosphofructokinase activity was routinely assayed at 25°C by monitoring change in absorbance at 340 nm due to NADH oxidation (Brooks and Storey, 1992). Optimal assay conditions for ground squirrel muscle PFK were 50 mM KH_2PO_4 , pH 8.0, 50 mM KCl, 5 mM MgCl_2 , 0.10 mM NADH, 0.5 mM ATP, 1.0 mM F6P, and 0.5 U aldolase, 0.5 U triosephosphate isomerase and 2 U α -glycerophosphate dehydrogenase. Ammonium sulfate was removed from the coupling enzymes by centrifugation through a small (5 ml) column of Sephadex G-25 equilibrated in 20 mM imidazole-HCl, pH 7.0, 5 mM MgCl_2 and 10 mM 2-mercaptoethanol before use (Helmerhost and Stokes, 1980). PFK was assayed using a Dynatech MR5000 microplate reader with Biolynx data capture software. Enzyme rates were analyzed with a Microplate Analysis Program (Brooks, 1994). All reactions were initiated with the addition of PFK.

Protein concentration was determined by the Coomassie blue G-250 dye binding method using the Bio-Rad prepared reagent and BSA as the standard (Bradford, 1976).

SDS-Polyacrylamide gel electrophoresis

Enzyme purity was judged using SDS-PAGE mini-gels (Laemmli, 1970); samples were run on an 8% w/v acrylamide lower gel and a 3.5% w/v stacking gel under at constant 200V. The running buffer was 24.8 mM Tris, 192 mM glycine, pH 8.8 containing 1% w/v SDS. The molecular weight (Da) of the standards were: rabbit

muscle myosin (210,000), *E. coli* β -galactosidase (116,000), glycogen phosphorylase *b* (97,400), BSA (66,000), ovalbumin (45,000), and bovine erythrocyte carbonic anhydrase (29,000). The gel was fixed overnight in 10% v/v acetic acid and 25% v/v methanol, stained for 1 h in 0.25% w/v Coomassie brilliant blue R, 50% v/v methanol and 7.5% v/v acetic acid, and then destained overnight in 10% v/v acetic acid and 25% v/v methanol.

Phosphofructokinase inactivation experiments

Inactivation experiments were conducted essentially as described by Bock and Frieden (1976a). The pH of the three stock incubation mixtures was fixed at 6.5, 7.2, or 7.5 at 23°C using potassium phosphate buffer. Additives, polyethylene glycol 8000 (PEG) or urea, were mixed with the stock incubation buffers, and the pH of the solutions was rechecked. The enzyme was diluted from a concentrated stock solution at pH 8.0 into the incubation mixture pre-equilibrated to the desired pH and temperature. After 1 h incubation or at timed intervals, aliquots were removed and diluted to 5 μ g/ml in 50 mM potassium phosphate buffer, pH 8.0 (containing 1 mM EDTA, 0.2 mM dithiothreitol) at 2°C. An aliquot of this enzyme solution was then further diluted 1:100 v/v into the pH 8.0 standard assay mixture and activity was determined. To avoid complications arising from the slow adsorption of the enzyme to glass (leading to an apparently greater enzyme inactivation), small volumes of incubation medium and PFK concentrations below 20 μ g/ml were avoided. A standard PFK concentration of 20 μ g/ml and an incubation volume of 2.0 ml were employed. The reference activity to which sample activities were compared was the activity of the enzyme incubated at pH 8.0 in phosphate buffer, diluted and assayed at pH 8.0 as described. The degree of inactivation is expressed as the fraction

of the observed activity compared with the reference activity under the same conditions at pH 8.0.

Kinetic analysis of phosphofructokinase at hibernating and euthermic temperatures

Purified PFK was assayed by the coupled enzyme procedure (Brooks and Storey, 1992). Enzyme rates were analyzed with the Microplate Analysis Program, and kinetic parameters were determined using a computer program (Brooks, 1992). Assay temperature was manipulated by using the microplate thermal controller (for 37°C assays) or by placing the entire microplate reader into a low temperature incubator for 5°C studies; in this case, thermistors placed in selected wells and attached to a YSI Model 42 SL telethermometer were used to confirm assay temperature. The effect of pH on *S. lateralis* PFK activity at euthermic, 37°C, and hibernating, 5°C, body temperatures was determined using imidazole-HCl buffer. The pH of the assay mixture was adjusted to set values at 23°C which were calculated to result in pH values of 7.5 at 5°C or 7.2 at 37°C based on a pH change of -0.017 pH units per 1°C increase for imidazole buffer (Somero, 1981). All reactions were initiated with the addition of purified PFK. Assay conditions were 20 mM imidazole-HCl buffer (pH 7.5 at 5°C and 7.2 at 37°C), 5 mM MgCl₂, 50 mM KCl, 0.2 mM F6P, 0.5 mM Mg.ATP, 10 mM β-mercaptoethanol, 0.15 mM NADH, and 1 U each of aldolase, triosephosphate isomerase and glycerol-3-phosphate dehydrogenase. Ammonium sulphate was removed from the coupling enzymes by centrifugation through a small (5 ml) column of Sephadex G-25 equilibrated in 20 mM imidazole-HCl, 5 mM MgCl₂, 10 mM 2-mercaptoethanol, pH 7.5 or 7.2, for assays at 5°C or 37°C respectively (Helmerhost and Stokes, 1980). Imidazole buffer pH values

were all adjusted at 23°C to values that would produce pH values of 7.5 or 7.2 at 5 or 37°C, respectively; this was calculated assuming a +0.017 unit increase in pH per 1°C decrease for imidazole buffer (Somero, 1981). Due to the sensitivity of PFK to minor pH variation, the pH of the inorganic phosphate solutions were also adjusted to be pH 7.5 at 5°C or pH 7.2 at 37°C.

Data and statistics

Data are presented as means $\pm$ S.E.M.. Statistical testing use the Student's t-test or one-way analysis of variance followed by the Student-Newman-Keuls test.

RESULTS

Enzyme purification

Table 2.1 shows the results of a typical purification of PFK from euthermic ground squirrel skeletal muscle. The enzyme was purified approximately 600-fold with a yield of 14 %. A single, sharp peak eluted from the ATP-Agarose affinity column. The preparation was judged to be homogeneous as demonstrated by the presence of a single 76 kDa protein band on SDS-PAGE (Figure 2.1) and showed a final specific activity of 110-115 units/mg protein when assayed at 23°C under optimal conditions at pH 8. The specific activity of the purified PFK preparation was comparable to values for mammalian muscle PFK from other sources: *Citellus beecheyi*, 130-135 units/ mg protein (Hand and Somero, 1983) and rabbit 180 units/ mg protein (Ling et al., 1966).

Stabilization of phosphofructokinase catalytic activity by molecular crowding agents

Cold lability of mammalian muscle PFK, from both rabbit and ground squirrel has been reported by other authors (Hand and Somero, 1983; Bock and Frieden, 1976a; 1976b). Cold lability, arising from the ionization of histidine residues that are responsible for the pH dependent loss of activity, has been used as the basis for the contention that acidification during entry into hibernation could be a critical factor in retarding energy metabolism. We felt that this issue was worthy of re-examination under conditions that approximate *in vivo* cellular conditions. Experiments were undertaken to reassess the pH-induced cold lability of PFK. Under *in vitro* incubation conditions of low pH and cold (pH 6.5, 5°C) in low ionic strength buffer, Hand and Somero (1983) reported that purified *C. beecheyi* PFK lost activity over time, and under our incubation conditions, the same phenomenon was reproduced. However, Figure 2.2 also shows that both the rate and the absolute amount of PFK activity lost during pH 6.5 incubation at 5°C could be substantially reduced by the addition of the molecular crowding agent, PEG. In the absence of PEG, 78 ± 3 % of the initial PFK activity was lost after 60 min incubation, the observed time course of inactivation approximating the results obtained for muscle PFK from both rabbit (Bock and Frieden, 1976a,21) and California ground squirrel, *C. beecheyi* (Hand and Somero, 1983). Incubation in the presence of 5% w/v PEG, however, significantly reduced enzyme activity loss and only 41 ± 3 % of activity was lost within the same period. Furthermore, in the presence of 10 % w/v PEG, PFK activity was highly stable with only 7 ± 1 % of total activity lost over the 1 h incubation. Thus, the addition of a protein crowding agent effectively stabilized the purified PFK.

Temperature and pH effects on PFK stability were further explored. Figure 2.3 shows the results of varying both pH and temperature on the activity of PFK remaining after 1 h incubation in the presence versus absence of 10 % w/v PEG. The results dramatically illustrate the effects that low pH and dilute solution have on PFK stability. At pH 6.5 PFK activity was strongly reduced when incubated without PEG and activity loss increased at higher incubation temperatures. However, addition of PEG at this pH greatly increased enzyme stability at all temperatures; for example, at 37°C only $5 \pm 0.7\%$ of PFK activity remained after 1 h incubation without PEG whereas $58 \pm 6\%$ of activity remained in the presence of 10% PEG. At pH 7.2 or 7.5, however, PFK showed high stability under all conditions with 80 % or more of activity remaining after the 1 h incubation in all cases. A tendency for greater activity loss at 5°C was seen along with the greatest affect of PEG in reversing low temperature effects on enzyme stability. However, statistical testing revealed no significant difference between any of the six experimental conditions at either pH 7.2 or pH 7.5.

Inactivation of phosphofructokinase by urea

The cold-temperature dependent loss of PFK catalytic ability has been suggested to be accentuated by the presence of low concentrations of urea (Hand and Somero, 1983; Lee and Lee, 1979). These results, coupled with conflicting data on urea accumulation during mammalian hibernation (Kristofferson, 1963; Riedesel and Steffen, 1980), led us to re-examine urea effects on PFK inactivation at 5°C under conditions of enzyme crowding with 10% w/v PEG. Consistent with previous reports, PFK stability at pH 6.5 and 5°C was compromised by the addition of urea (Figure 2.4). Some reduction

of PFK activity was indicated in the presence of 50 mM urea but the addition of 400 mM urea reduced PFK activity significantly to $13 \pm 3\%$ of the original activity, or less than half of the activity remaining in the absence of urea ($29 \pm 5\%$). However, the addition of 10 % PEG reversed all of the negative effects by cold, low pH and urea; in the presence of PEG, there were no significant differences in the amount of PFK activity remaining after incubation with 0, 50 or 400 mM urea and PFK activity remaining ranged from 87-92 % of initial activity. There were no significant differences between the 3 urea groups in the presence of PEG, so actually PEG did a proportionally better job of overriding inactivating conditions at high urea than at 0 urea – i.e. both the low pH and the urea effects were reversed. When PFK was incubated at pH 7.5, PEG appeared to improve the retention of PFK activity at 0 mM urea; however, the addition of PEG had no influence on PFK activity remaining when incubations were conducted in the presence of 50 or 400 mM urea.

Temperature effects on the pH dependence of phosphofructokinase

Figure 2.5 shows pH versus activity curves for PFK at 5 and 37°C with saturating ATP and F6P. At 37°C ground squirrel PFK showed a narrow pH optimum between pH 7.4 and 7.6 and enzyme activity declined sharply at both higher and lower pH values. When assayed at 5°C, however, the optimum pH shifted to a higher value, pH 7.7-8.1, and the curve became somewhat broader.

Kinetic properties of PFK at hibernating and euthermic temperatures

An analysis of selected kinetic properties of PFK purified from *S. lateralis* muscle is presented in Table 2.2. Enzyme affinity for substrates F6P ($S_{0.5}$) and ATP (K_m), K_a values for allosteric activators (ADP, AMP, F2,6P₂, and NH₄⁺), and I_{50} for inhibitors (ATP, citrate, PEP, and urea) were compared at assay temperatures that mimic the hibernating (5°C) versus euthermic (37°C) body temperatures of the animal. The pH of the assay mixture was fixed using imidazole-HCl buffer and allowed to vary with temperature so that the pH at 5°C was 7.5 and the pH at 37°C was 7.2. The pH of the imidazole buffer used at 37°C was set independently of the buffer used at 5°C. The buffer used at 5°C was set to pH 7.2 (23°C) to give a pH value at 5° of 7.5 ($+18 \times 0.017 = 0.306 + 7.20 = 7.5$); the buffer used at 37°C was fixed at pH 7.44 (23°) to give a pH at 37°C of 7.2 ($-14 \times 0.017 = -0.238 + 7.44 = 7.2$). The enzyme showed reduced sensitivity to all activators at 5°C, the K_a values for AMP, ADP, NH₄⁺, and F2,6P₂ increasing by 5.3-, 3.4-, 2.6- and 11.3-fold, respectively, compared with 37°C values. By contrast, inhibitor constants were differentially affected by temperature change. Inhibition by Mg.citrate was not affected by temperature change whereas the I_{50} value for Mg.ATP increased by 70% at 5°C. However, sensitivity to inhibition by PEP and urea increased at low temperature, the I_{50} values dropping to 53 and 60 %, respectively, of the corresponding values at 37°C.

Figure 2.6 shows the effect of increasing the concentration of inorganic phosphate on the activity of PFK at 5°C. At pH 7.0 phosphate acted as a potent activator of PFK and raised maximal activity 5.1-fold. The calculated K_a for phosphate was 1.7 ± 0.1 mM, $n = 3$. However, increasing the pH slightly to 7.3 dramatically changed the effect of phosphate on enzyme activity; phosphate was an activator only at low concentrations

with a maximal 1.7-fold activation at 2 mM phosphate. At concentrations higher than 10 mM phosphate became inhibitory. A further increase in pH to 7.5 removed all activating characteristics of phosphate and the compound inhibited PFK activity with an I_{50} value of 8.5 ± 0.2 mM. An additional increase to pH 8.0 resulted in even stronger inhibition of the enzyme with a decrease in the I_{50} to 4.0 ± 0.4 mM.

PFK exhibited significantly different substrate saturation kinetics at the two temperatures (Tables 2.3 and 2.4). The enzyme showed sigmoidal F6P kinetics at both temperatures, with the n_H being significantly greater than 1.0 in both instances. The $S_{0.5}$ for F6P decreased by 39 % at 5°C whereas the K_m Mg.ATP was reduced by 85 % at the lower temperature, thus, affinity for both substrates increased at low temperature; compared with 37°C. PFK exhibits sigmoidal F6P kinetics at lower pH values but converts to hyperbolic kinetics with the addition of allosteric activators (F-1,6-P₂, F-2,6-P₂, AMP, inorganic phosphate, and NH_4^+) or a rise in pH values near 8 (Ramaiah, 1974; Lowry and Passonneau, 1966; Turnheim and Lowenstein, 1976). As predicted then, at 37°C, the addition of 10 mM phosphate to *S. lateralis* PFK increased the maximal velocity by 1.5-fold and shifted the velocity versus [F6P] curve to the left (from sigmoidal to hyperbolic). The $S_{0.5}$ shifted from 0.12 ± 0.004 mM F6P to 0.088 ± 0.007 mM (n=4). A change in the sigmoidal nature of the curve was also observed; the Hill coefficient was 2.03 ± 0.21 in the absence of phosphate and 1.21 ± 0.01 (n=4) in the presence of phosphate (Table 2.3).

The action of inorganic phosphate on F6P kinetics at 5°C is shown in Figure 2.7 and kinetic parameters are summarized in Table 2.3. The F6P saturation curve was strongly shifted to the left with the addition of as little as 5 mM inorganic phosphate

(Figure 2.7) and $S_{0.5}$ was reduced by 65 %. The addition of phosphate also changed the V vs $[S]$ relationship from sigmoidal to hyperbolic, the Hill coefficient dropping by one-half in the presence of 5 mM phosphate. However, phosphate caused a strong reduction in enzyme maximal velocity; for example, V_{max} was reduced by 64% with the addition of 20 mM phosphate. The $S_{0.5}$ increased, as has been reported with temperature studies on muscle PFK from the Alaskan King Crab (Freed, 1971), from 0.071 ± 0.009 mM at 5°C to 0.121 ± 0.004 at 37°C .

For comparison, Table 2.6 also shows the effects of 10 mM phosphate on F6P kinetics at 37°C and pH 7.2. Phosphate had the same pattern of effect on F6P affinity and Hill coefficient although the proportional change in $S_{0.5}$ was not as great; $S_{0.5}$ decreased by 27 % in the presence of 10 mM phosphate and Hill coefficient again dropped by nearly 50% to 1.2. The effect of phosphate on V_{max} was the opposite of that seen at low temperature. At 37°C , the addition of 10 mM phosphate increased V_{max} by 46 %.

S. lateralis PFK showed hyperbolic substrate saturation kinetics with respect to Mg.ATP concentration at both 5 and 37°C . Table 2.4 shows that although phosphate did not affect the K_m of Mg.ATP it appeared to alleviate ATP inhibition at 5°C . The I_{50} for Mg.ATP increased by 2-fold in the presence of 5 mM phosphate and by 5-fold with 20 mM phosphate. However, as noted previously, the maximum velocity was again reduced at 5°C in the presence of phosphate by 49 and 79%, respectively at 5 and 20 mM phosphate (Table 2.4). Figure 2.8 shows a Hanes-Wolf plot of phosphate inhibition. Phosphate inhibition was found to be non-competitive with respect to Mg.ATP and the K_i was determined to be 0.15 ± 0.01 mM ($n=4$).

The effects of temperature and phosphate on PFK activity were also investigated under optimal (0.5 mM) and inhibitory (5.0 mM) Mg.ATP concentrations. Arrhenius plots were constructed from $V_{\max}$ assays conducted in buffer only (50 mM imidazole) and in buffer plus phosphate (50 mM imidazole plus 10 mM phosphate). When assayed in imidazole under optimal ATP concentrations (Figure 2.9a), the relationship was linear to approximately 22°C and the E_a was 54 ± 1 kJ/mol ($n=4$). When PFK was assayed in 10 mM phosphate under optimal Mg.ATP conditions there was a break in the Arrhenius plot at 10°C; the E_a was 122 ± 6 kJ/mol ($n=4$) for the lower portion of the plot, that is 2-10°C, and was 44 ± 0.2 kJ/mol from 10-45°C. Under inhibitory Mg.ATP concentrations (Figure 2.9b), phosphate inhibition of PFK activity occurred at temperatures less than 24°C with the maximal inhibition at the lowest temperature examined. Activation of enzyme activity above the level observed in imidazole occurred at temperatures greater than 24°C. This plot also exhibited a break at 10°C; the E_a for the lower portion of the plot was 102.8 ± 1.7 kJ/mol ($n=4$) and the E_a for the top portion was 61.9 ± 0.9 kJ/mol. Temperature exhibited little effect on PFK activity under inhibitory Mg.ATP concentrations when assayed in the absence of phosphate; a slight decline in activity was observed with increased temperature. Figure 2.10 represents a plot of $\log W$ vs $1/T$, where W is the ratio of maximal velocity with 10 mM phosphate to maximal velocity in the absence of phosphate (Braxton et al., 1994). Under optimal Mg.ATP levels, the data was characterized by a roughly linear relationship with a slightly negative slope (-0.8) and a sharp break at approximately 9°C so that W was zero at $6.6 \pm 0.04^\circ\text{C}$ ($n=4$). However, when the enzyme was assayed in the presence of 5 mM Mg.ATP, the relationship had a sharp negative slope (-5.3) that crossed through zero at $28.1 \pm 0.1^\circ\text{C}$, ($n=4$).

Table 2.1. Purification of PFK from euthermic ground squirrel skeletal muscle. Enzyme was isolated from 2.13 g skeletal muscle.

Stage	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Crude extract	184.4	35.4	0.2	100	1
Sephadex DE-52	5.5	29.0	5.2	82	28
ATP-Agarose	0.05	5.3	113	15	594
Concentrated enzyme	0.05	5.0	110	14	581

Table 2.2. Effects of temperature on the kinetic properties of skeletal muscle PFK from the euthermic golden mantled ground squirrel, *Spermophilus lateralis*.

	37°C	5°C
K _a AMP, μ M	35 $\pm$ 2	184 $\pm$ 6 ^a
K _a ADP, μ M	36 $\pm$ 1	122 $\pm$ 6 ^a
K _a Fructose-2,6-bisphosphate, nM	40 $\pm$ 3	449 $\pm$ 15 ^a
K _a NH ₄ ⁺ , mM	1.4 $\pm$ 0.1	3.6 $\pm$ 0.1 ^a
I ₅₀ Phosphoenolpyruvate, mM	1.2 $\pm$ 0.1	0.6 $\pm$ 0.04 ^a
I ₅₀ Urea, mM	314 $\pm$ 30	189 $\pm$ 13 ^b
I ₅₀ Mg.Citrate, μ M	52 $\pm$ 5	48 $\pm$ 2

NOTE: Values are means $\pm$ SEM for n=4 separate preparations of purified enzyme. F6P concentrations were subsaturating (0.1 mM) for K_a and I₅₀ determinations. For I₅₀ determinations, inhibitor stock solutions were prepared with added magnesium, 1:1 molar ratio for Mg.ATP and 2:1 for Mg:citrate. The pH of the assay mixtures were fixed at 23°C using imidazole-HCl buffer and allowed to vary with temperature so that the pH at 5°C was 7.5 and the pH at 37°C was 7.2. ^a -Significantly different from the corresponding value at 37°C, P < 0.005; ^b - P < 0.025 (Student's t-test, two-tailed).

Table 2.3. The effect of inorganic phosphate concentration on fructose 6-phosphate kinetics of *S. lateralis* muscle PFK at two assay temperatures

Temperature	Phosphate (mM)	S _{0.5} F6P (mM)	Hill Coefficient (n _H)	Maximal Velocity (mU)
5°C	0	0.071 ± 0.009	2.30 ± 0.43	9.4 ± 0.3
	5	0.025 ± 0.002 ^a	1.23 ± 0.13 ^b	6.7 ± 0.2 ^a
	15	0.032 ± 0.004 ^a	1.12 ± 0.11 ^b	3.7 ± 0.1 ^a
	20	0.022 ± 0.002 ^a	0.82 ± 0.15 ^b	3.4 ± 0.1 ^a
37°C	0	0.121 ± 0.004	2.03 ± 0.21	6.21 ± 0.2
	10	0.088 ± 0.007 ^b	1.21 ± 0.06 ^b	9.09 ± 0.4 ^a

Maximal velocity values are milliunits of enzyme activity obtained for 0.2 µg (at 5°C) or 0.03 µg (at 37°C) of purified *S. lateralis* skeletal muscle phosphofructokinase. The pH of the assay mixtures were fixed at 23°C using 50 mM imidazole-HCl buffer and allowed to vary with temperature so that the pH at 5°C was 7.5 and the pH at 37°C was 7.2. The concentration of Mg.ATP was held at 0.5 mM. All other assay conditions are detailed in the Materials and Methods. Data are means ± SEM for n=4 separate preparations of purified enzyme. ^a - Significantly different from the corresponding 0 mM phosphate value using the Student's t-test (2-tailed) or one-way analysis of variance followed by the Student-Newman-Keuls test (2-tailed), P < 0.005, ^b - P < 0.05.

Table 2.4. The effect of inorganic phosphate concentration on Mg.ATP kinetics of *S. lateralis* muscle PFK at two different temperatures.

Temperature	Phosphate (mM)	K _m Mg.ATP (μM)	I ₅₀ Mg.ATP (mM)	Maximal Velocity (mU)
5°C	0	19.6 ± 0.37	2.13 ± 0.22	9.25 ± 0.37
	5	17.7 ± 0.23 ^a	4.70 ± 0.50 ^a	4.72 ± 0.13 ^a
	10	18.9 ± 0.37	10.72 ± 0.64 ^{a,b}	1.92 ± 0.31 ^{a,b}
37°C	0	127.0 ± 6.6	1.29 ± 0.19	7.23 ± 0.51
	10	53.7 ± 3.2 ^a	2.79 ± 0.09 ^a	7.04 ± 0.18

Maximal velocity values are milliunits of enzyme activity obtained for 0.2 μg (at 5°C) or 0.03 μg (at 37°C) of purified *S. lateralis* skeletal muscle phosphofructokinase. The pH of the assay mixture was fixed at 23°C using imidazole-HCl buffer and allowed to vary with temperature so that the pH at 5°C was 7.5 and the pH at 37°C was 7.2. The concentration of F6P was held at 0.5 mM. All other assay conditions are detailed in the Materials and Methods. Data are means ± SEM for n=4 separate preparations of purified enzyme. ^a - Significantly different from the corresponding 0 mM phosphate value using one-way analysis of variance with the Student-Newman-Keuls test (2-tailed), P < 0.01; ^b - significantly different from the value with 5 mM phosphate.

Figure 2.1. Coomassie Blue stained SDS-PAGE gel of purified PFK from *S. lateralis* skeletal muscle. Lane A, crude muscle homogenate, Lane B, DE-52 salt eluate; Lanes C1-C3, peak PFK activity fractions from ATP-agarose affinity chromatography; Lane D, MW standards. The MW of standards used are: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase $\bar{b}$ (97 kDa), serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa).

Figure 2.2. Effects of PEG in counteracting the pH and temperature induced inactivation of *S. lateralis* skeletal muscle PFK (20 µg/ml) incubated at 5°C in 50 mM potassium phosphate buffer, pH 6.5 containing 1 mM EDTA, 10 mM dithiothreitol. Data are means $\pm$ SEM for n = 3 separate preparations of purified enzyme. PEG additions are: none (●), 5 % w/v (▲), 10 % w/v (■).

Figure 2.3. The effect of PEG on the pH and cold induced inactivation of *S. lateralis* skeletal muscle PFK (20 µg/ml) incubated for 1 h at pH 6.5, pH 7.2 or pH 7.5 in 50 mM potassium phosphate buffer, 1 mM EDTA, 10 mM dithiothreitol at 5, 25 or 37°C. Bars are: no PEG (solid); plus 10% w/v PEG (open). Data are means ± SEM for n=3 separate preparations of purified enzyme. a - Significantly different from the corresponding value at the same temperature in the absence of PEG using one-way analysis of variance followed by the Student-Newman-Keuls test, two-tailed, $P < 0.005$; b- significantly different from the corresponding values at lower temperatures, $P < 0.05$.

Figure 2.4. The effect PEG on the pH-induced inactivation of *S. lateralis* skeletal muscle PFK (20 µg/ml) incubated for 1 h at pH 6.5 and pH 7.5 (in 50 mM potassium phosphate buffer, 1 mM EDTA, 10 mM dithiothreitol at 5°C) and the influence of added urea (0, 50 or 400 mM) on the process. Bars are no PEG (solid); plus 10% w/v PEG (open). Data are means ± SEM for n=3 separate preparations of purified enzyme. a - Significantly different from the corresponding value at the same urea concentration in the absence of PEG using one-way analysis of variance followed by the Student-Newman-Keuls test, two-tailed, $P < 0.005$; b- significantly different from the corresponding values at lower urea concentrations, $P < 0.05$.

Figure 2.5. The effect of pH on *S. lateralis* PFK activity at euthermic (37°C; ●) and hibernating (5°C; ■) body temperatures. Enzyme activity was assayed in 30 mM imidazole-HCl, 5 mM MgCl₂, 50 mM KCl and 0.1 mM NADH at saturating substrate concentrations (1.0 mM F6P and 0.5 mM ATP). Data are means $\pm$ SEM of n=4 separate preparations of purified euthermic enzyme. The pH values of assay mixtures were measured at room temperature (23 $\pm$ 0.1°C) and the pH at the experimental temperatures was then calculated assuming an increase in pH of 0.017 pH unit per 1°C decrease in temperature (Somero, 1981).

Figure 2.6. Interacting effects of pH and inorganic phosphate concentration on the activity of *S. lateralis* phosphofructokinase at 5°C. Activities are expressed relative to the PFK activity at each pH value in the absence of phosphate. Symbols are: pH 7.0 (■); pH 7.3 (●); pH 7.6 (Δ); and pH 8.0 (▲). Data shown is the result of one trial but are representative of n=3 determinations from separate preparations of purified euthermic enzyme.

Figure 2.7. The effect of inorganic phosphate on F6P kinetics of *S. lateralis* PFK at 5°C and pH 7.5. PFK activity was measured as described in the Materials and Methods with phosphate at: 0 mM (●), 5 mM (■), 15 mM (▲), or 20 mM (◆). Data shown are the result of one trial but are representative of n=4 determinations from separate preparations of euthermic enzyme.

Figure 2.8. Hanes-Wolff plot showing the effect of phosphate on the reaction velocity of *S. lateralis* skeletal muscle PFK at varying Mg.ATP concentrations at 5°C. PFK activity was measured as in the Materials and Methods with additions of phosphate at: 0 mM (●), 5 mM (■), or 10 mM (▲). Inset: Secondary plot of PFK maximal velocity versus phosphate concentration at 5°C. Data are means $\pm$ SEM for n=4 separate preparations of euthermic purified enzyme.

Figure 2.9. Arrhenius plots of ground squirrel skeletal muscle PFK activity versus temperature. **A.** PFK measured under optimal substrate conditions: 1.0 mM F6P, 0.5 mM Mg.ATP, 5 mM MgCl₂, 50 mM KCl, and 0.15 mM NADH with buffer (●) 50 mM imidazole, pH 7.5 at 5°C or (○) 50 mM imidazole + 10 mM K₂HPO₄/KH₂PO₄, pH 7.5 at 5°C. **B.** PFK measured under conditions of inhibitory Mg.ATP: 5.0 mM F6P, 5.0 mM Mg.ATP, 5 mM MgCl₂, 50 mM KCl, and 0.15 mM NADH with buffer (●) 50 mM imidazole, pH 7.5 at 5°C or (○) 50 mM imidazole + 10 mM K₂HPO₄/ KH₂PO₄, pH 7.5 at 5°C. Data are means ± SEM for n=4 separate preparations of euthermic purified enzyme.

Figure 2.10. Plot of $\log W$ versus $1/T$ summarizing the effect of 10 mM phosphate on $V_{\max}$ for *S. lateralis* PFK under optimal ($\square$) and inhibitory ($\blacksquare$) Mg.ATP levels. W is the ratio of maximal velocity with 10 mM phosphate to maximal velocity in the absence of phosphate. Data are means $\pm$ SEM for $n=4$ separate preparations of euthermic purified enzyme.

DISCUSSION

A reduction in glycolytic flux is known to occur during hibernation with the putative locus of major control proposed to be PFK. Considerable work has been completed in an effort to determine the mechanism by which regulation of PFK occurs in mammalian hibernation. PFK kinetics constants in *S. lateralis* skeletal muscle showed no differences when euthermic and hibernating animals were compared (Brooks and Storey, 1992). These results eliminated covalent modification as a direct suppressor of enzyme activity. Another approach has focused on temperature and pH dependence in enzyme activity and subunit assembly (Hand and Somero, 1982; 1983; Bock and Frieden, 1976a; 1976b; Somero et al., 1991). The effects of lowered pH and temperature on PFK have been proposed to contribute at least partially to the change in glycolytic contribution to energy metabolism in mammalian hibernation (Hand and Somero, 1983; Malan et al., 1985). PFK is highly sensitive to pH change; assays of PFK isolated from *Citellus beecheyi* showed a 60% decrease in activity at 6°C and pH 6.5. The occurrence of a relative intracellular acidification during hibernation has been demonstrated (McArthur et al., 1990) in Columbian ground squirrel. That study reported a muscle pH of 7.24 at 37°C and 7.45 at 5°C based on ^{31}P NMR spectroscopy. The pH in muscle of hibernators was determined to be 0.2-0.4 units lower than predicted by a slope of -0.017 pH units/°C if the euthermic pH was the starting point. Based on these results, Malan et al. (1985) predicted that hibernators would experience about 50% inhibition of muscle PFK *in vivo* and a significant inhibition of muscle glycolytic flux during hibernation.

Two problems arise with these studies of temperature and pH regulation on PFK: a) extremely low protein concentrations were employed in the *in vitro* inactivation experiments and b) the ionic strength of the incubation media was (usually) much lower than that found *in vivo*. To our knowledge, no experiments have been undertaken to determine if these same inactivation qualities are observed under conditions approximating the *in vivo* tissue protein concentrations of 330 mg/ml (Fulton, 1982). These differences are difficult to resolve because of the inability to reproduce cytoplasmic conditions of high ionic strength and high protein concentration *in vitro*; however, we have circumvented this problem by manipulating the *in vitro* system through the addition of a molecular crowding agent, PEG.

The experiments of Figure 2.2 demonstrate the effects of protein crowding on the cold inactivation of PFK, inactivation apparently being due to enzyme conversion from an active tetramer into an inactive dimer. At a PEG concentration of 5% w/v, 60% of the initial PFK activity was recovered after a 60 min incubation, an increase from the control value of 20% recorded in the absence of PEG. The addition of 10% w/v PEG further reduced the inactivation so that 93% of the initially recorded activity was recovered. This latter response did not vary when conditions of pH or urea concentration were changed, and apparently was the result of a general stabilization of protein due to high PEG concentrations. PEG apparently acts by crowding proteins together, and by limiting H₂O availability mimics the high protein concentrations found within a cell. The decrease in PFK stability with increased temperature observed in Figure 2.3 is likely a result of more rapid denaturation of PFK in dilute solution at 37°C than at 5°C. PFK stability at

5°C in the presence of PEG was not affected by pH change. This illustrates that the instability associated with PFK at low pH was emphasized in dilute solution and suggests the hibernating enzyme is stable at 5°C under physiological protein crowding conditions regardless of the pH.

The pH vs activity plots performed at euthermic and hibernating temperatures (Figure 2.5), indicate the *S. lateralis* homologue of muscle PFK is effectively inactivated (when assayed in dilute solution) at pH values less than 7 at both temperatures; although, clearly the pH profile would be much different if stabilizers like PEG were present. The overall pH temperature coefficient determined for Columbian ground squirrel shoulder muscle (McArthur et al., 1990) was -0.009 pH units/ °C with a hibernating pH value of 7.45 (T_b 7-9°C) and a euthermic pH value of 7.24 (T_b 37°C). Since the pH of neutrality of water changes by an amount similar to that of the temperature dependency of the pK of imidazole, a relative acidification of the muscle in hibernation is produced. My results show that purified PFK behaved as most proteins do when exposed to changing temperature in imidazole buffer; the pH optima curve shifted to a more basic pH by 0.015 units /°C, an amount that was virtually equal to the 0.017 units/ °C found for imidazole. If pH approximated 7.24 in euthermic cells at 37°C, then enzyme activity was 85% of maximal. If the drop in temperature pushed cellular pH to about 7.45, then enzyme activity dropped to 70% of maximal according to Figure 2.5. This is in direct conflict with the perturbing effects of low pH and low temperature on PFK activity reported by other authors (Hand and Somero, 1983; Bock and Frieden, 1976a; Lee and Lee, 1979). These studies reported that the pH at which substantial inhibition of PFK activity

occurred shifted to higher values at lower temperatures. At hibernating temperatures, significant activity loss was reported below pH values of 7.5 and they argued that a hibernating muscle pH near 7.0 at 6°C would be sufficient to promote virtually total loss of PFK activity. When the apparent pK of imidazole is used, calculations for the adjustment of the hibernating pH gave a value of 7.73 at 8°C. PFK activity at this pH corresponds to 95% of the maximal activity obtained at 5°C. From my investigations it appears that the amount of enzyme inactivation due to pH effects is minor when conditions are used that approximate the intracellular crowding. The low temperature and low pH PFK lability observed in vitro was exacerbated by the effects of low protein concentration and lack of crowding.

As well, solute interaction has been proposed to act synergistically with the pH and temperature interactions detailed above. Significant inactivation of PFK has been suggested to occur at physiological levels of urea, and inactivation is proposed via unfolding of native macromolecules through increased solvent exposure of subunit interaction sites (Hand and Somero, 1982; 1983). However, the counteracting solute theory proposed by Somero and coworkers has not been shown to have major effects on the properties of PFK either from estivating (Grundy and Storey, 1994; Cowen and Storey, unpublished) or hibernating species (this study).

For these reasons, I suggest that some other combination of effects acts to modulate muscle PFK activity during hibernation in *S. lateralis*. Previous studies (Brooks and Storey, 1992) failed to find differences in PFK kinetic parameters between hibernating and euthermic ground squirrels that would indicate post-translational modification by reversible protein phosphorylation during transitions between the two

states. PFK regulation in the hibernating state may result from a temperature induced inversion of allosteric behaviour (Braxton et al., 1994), from changes in the amounts of PFK associated with myofibrils (Somero et al., 1991) and/or from temperature influences on the catalytic, activating and inhibitory sites (Freed, 1971). Since the effects of PEG on PFK tetramer-dimer equilibrium indicated that inactivation due to pH and temperature sensitivity was eliminated when the protein was removed from very dilute solution and preliminary studies in our lab indicated no differences in the amount of particulate-associated muscle PFK between hibernating or euthermic *S. lateralis*, we investigated mechanisms whereby temperature acts as a modulator of allosteric effectors of PFK during hibernation.

Allosteric activators and inhibitors are generally considered to induce or bind to distinctly different enzyme conformations and thereby convey altered functionality to a regulatory enzyme. Allosteric modifiers act either by changing the strength of the subsequent binding that the enzyme displays for its substrate or by changing the activation energy of the enzyme catalyzed reaction induced by the allosteric ligand (Braxton et al., 1994). The tissue-specific responses of *S. lateralis* during hibernation illustrated the importance of allosteric activators in regulating PFK activity in heart and leg muscle (Brooks and Storey, 1992). In the case of leg-muscle, the concentration of F2,6P₂ decreased from a level five times the K_a value in euthermic animals to one-half the K_a value in hibernating animals, suggesting that F2,6P₂ levels may regulate glycolytic rates by directly influencing PFK activity in these tissues.

Kinetic findings interpreted for mammalian muscle PFK have indicated the presence of not less than seven substrate, inhibitor and de-inhibitor sites (Kemp and Foe,

1983). ATP inhibition is overcome by small increases in ADP, AMP and inorganic phosphate, all of which must increase in the cell whenever ATP use exceeds ATP production. This effect is partially expressed in the low temperature F6P kinetics of hibernator PFK; increasing phosphate levels initially result in a hyperbolic shift in the F6P substrate curve but with an unusual decrease in V_{max} . However, further phosphate addition does not have an effect on substrate affinity and continues to lower V_{max} . A similar phenomenon was seen with respect to the allosteric influences of phosphate on PFK Mg.ATP kinetics. The drop in temperature and hence increased pH could account for the difference in F6P substrate affinity between the two temperatures (i.e. smaller at higher pH), and the effect of phosphate is pH dependent since, as Figure 2.6 shows, lowering the pH changes inhibition to activation. It would appear that phosphate inhibition is induced by low temperature since the addition of phosphate at 37°C showed strong activation at a pH that showed inhibition at 5°C.

Temperature-induced changes in PFK have been previously described for Mg.ADP allosteric effects seen for F6P kinetics for *Bacillus stearothermophilus* PFK (Braxton et al., 1994). However, this is the first reported occurrence of temperature induced changes in allosteric regulation for mammalian PFK. The temperature induced inversion of phosphate allosteric regulation was at its greatest when observed under inhibitory Mg.ATP concentration. As seen in Table 2.4, the binding of the allosteric ligand at low temperature does not modify the affinity of enzyme for substrate. The Hanes-Wolf plot (Figure 2.8) also demonstrates that the Mg.ATP K_m value was not affected by increasing phosphate levels at low temperature since the data set was typical of non-competitive inhibition. However, a significant decrease in V_{max} was induced by phosphate at low

temperatures indicating an effect on the catalytic efficiency (k_{cat}) of the enzyme.

Therefore, the low temperature effects on hibernator PFK are due to a change in the activation energy of the enzyme catalyzed reaction induced by the allosteric ligand.

Braxton et al. (1994) have previously defined the effect that an allosteric ligand has on V_{max} by expressing Arrhenius plots as a ratio of maximal velocities when the allosteric ligand is saturating and when the allosteric ligand is absent. The plot of W vs $1/T$ for *S. lateralis* PFK under optimal conditions is relatively horizontal and only crosses below 0 at temperatures below 7°C. This indicates that the allosteric effect of the ligand is consistent throughout the temperature range investigated and has little effect on V_{max} . However, under inhibitory concentrations of Mg.ATP, the plot of $\log W$ vs $1/T$ is a linear relationship with a negative slope such that $\log W$ is equal to zero at 29°C. A comparison of the two relationships in Figure 2.10 indicates that the inhibitory effect of the allosteric ligand is most potent at lower temperatures under inhibitory ATP levels.

Although a 0.3 unit pH difference exists between the 5 and 37°C assay environments, we do not feel that this alone explains the different kinetics observed. It is well known that as PFK is subjected to higher pH values, the $S_{0.5}$ for F6P decreases and the enzyme loses allosteric properties (Kemp and Foe, 1983); however, our results show that the sigmoidal character of the F6P kinetics is retained at 5°C at pH 7.5 and indicate that changes in kinetic parameters were most likely due to temperature alone.

Temperature effects on PFK kinetics indicate a higher substrate affinity at low temperature, especially for ATP (even when accounting for reduced ATP levels in hibernating muscle. Overall, sensitivity to adenylates (ATP inhibition, AMP and ADP activation) was reduced when the enzyme was assayed at hibernating temperatures, as

was sensitivity to F2,6P₂. PFK is generally thought of as being sensitive to 2 messages - 1) overall cellular energy status, via adenylate and NH₄⁺ levels, and 2) biosynthetic demands for carbohydrates, via F2,6P₂ which is the way that extracellular hormones can influence PFK (via phosphorylation/ dephosphorylation control of PFK-2). In a recent paper, Brooks and Storey (3) observed that the concentration of F2,6P₂ in hibernating golden mantled ground squirrel leg muscle decreased to 20% of the euthermic value. The 11-fold increase in F2,6P₂ K_a value at 5°C when coupled with a potential decrease in tissue levels would effectively eliminate PFK activation via this potent allosteric effector. The results indicate that the enzyme is also less likely to be activated by rising AMP and ADP in the torpid muscle (hence, muscle glycolysis is less sensitive to changing energetic state when torpid) and is clearly much less sensitive to biosynthetic signals which would help to preserve carbohydrate reserves during torpor. This enzyme is particularly well suited to the metabolic conditions of hibernation since a decreased response to adenylates and to F2,6P₂ and the increased citrate that accompanies a switch to fatty acid oxidation serve to depress PFK activity. These properties serve to link enzyme activity to allosteric activator concentrations such as F2,6P₂ and adenylates to permit carbohydrate sparing during hibernation.

One may surmise the potential effects of adenylate levels on the maintenance of a hibernation bout. Although ATP concentrations drop during hibernation in *S. lateralis* muscle (Chapter 3), the levels appear to remain inhibitory, at least in the short term, with respect to temperature induced phosphate inhibition of PFK. The data from the plot of W vs $1/T$ demonstrate that, when faced with inhibitory ATP levels and low temperature, PFK

activity is about 32-fold lower than at euthermic temperatures. It appears that ground squirrel PFK is adapted to function under two conditions: 1) under maximal flux generating conditions (low ATP, moderate phosphate, higher temperatures), and 2) under normal physiological conditions of hibernation (moderate ATP, high phosphate, lower temperatures). The relative amounts of phosphate and ATP interact with temperature to dictate the level of glycolytic flux through PFK.

PFK activity could increase by 15-fold with a drop in ATP concentrations to levels approaching 1 mM. This is apparent from the data of Figure 2.10 where a switch from optimal to inhibitory ATP levels significantly decreased PFK activity in the presence of phosphate at temperatures approximating those found during hibernation. The re-establishment of cellular ionic balance is currently suggested for the arousal from of a hibernation bout (Willis, 1982). The active transport of ions across cellular membranes is a large consumer of ATP. An increase in ATP consumption as a consequence of increased ion pumping would relieve ATP inhibition of PFK and thereby facilitate an increase in glycolytic flux through PFK during re-awakening.

The results of these experiments suggest that, under conditions that mimic the intracellular protein crowding, an acidification during hibernation does not act in a synergistic manner with falling body temperature to inhibit PFK activity. The data presented here also conflict with the temperature and pH induced tetramer-dimer regulation proposed by Hand and Somero (1983); in fact, phosphate inhibition of PFK at low temperature is actually counteracted when pH drops below 7.0 (Figure 2.6). The *in vitro* pH and temperature effects on PFK subunit assembly equilibria, and thereby, on

catalytic activity potential have been applied perhaps somewhat hastily by Somero and colleagues to account for *in vivo* modulation of glycolytic flux during hibernation. The thermal modulation of allosteric effects on PFK activity provides a better mechanism for the regulation of glycolytic flux.

CHAPTER 3

**Regulation of ground squirrel Na^+K^+ -ATPase activity
by reversible phosphorylation during hibernation.**

INTRODUCTION

The cold winter temperatures found in northern terrestrial latitudes are one of the most severe environmental stresses which animals must endure to ensure their survival. Many small mammals cope with the stress of a restricted food supply and/or extreme cold temperatures by using the physiological strategy of hibernation. These species have well developed biochemical and physiological mechanisms in place so that 1) metabolic fuel can be conserved throughout the winter, for example, by inhibiting glycolysis (Chapter 2), 2) shivering and non-shivering thermogenesis can be reduced in all cells as the animal enters torpor, and 3) ATP-producing and ATP-consuming pathways can be rebalanced and homeostasis can be re-established and maintained at a lower body temperature.

Recent work has shown that regulation of membrane ion channels and ion pumps and the transmembrane gradients of ions that they control, including Na^+ and K^+ gradients, is a key factor in achieving the energy conservation that results in metabolic rate depression (Flanigan et al, 1993; Buck and Hochachka, 1993; Staples and Hochachka, 1997). The sodium-potassium activated adenosine triphosphatase (Na^+K^+ -ATPase; EC 3.6.1.37) is a highly conserved plasma membrane enzyme essential for cellular homeostasis. The enzyme helps to maintain the low Na^+ and high K^+ concentrations of the intracellular milieu. This role is particularly important in excitable cells such as muscle and nerve. The Na^+K^+ -ATPase is also central in the control of cell volume and has a major function in the recovery of the membrane potential and ionic gradients. To carry out its various functions, the Na^+K^+ -ATPase ion pump utilizes cytosolic ATP. It is one of the single greatest users of cellular energy, particularly in

endotherms, and is responsible for 5-40% of steady state energy consumption depending on the cell type (Clausen, 1986).

Cellular homeostasis depends on the maintenance of adequate ATP levels and a near-continuous balance between ATP-generating and ATP-utilizing processes. Since overall net metabolic rate falls dramatically during hibernation (usually to < 5% of euthermic resting rate) and since ATP-generating mechanisms are known to be suppressed during hibernation (e.g. oxygen consumption is reduced, carbohydrate catabolism is suppressed), then it follows that ATP-utilizing processes must also be suppressed during hibernation. I hypothesized, therefore, that regulated suppression of the activity of the cellular Na^+K^+ -ATPase ion pumps would have to occur in order to achieve a stable state of torpor. In the present chapter, I describe experiments that quantified the maximal activities of the Na^+K^+ -ATPase and levels of adenylates in organs of the euthermic and hibernating golden-mantled ground squirrels and that assess the role of apparent reversible protein phosphorylation in the regulation of the enzyme during hibernation.

MATERIALS AND METHODS

Animals and Chemicals

Adult golden mantled ground squirrels, *Spermophilus lateralis*, were obtained in the Crooked Creek area of the White Mountains of California. The animals were treated as previously described (see Chapter 2). All biochemicals were obtained from Boehringer Mannheim, Montreal, PQ, or Sigma Chemical Co., St Louis, MO.

Muscle Tissue Adenylate Levels

Perchloric acid extracts were prepared from frozen tissue samples for the measurement of adenylate levels. For metabolite extraction, frozen tissue samples were ground to a fine powder under liquid nitrogen with a mortar and pestle. The powder was transferred to precooled 2 mL eppendorf tubes. Five volumes of ice-cold 6% w/v PCA containing 1 mM EDTA were then added and the sample was immediately homogenized on ice with 2 x 15s bursts of a Bio-Max homogenizer at full speed. Homogenates were centrifuged at 20,000x g for 20 min at 4°C. Protein free supernatants were removed and then neutralized on ice with a KOH solution (3 N KOH, 0.4 M KCl, 0.3 M Tris base). Precipitated salts were removed by centrifugation at 20,000 x g for 20 min at 4°C and the neutralized supernatant was stored at -80°C until analysis.

The adenylate contents and IMP levels of extracts were determined with the ion-exchange HPLC methodology of Sayos et al. (1994). The system consisted of a Beckman 110B solvent delivery system with a Bio-Rad C18 HL 90-5S column (250 x 4.6 mm) and a photodiode array detector set at 254 nm. Elution was by binary gradient using mobile phases of 30 mM KH₂PO₄ (pH 5.45), 7.5 mM tetrabutylammonium phosphate (TBAP) and 30 mM KH₂PO₄ (pH 7.0), 7.5 mM TBAP and 50% v/v acetonitrile.

Preparation of samples for Na⁺K⁺-ATPase assay

Tissue samples (0.2 g) from euthermic or hibernating *S. lateralis* were homogenized 1:5 w/v in homogenization buffer (25 mM imidazole, pH 7.4, 25 mM NaF, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 0.2% w/v deoxycholate, 1 mM dithiothreitol) containing 1 mM PMSF. The homogenate was allowed to sit on ice for 30 min prior to

centrifugation at 10,000g for 10 min at 5°C. Endogenous phosphate was removed from a 0.5 mL aliquot of supernatant by centrifugation through a 10 ml column of Sephadex G-25 equilibrated in homogenization buffer (Helmerhost and Stokes, 1980). Samples were stored on ice until assay.

Measurement of Na⁺K⁺-ATPase Activity

The assay method was based on that described by Crombie et al. (1996). Each extract was assayed in triplicate in a control buffer lacking K⁺ (120 mM NaCl; 5 mM MgCl₂, 25 mM imidazole-HCl; 1 mM ouabain, pH 7.4) to measure non-specific ATPase activity and in an experimental buffer (100 mM NaCl; 20 mM KCl; 5 mM MgCl₂, 25 mM imidazole-HCl; pH 7.4) to measure total ATPase activity, the difference between the two giving the activity due to Na⁺-K⁺-ATPase. A 12.5 µL aliquot of enzyme extract was assayed in a final volume of 0.25 mL, and the assay started by the addition of ATP to give a final concentration of 3 mM. The assay mixture was incubated at 25°C for 15 min. Initial experiments determined that at a temperature of 25°C, production of phosphate was linear with respect to time and the volume of homogenate assayed. Aliquots of the assay mixture were taken for inorganic phosphate analysis by the malachite green-molybdate dye binding method (Ekman and Jager, 1993). Absorbance at 660 nm was compared with the absorbances of standard solutions of sodium phosphate. One unit is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate per hour. Data were expressed as means ± S.E.M. and were analyzed using the Student's t-test (two-tailed).

Phosphorylation of muscle and kidney Na^+K^+ -ATPase

Kidney or skeletal muscle samples from either euthermic or hibernating animals were homogenized 1:2 (w/v) in 25 mM imidazole, pH 7.4, 25 mM NaF, 2 mM EDTA, 2 mM EGTA, 250 mM sucrose, 0.2% w/v deoxycholate, 1 mM dithiothreitol, 1 mM PMSF using a Polytron PT 10 homogenizer. Homogenates were left on ice for 30 min and then centrifuged at 10,000 x g for 10 min. The supernatant was desalted by centrifugation through a Sephadex G-25 column pre-equilibrated in 50 mM imidazole, pH 7.0, 25% (v/v) glycerol, 0.2% (w/v) deoxycholate, 10 mM β -mercaptoethanol and 0.1 mM EDTA (Incubation Buffer). A 40 μL aliquot of desalted homogenate was added to 80 μL Incubation Buffer, and the appropriate effectors were added to give a final total volume of 150 μL . This procedure relied on endogenous protein kinases and phosphatases to alter the Na^+K^+ -ATPase activity. All samples for phosphorylation time-course determinations contained 30 mM NaF to inhibit protein phosphatases and 10 mM Mg.ATP. Experimental samples also contained other effectors as noted in individual figure and table legends. Reactions were incubated for specified times at 25°C and then Na^+K^+ -ATPase activity was measured.

Dephosphorylation of inactivated muscle Na^+K^+ -ATPase

Dephosphorylation of muscle Na^+K^+ -ATPase was measured in desalted extracts from either euthermic or hibernating animals. In order to measure dephosphorylation in muscle homogenates, an enzyme sample was first experimentally phosphorylated by

incubating 40 μL desalted supernatant with 30 mM NaF, 15 mM MgCl_2 , 10 mM ATP, and 1 μM cAMP (total volume = 150 μL). The mixture was incubated for 2 h at 25°C to phosphorylate $\text{Na}^+\text{-K}^+\text{-ATPase}$, and then desalted through Sephadex G-25 columns equilibrated in incubation buffer to remove Mg^{2+} and ATP. Dephosphorylation was then initiated by adding 10 U of alkaline phosphatase. Samples were removed after a 2 h incubation period and assayed for $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity.

RESULTS

Muscle Adenylate Levels

Mean ATP levels in skeletal muscle were 4.1 ± 0.3 $\mu\text{mol/g}$ wet mass in euthermic *S. lateralis* and 30 % lower at 2.9 ± 0.3 $\mu\text{mol/g}$ wet mass when measured in hibernating animals (Table 3.1). ADP and AMP were similarly reduced in hibernating individuals so that, overall, total adenylates decreased significantly by 32 %. No change in energy charge, $[\text{ATP} + 0.5 \text{ADP}] / [\text{ATP} + \text{ADP} + \text{AMP}]$, was observed between euthermic and hibernating states indicating that a regulated reduction in all adenylates occurred. A common product of adenylate degradation, such as during burst muscle work, is IMP which is produced by the action of AMP deaminase. However, IMP levels were not altered in muscle of hibernating individuals.

$\text{Na}^+\text{K}^+\text{-ATPase}$ Activity in *S. lateralis* tissues

Maximal activities of $\text{Na}^+\text{K}^+\text{-ATPase}$ in membrane extracts from euthermic and hibernating *S. lateralis* are shown in Figure 3.1. Activity was highest in kidney with

euthermic values 5-fold or more higher than activities in liver, heart and skeletal muscle. Na^+K^+ -ATPase activities in skeletal muscle, kidney, and liver all decreased significantly during hibernation, falling by 60, 41, and 46% respectively, compared with euthermic values. Activity in heart was unaffected by hibernation.

Putative phosphorylation and dephosphorylation of muscle Na^+K^+ -ATPase

To determine whether post-translational modification via reversible phosphorylation could be responsible for the changes in muscle Na^+K^+ -ATPase activity observed during hibernation, extracts from skeletal muscle of euthermic and hibernating *S. lateralis* were incubated *in vitro* under conditions that would stimulate the activities of endogenous protein kinases or protein phosphatases. Activity of the enzyme from euthermic squirrels was not affected when incubated under conditions that promoted protein phosphatase activity (minus ATP and NaF, plus Ca^{2+} and Mg^{2+}) but incubations under conditions that promoted protein kinase activity strongly reduced activity (Figure 3.2). Conditions stimulating the activity of cAMP-dependent protein kinase (ATP + cAMP) reduced enzyme activity from euthermic animals by 85% whereas incubations that promoted cGMP-dependent protein kinase or protein kinase C, reduced Na^+K^+ -ATPase maximal activity by 72 and 65 %, respectively. By contrast, when the enzyme from skeletal muscle of hibernating individuals was assessed, incubation under conditions that promoted protein kinase action had no significant effect on enzyme activity but conditions that promoted phosphatase action raised maximal activity of the enzyme by 30%.

To further investigate the role of putative reversible phosphorylation in enzyme control, muscle extracts from euthermic versus hibernating squirrels were incubated *in vitro* for 2 h in the presence of Mg.ATP + cAMP (Figure 3.3). These conditions were chosen in order to maximize enzyme inactivation by phosphorylation, to facilitate comparisons by balancing protein content, and to determine if the recovery of enzyme activity by dephosphorylation could be increased by decreasing the incubation time at higher temperature. Na⁺K⁺-ATPase activity in extracts of euthermic muscle declined from 9.1 U/mg protein in incubations with no additions to 1.9 U/mg protein in incubations with Mg.ATP + cAMP, a value identical with that in muscle extracts from hibernators. However, when the phosphorylation-treated euthermic enzyme was subsequently incubated for 2 h with alkaline phosphatase, Na⁺K⁺-ATPase activity was restored, increasing 11.5-fold to 10.4 U/mg protein, a value not significantly different from the original activity. Different results occurred when membrane extracts from hibernating muscle was tested. Incubation with Mg.ATP + cAMP had no measurable effect on Na⁺K⁺-ATPase activity but the subsequent treatment with alkaline phosphatase raised enzyme activity by 3.5-fold.

Reversible phosphorylation often alters substrate affinity of an enzyme. To determine if this was the case with Na⁺K⁺-ATPase, the velocity versus [ATP] relationship was examined for the enzyme in membrane extracts from both euthermic and hibernating muscle (Figure 3.4). The ATP substrate dependence for Na⁺K⁺-ATPase is described by a curve with an intermediary plateau (Boldyrev and Kurella, 1996). The substrate relationships for the euthermic and hibernating enzymes had undefined intermediary

Figure 3.1. Maximal activities of Na^+K^+ -ATPase in four organs of euthermic and hibernating *S. lateralis*. Values are means $\pm$ S.E.M. for n=4 determinations on separate individuals. *-Significantly different from the corresponding euthermic value, $P<0.05$.

Figure 3.2. Effect of stimulating the activities of endogenous protein kinases on skeletal muscle Na^+K^+ -ATPase activity *in vitro*. Values are means $\pm$ S.E.M. (n=4). Samples were incubated at 30°C for 5 h before assay. Incubations contained 10 mM ATP, 30 mM NaF, and 15 mM MgCl_2 (control) with 0.3 mM cAMP (+cAMP), 0.3 mM cGMP (+ cGMP), or 1.3 mM CaCl_2 + 7 $\mu\text{g/mL}$ phorbol 12-myristate 13-acetate (+ Ca^{2+} /PMA). The final condition contained 15 mM MgCl_2 and 1.3 mM CaCl_2 in the absence of ATP and NaF (+ Mg^{2+} /-NaF). *- Significantly different from corresponding control (no addition) value using the Student's t-test, $P < 0.05$.

Figure 3.3. Phosphorylation and subsequent dephosphorylation of skeletal muscle Na^+K^+ -ATPase from euthermic and hibernating *S. lateralis*. Maximal activity was measured after 2 h of *in vitro* incubation with no additions (no fill), after 2 h of incubation in the presence of 10 mM ATP, 10 mM MgCl_2 , and 0.3 mM cAMP (hatched fill), and after a subsequent 2 h of incubation of the *in vitro* phosphorylated enzyme in the presence of 10 U alkaline phosphatase (black fill). Values are means $\pm$ S.E.M. for n=4 separate determinations. ^a - Significantly different from control incubations without added effectors, $P < 0.05$; ^b - significantly different from incubations in the presence of kinase effectors.

Figure 3.4. ATP concentration dependence of skeletal muscle Na^+K^+ -ATPase activity from euthermic (O) and hibernating (●) *S. lateralis*. Enzyme activity is expressed as $\mu\text{mole Pi released/h/mg protein}$.

Table 3.1. Adenylate and IMP levels in skeletal muscle from *Spermophilus lateralis* under hibernating and euthermic conditions.

[Metabolite] ($\mu\text{mol/g}$ wet mass)		
	Euthermic	Hibernating
ATP	4.1 ± 0.3	$2.9 \pm 0.3^*$
ADP	0.8 ± 0.1	$0.5 \pm 0.1^*$
AMP	0.2 ± 0.03	$0.1 \pm 0.03^*$
IMP	0.3 ± 0.1	0.2 ± 0.04
Total Adenylates	5.14 ± 0.4	$3.5 \pm 0.4^*$
Energy charge	0.87 ± 0.01	0.90 ± 0.01

Values are means $\pm$ SEM, n=5-8 individual samples. Energy charge is $[\text{ATP} + 0.5 \text{ ADP}] / [\text{ATP} + \text{ADP} + \text{AMP}]$. * Significantly different from the corresponding euthermic value using the Student's t-test (2-tailed), $P < 0.05$.

plateaus. The ATP substrate dependency for the hibernator enzyme had a reduced maximal velocity.

DISCUSSION

Among other remarkable properties, hibernating mammals display the ability to maintain appreciable cell function at low body temperatures. The ability to maintain the energy status of the cell during hibernation requires a suppression and coordination of both ATP-utilizing and ATP-consuming processes such that energy charge is maintained at a high and constant level (T. English, Ph.D. thesis, 1997). In *S. lateralis*, ATP levels are reduced in hibernation, but at the expense of the total adenylate pool rather than the energy charge. A similar conservation of energy charge but with a drop in total adenylates has been reported in other hibernators, meadow jumping mice, *Zapus hudsonius* (Storey and Kelly, 1995), and Chinese hamsters (El Hachimi et al., 1990). Comparable results were also found when adenylates were measured in skeletal muscle of Richardson's ground squirrels (*S. richardsonii*) (MacDonald and Storey, unpublished data) and white-tailed prairie dogs (*Cynomys leucurus*) (T. English, Ph.D. Thesis, 1997). For example, *S. richardsonii* ATP levels decreased in hibernation from 6.19 ± 0.20 to 4.91 ± 0.01 $\mu\text{mol/g}$ wet mass, and total adenylate levels were 14 % lower in hibernating muscle than in euthermic muscle, 7.30 ± 0.19 versus 6.31 ± 0.13 $\mu\text{mol/g}$ wet mass. Hibernation bout length also appears to have effect on the level of adenylate loss; hibernation bouts of 10 d for *S. richardsoni* (MacDonald and Storey, unpublished data), and 30 days for both of *C. leucurus* (T. English, Ph.D. Thesis, 1997), and *S. lateralis* (this

study) resulted in 13, 27, and 32% decreases in total adenylate levels with no change in the energy charge. The maintenance of the energy charge in skeletal muscle during hibernation in ground squirrels and other hibernators implies that adenylate metabolism is regulated during hibernation. The lack of IMP build-up implies a mechanism of adenylate depletion different from the one that often acts in skeletal muscle (Mommensen and Hochachka, 1988). During burst muscle work AMP deaminase can help to stabilize elevated energy charge by pulling AMP out of the pool as it builds up and converting it to IMP and ammonium ion. The formation of the latter is also a sink for protons that are accumulated during anaerobic glycolysis

Ion pumping (via Na^+K^+ -ATPase) is known to require a large fraction of the total cellular energy supply (Clausen, 1986). Several mechanisms of control exist for the regulation of Na^+K^+ -ATPase activity. The enzyme in skeletal muscle is under the control of a number of circulating hormones that impart both short- and long-term control over its activity (Clausen, 1986). The molecular mechanisms by which hormones alter Na^+K^+ -ATPase activity include: long-term regulation exerted by thyroid hormone and aldosterone, mediated by changes in gene expression, whereas short term regulation is exerted by catecholamines, mediated by reversible phosphorylation of the pump catalytic subunit (Ewart and Klip, 1995; Bertorello and Katz, 1995).

Kinase mediated phosphorylation and pump inhibition may occur through several mechanisms. Phosphorylation by PKC and PKA mediated events occurs on the catalytic α -subunit (Beguin et al, 1994; Bertorello et al, 1991). α -Subunit phosphorylation could cause a conformational change in subunit structure with resulting altered affinity for one

or more of its major substrates. Phosphorylation may also trigger the α -subunit to be internalized by endocytosis and eventually degraded (Ewart and Klip, 1995). Our results suggest that the depressed Na^+K^+ -ATPase activity seen during hibernation in *S. lateralis* tissues may be largely a function of altered substrate affinity characteristics. Hence, all of the protein kinase mediated suppression of enzyme activity in euthermic extracts was recovered after subsequent incubation of the enzyme with alkaline phosphatase (Fig. 3.3) and with extracts from hibernating animals, Na^+K^+ -ATPase was raised 3.5-fold after alkaline phosphatase treatment, reaching an activity that was about two-thirds of the value in the corresponding euthermic incubation. *In vitro* incubations under conditions that stimulated endogenous kinases and phosphatases also support this conclusion (Fig. 3.2). However, treatment with alkaline phosphatase or stimulators of endogenous phosphatases did not fully restore the maximal Na^+K^+ -ATPase activities seen in euthermic muscle so additional factors may also play a role such as a cold-acclimation, a hibernation-dependent decrease in total Na^+K^+ -ATPase (resulting from membrane vesicularization and/or proteolytic degradation, or from enzyme oxidation in hibernator tissues). I cannot rule out any of these possibilities as partial contributors to enzyme control since the two animal groups used in the present study were euthermic animals acclimated at 22°C versus hibernating animals that were sampled 30 days later while torpid at 3.5°C. However, given the key role of Na^+K^+ -ATPase in the heat generation associated with shivering thermogenesis, it is logical to assume that hibernating animals would retain a high potential Na^+K^+ -ATPase activity while torpid so that the enzyme could be rapidly activated during the arousal process. Hence, a rapidly reversible control

mechanism such as protein phosphorylation would be highly effective for masking or inhibiting enzyme activity during torpor without changing total enzyme potential.

The substrate dependence for euthermic ground squirrel Na^+K^+ -ATPase was described by a curve with an undefined intermediary plateau (Figure 3.4). The ATP substrate dependency for the hibernator had a reduced maximal velocity and also lacked the intermediary plateau that is characteristic of the regulating effect of ATP on Na^+K^+ -ATPase activity (Lopina et al, 1995; Glynn and Karlish, 1990). However, solubilization of membrane bound enzyme by non-ionic detergents converts a complex substrate-velocity dependence into a hyperbolic curve (Boldyrev et al., 1990). Recently, it has been demonstrated that oxidative damage modifies the affinity of the enzyme for ATP, and suppressed Na-dependent ATP hydrolysis and Rb^+ occlusion whereas Na-activated ATP/ADP exchange and enzyme phosphorylation were both almost unaffected (Huang, et al., 1994). The oxidized, modified enzyme is characterized by both decreased enzyme activity and transformed kinetic behavior, namely an increase in substrate concentration does not result in an additional increase in enzyme activity (Boldyrev and Kurella, 1996). Laboratory experiments with ground squirrels (*Spermophilus citellus*) revealed that autooxidation increased during torpor (Buzadzic et al., 1990), and when the polyunsaturated fatty acid content (PUFA) of the diet was increased, the rate of lipid peroxidation during hibernation was further increased (Frank and Storey, 1995). Hibernating animals may thus be particularly susceptible to enzyme damage by lipid peroxidation due to both torpor and the high PUFA content of their tissues.

Rigorous control of cellular ATP hydrolysis would be beneficial during hibernation when ATP consumption needs to be suppressed. Post-translational modification of

metabolism by phosphorylation (Storey, 1987) and allosteric control (Brooks and Storey, 1992; this thesis, Chapter 2) of key glycolytic enzymes have been demonstrated as mechanisms that control key catabolic, i.e. ATP-producing enzymes during mammalian hibernation. A reduction in Na^+/K^+ -pumping, a key ATP-consuming process, would not cause permanent cell damage but may temporarily compromise normal tissue function during the metabolic depression that characterizes mammalian hibernation. Excessive dissipation of ion gradients could cause cell swelling, loss of membrane potential and cell damage. There is evidence that in many tissues ion gradients are maintained, or at least are more slowly dissipated, in successful metabolic depression during hibernation (Ikomi-Kumm et al, 1994; Willis and Marjanovic, 1992; Zhao and Willis 1989). For example, erythrocytes of hibernating species, notably ground squirrels, maintain their Na^+ and K^+ gradients better at 5°C partly by decreases in the passive loss of K^+ and gain of Na^+ and partly by a relatively faster Na^+K^+ -pump activity at 5°C than found in cells of a mammal with typical cold sensitivity (e.g. guinea-pig or human) (Willis, 1986). The ability to maintain ion gradients at hibernating body temperatures and the suppression of Na^+K^+ -pump activity may protect tissues from the deleterious effects of ATP starvation. The key is the coordination of all factors to produce a net balanced metabolic suppression by targeting may different key points of metabolism. Interestingly, this can all apparently be achieved by reversible phosphorylation acting on a wide variety of different cellular targets.

CHAPTER 4

**Temperature effects on brown adipose cAMP-dependent protein kinase
kinetic properties and enzyme role in hibernating ground squirrels**

INTRODUCTION

Arousal from torpor is an explosive event during which substrate mobilization for energy production and non-shivering thermogenesis for heat production are at their maxima (Wang 1989). Non-shivering thermogenesis in brown adipose tissue (BAT) is the primary source of heat production for rewarming the animal during arousal (Nedergaard and Cannon 1990). Thermogenesis in BAT can be increased with the administration of thyroxine, norepinephrine (NE) or epinephrine (Wunder 1979). Although both adrenal epinephrine and neuronal NE seem equipotent in activating metabolic responses from brown fat cells *in vitro*, NE is now accepted as the primary physiological effector of BAT thermogenesis *in vivo* (Jansky 1973; Wunder 1979). Its effects are mediated via stimulation of β_3 -adrenergic receptors (Dicker et al. 1996) which, in turn, activate adenylate cyclase leading to the release of 3',5' cyclic adenosine monophosphate (cAMP) into the cytoplasm. This cAMP allosterically activates protein kinase A (PKA) causing catalytic and regulatory subunits of the enzyme to dissociate and freeing the catalytic subunits to phosphorylate a variety of target proteins (Taylor et al. 1990). One of the targets in BAT is hormone sensitive lipase which is activated by PKA-mediated phosphorylation and catalyzes the hydrolysis of stored triglycerides to provide free fatty acids as fuel for mitochondrial oxidation or as uncouplers of oxidative phosphorylation through the GDP-binding protein (Nicholls and Locke 1984). Numerous studies have established that lipid oxidation, uncoupled from oxidative phosphorylation, is the primary thermogenic pathway in BAT (Cannon and Nedergaard 1985; Klaus et al. 1991; Nicholls and Locke 1984; Nicholls et al. 1986). β -Adrenergic signal transduction is

also involved in the stimulation of gene expression that results in adipocyte proliferation and differentiation (Cannon et al., 1996).

Information regarding the role of protein kinase cascade systems in the hibernation process is still limited although some studies have investigated reversible phosphorylation control of glycolytic enzymes during hibernation (Storey 1987a,b; Brooks and Storey 1992a; El Hachimi et al. 1990). In the present study, kinetic and structural studies of the purified catalytic subunit of cAMP-dependent protein kinase (PKAc) from brown adipose tissue of the Richardson's ground squirrel, *Spermophilus richardsonii*, were undertaken at both 37 and 5°C to explore enzyme properties and the possible role of temperature in modulating enzyme function in hibernation. Kinetic studies included a comparison of the ground squirrel BAT enzyme with purified muscle PKAc from rabbit (a non-hibernating mammal) to determine if a specially adapted enzyme is present in the hibernator with kinetic properties optimized for low temperature function.

MATERIALS AND METHODS

Chemicals and animals

Richardson's ground squirrels, *S. richardsonii*, were captured in the foothills of the Rocky Mountains near Calgary, Alberta in September, 1995. All animals were individually housed in rat cages at the animal care facility of the University of Calgary, Calgary, Alberta and maintained at 22°C on a fall (10L: 14D) photoperiod. At the end of an 8 week feeding period, animals were induced to hibernate by placing their cages in a cold room at 4°C on a 8L:16D photoperiod. After a 24 h habituation period, all food (but

not water) was withheld. During the first week of fasting, the activity of the animals was monitored; those that did not hibernate were removed from the cold room at the end of the week. Those that were hibernating at the end of 7 days were maintained at 4°C for an additional 2 days so that the minimum time that animals were torpid before sampling was 2 days and the maximum time of torpor before sampling was 9 days. Control animals were kept at 22°C and were sacrificed by decapitation. Tissues from both euthermic and hibernating squirrels were immediately excised, immersed in liquid nitrogen, shipped to Carleton University on dry ice, and subsequently stored at -80°C until use.

[$\gamma^{32}\text{P}$] ATP (3000 Ci/mmol) was obtained from New England Nuclear (Montreal, PQ). Lavendustin A, KT-5823, Calphostin C and H-89 were from Biomol (Plymouth Meeting, PA). Protein kinase C inhibitor peptide (19-36; PKC-I) and PKA inhibitor peptide (5-24; PKA-I) were purchased from Peninsula Labs Inc. (Belmont, CA). The cAMP radioimmunoassay kit was purchased from Amersham Life Sciences Inc. (Oakville, ON). Phosphocellulose paper (P81) was obtained from Whatman (Canlab; VWR, Mississauga, Ont). Rabbit muscle "Peak 1" cAMP-dependent protein kinase was from Sigma Chemical Co. (St. Louis, MO). Before use in kinetic studies, the regulatory subunit was removed by passing the preparation through a column of Sephadex DE-52 previously equilibrated in PEM buffer (20 mM potassium phosphate, pH 6.8, 2 mM EDTA, and 10 mM 2-mercaptoethanol); the regulatory subunits bound to the column and the catalytic subunits were eluted with 0.1 mM cAMP in PEM buffer. All other chemicals and column materials were purchased from Sigma Chemical Co. or Boehringer Mannheim (Montreal, PQ).

In vitro phosphorylation incubations with endogenous PKAc

Studies were designed to determine the effect of temperature on phosphorylation events specific to PKAc in ground squirrel BAT. Frozen samples of interscapular BAT (0.3-0.5 g) were homogenized 1:4 (w/v) in ice-cold dephosphorylation buffer (20 mM potassium phosphate, 5 mM MgCl_2 , 0.1 mM EDTA, 2 mM 2-mercaptoethanol, pH 7.2) containing 1 $\mu\text{g/mL}$ aprotinin, 10 $\mu\text{g/mL}$ leupeptin and a few crystals of phenylmethylsulphonyl fluoride (PMSF). The homogenate was centrifuged at 20,000 g for 20 min at 4°C. The supernatant was removed and stored on ice for 5 h to allow dephosphorylation of endogenous phosphoproteins by protein phosphatases to occur. The samples were then desalted by centrifugation through a column of Sephadex G-25 (1 x 5 cm) pre-equilibrated in incubation buffer (40 mM imidazole-HCl, 10 mM potassium phosphate, 0.1 mM EDTA, 15 mM 2-mercaptoethanol and 20% v/v glycerol, pH 7.2) (Helmerhorst and Stokes 1980). Aliquots (100 μL) of desalted supernatant were then added to each of four incubation solutions to give a final volume of 200 μL . The four incubation solutions all contained 10 mM ATP, 60 $\mu\text{Ci/mL}$ ^{32}P ATP, 20 mM MgCl_2 and 30 mM NaF. Other additives were: solution I, 20 mM EDTA; solution II, incubation solution only with no additions; solution III, 10 μM cAMP; and solution IV, 10 μM cAMP plus 2.5 μM PKA-I inhibitor peptide. The final calculated ATP species concentrations in each incubation solution were determined by computer program (Brooks and Storey 1992b): Solution I, 5.2 mM free ATP, 0.03 mM free Mg^{2+} and 1.4 mM $\text{Mg} \cdot \text{ATP}$; Solution II-IV, 0.13 mM free ATP, 5.0 mM free Mg^{2+} and 9.7 mM

Mg.ATP. Incubation mixtures were vortexed and left overnight (16 h) at either 5°C or 37°C. After this time, protein bound ^{32}P was separated from free ^{32}P -ATP by passage through Sephadex G-25, as described above. An aliquot of the incubation sample was then removed, mixed 1:1 with SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and run on SDS-PAGE by the method of Laemmli (1970) with 12% w/v acrylamide separating gels and 5% w/v stacking gels. Protein standards were rabbit muscle myosin (205 kDa), *E. coli* β -galactosidase (116 kDa), rabbit muscle phosphorylase α (97 kDa), BSA (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa). Gels were dried using a Bio-Rad Model 583 gel dryer and exposed to X-ray film for 12 days at -80°C. Developed autoradiograms were converted into densitometry scans using a Hewlett Packard Scanjet 3c with Corel Photo-Paint v5.0 and Molecular Dynamics ImageQuant v3.22 software.

Determination of tissue cAMP levels

For the determination of cAMP levels, perchloric acid extracts of frozen ground squirrel tissues were prepared according to Storey and Storey (1984). In brief, frozen tissue samples were ground to a powder under liquid nitrogen using a mortar and pestle. The frozen tissue powder was then added to a chilled, preweighed 2 mL Eppendorf tube which was then quickly weighed a second time to give tissue weight by subtraction. Five volumes of ice-cold 6% w/v perchloric acid (PCA) containing 1 mM EDTA were then added, and the sample was immediately homogenized on ice. Samples were centrifuged at 20,000 g at 5°C in a Biofuge 15 for 20 min to precipitate denatured protein. A

measured aliquot of the supernatant was removed and 0.25 vol of neutralization solution (3 N KOH, 0.4 M KCl, 0.3 M Tris base) was added and mixed for 1 min. Further adjustments of the extract to a pH between 7 and 8 was achieved with subsequent additions of small volumes (1-3 μ L) of the solution. Neutralized solutions were then centrifuged for 20 min at 5°C. The supernatant was removed and stored at -80°C until used to measure cAMP levels. Concentrations of cAMP were determined via competitive protein binding assays using the commercial RIA kit from Amersham.

Enzyme assay

cAMP-Dependent protein kinase activity was assayed in a 60 μ L volume as described by Corbin and Reimann (1974). The standard reaction mixture contained 20 mM potassium phosphate (pH 6.8), 0.15 mM Kemptide, 50 mM KCl, 1 mM EDTA, 2.08 mM magnesium acetate, 0.237 mM unlabelled ATP and 10 μ Ci/mL radioactive ^{32} P -ATP (0.6 μ Ci per assay). A 10-20 μ L aliquot of enzyme (or enzyme + water) was added to initiate the reaction. When holoenzyme was being assayed, the reaction mixture also contained 10 μ M cAMP. These concentrations gave approximately 0.2 mM Mg.ATP and 0.74 mM free Mg^{2+} in the assay mixture (Brooks and Storey, 1992b), values that were determined to be optimal for the ground squirrel interscapular BAT enzyme. When measuring the Michaelis constant for Mg.ATP the total Mg^{2+} concentration was adjusted to keep free Mg^{2+} concentration at an optimal level. For determination of the pH dependence of PKAc, activity at different pH values was measured by adding 20 mM potassium phosphate pre-adjusted to the appropriate pH value. The pH of 'dummy'

reactions containing all components except the radioactive ^{32}P -ATP was measured at $22 \pm 1^\circ\text{C}$ and corresponding pH values at 37 or 5°C were calculated based on the known change in buffer pH with temperature. Assays were run for 5 -10 min and were linear under these conditions as determined by preliminary time courses and by specific activity curves. Reactions were stopped by spotting 40 μL aliquots of each assay mixture onto individual 2 x 2 cm pieces of Whatman P81 filter paper. The papers were washed for five min intervals with four changes (75 mL each) of 75 mM H_3PO_4 . Papers were then counted using Cerenkov methodology on a Packard Model 1900CA scintillation counter. When measuring activity with Histone IIA, incubations were stopped by adding 1 mL of ice-cold 5% w/v TCA + 10 mM H_3PO_4 and incubating for 10 min on ice. Samples were then filtered through Whatman GF/c filter papers, washed 4 times with the TCA/ H_3PO_4 solution and counted using Cerenkov methodology. One unit of PKAc activity was defined as the amount of enzyme that catalyzes the incorporation of 1 μmol of ^{32}P from ATP onto kemptide per min. Routine assays such as those used to find enzyme activity during purification procedures were done at 22°C whereas assays done for kinetic analysis were conducted at 37 or 5°C or over a range of temperatures (Arrhenius plots).

Purification of brown adipose cAMP-dependent protein kinase catalytic subunit

The method of Rubin et al. (1974) was used with modifications. All purification procedures unless noted were performed at room temperature. Frozen interscapular BAT (1.6 g) from control animals was homogenized 1:7 w/v in ice-cold buffer A (10 mM KH_2PO_4 , 10 mM K_2HPO_4 , 2 mM 2-mercaptoethanol, 1 mM EDTA, pH 6.8) with 1

$\mu\text{g/mL}$ aprotinin and $10\ \mu\text{g/mL}$ leupeptin and a few crystals of PMSF added. The homogenate was centrifuged at 20,000 g for 20 min; the supernatant was saved and applied directly onto a Sephadex DE-52 column (1.5 x 5 cm) which had been previously equilibrated in buffer A. The column was washed with 50 mL of buffer A and then the enzyme was eluted using 50 mL of a linear gradient of 0-1.0 M KCl in buffer A. One mL fractions were collected and assayed for PKA activity. Peak fractions were pooled and concentrated using Amicon-10 centrifugal concentrators to a volume less than or equal to 2 mL. The concentrated sample was loaded onto a Sephacryl S-300 gel filtration column (2 x 70 cm) equilibrated in buffer A; 1 mL fractions were collected and those containing peak enzyme activity were pooled. The pooled sample was then applied to a second DE-52 column (1 x 5 cm) in buffer A. The column was washed with 50 mL of buffer A and then the PKA catalytic subunit was eluted with 10 mL of a 0.1 mM cAMP solution. Fractions (1 mL) were collected and those containing peak enzyme activity were pooled and concentrated using Amicon-10 concentrators to a final volume of 0.75 mL. The enzyme was dialyzed for 2 h against 2 L of buffer A and then used for kinetic analysis. Purified PKAc was stored for up to three weeks at 5°C in buffer A plus 200 mM K_2HPO_4 , pH 7.0. Purity was judged by SDS-PAGE (as described above) with staining by Coomassie blue. Protein concentrations were determined by the method of Bradford (1976) using the Bio-Rad Laboratories prepared reagent with BSA as the standard.

PKA holoenzyme isolation and activation studies

A sample of previously frozen euthermic ground squirrel interscapular BAT (0.25g) was homogenized 1:5 (w/v) in PEM buffer containing 1 $\mu\text{g/mL}$ aprotinin and 10 $\mu\text{g/mL}$ leupeptin with a few crystals of PMSF. The homogenate was centrifuged at 22,000g for 20 min in a Sorvall RC2-B refrigerated centrifuge. The supernatant was removed and applied to a Sephadex DE-52 column (1.5 x 5 cm) pre-equilibrated in PEM buffer. The column was washed with 40 mL of PEM buffer and the peak of PKA holozyme was eluted with 40 mL of a 0-750 mM KCl gradient. Aliquots of column fractions were assayed under standard conditions $\pm 1 \mu\text{M}$ cAMP to locate the peak column fractions containing the holoenzyme. These fractions were then pooled and stored for up to 2 h at 5°C until use. Activation constants for cAMP dissociation of the holoenzyme were determined at both 5 and 37°C by incubating aliquots of PKA holoenzyme with different concentrations of cAMP (0-20 μM) for 30 min. After incubation, the percentage of enzyme present as the free catalytic subunit in each sample was determined via standard activity assays ($\pm 10 \mu\text{M}$ cAMP) with kemptide as the substrate.

CM-Cellulose Chromatography and Isoelectrofocusing

Isoforms of ground squirrel PKAc were assessed using chromatography on CM-52 cellulose and isoelectric focusing. We found that fully purified BAT PKAc did not survive isoelectrofocusing and so a partially purified enzyme was used for both these studies. Tissue samples (0.3 g) from euthermic, control animals were homogenized 1:5 w/v in buffer A containing protease inhibitors (leupeptin, aprotinin and PMSF) (as

described previously) and then centrifuged for 20 min at 20,000 g at 4°C. The supernatant was applied directly onto a DE-52 column (1.5 x 5 cm) which had been previously equilibrated in buffer A. The column was washed with 50 mL of buffer A and then the PKA catalytic subunit was eluted with 10 mL of a 0.1 mM cAMP solution in buffer A. One mL fractions were collected and assayed for PKA activity; peak fractions were pooled and aliquots were used for isoform analysis. For CM-52 cellulose chromatography, an aliquot of enzyme (2 mL) was applied to a 1 x 2 cm column of CM-cellulose equilibrated in PEM buffer, pH 7.5. After washing with PEM buffer, enzyme was eluted with 30 ml of a 0-300 mM KCl gradient in PEM buffer. Fractions (1 mL) were collected and assayed for PKA activity. For isofocusing, a 2 mL aliquot was placed in the middle of a 110 mL isofocusing column containing pH 3.5-10 ampholytes stabilized by a 0-30% w/v linear sucrose gradient (Vesterberg, 1971). The column was developed at 4°C for 12 h at 300 V at 5°C and then 2 mL fractions were collected, assayed for activity, and pH measured.

Kinetic studies of purified *S. richardsonii* BAT PKAc and rabbit muscle PKAc

All kinetic studies involving purified ground squirrel BAT or rabbit muscle PKAc were performed at 37°C and 5°C using the basic assay procedure outlined above. The former temperature represents the euthermic body temperature of both animals whereas the lower temperature is typical of body temperature in hibernating ground squirrels (Wang 1989). Water baths set at 37°C or 5°C (or other temperatures for Arrhenius plots) were used to maintain constant assay temperature and reaction mixtures were pre-incubated at each temperature for 10 min prior to the addition of enzyme to ensure

thermal equilibration. Substrate affinity constants (K_m values) for Kemptide, histone IIA, and Mg.ATP as well as the inhibitory effects (I_{50} values) of different neutral salts were determined under these conditions with PKAc from both sources. All kinetic data for substrate binding was initially analyzed using Hill plots; however, when the Hill coefficient was equal to 1 the data was described using the Michaelis-Menten equation. I_{50} values for inhibitors were determined by Job plots, V_o/V_i versus $[I]$, where V_o is the enzyme velocity in the absence of inhibitor, V_i is the enzyme velocity in the presence of inhibitor and $[I]$ is the concentration of inhibitor (Brooks and Storey 1992c). Arrhenius plots and pH activity profiles for both enzymes were determined under V_{max} conditions. Kinetic data was analyzed using the Student's t-test (2-tailed).

Fluorescence studies of *S. richardsonii* PKAc

Fluorescence spectra were recorded on a Perkin-Elmer LS-50 fluorescence spectrophotometer. Cuvette temperature was controlled with a water jacketed cell holder attached to a Neslab RTE-210 water bath. Assay conditions included an enzyme concentration of 8 $\mu\text{g/mL}$ in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol in a 3 mL quartz cuvette with a 1 x 1 cm path length. The excitation monochromator was set at 280 nm and the emission monochromator was set to scan the wavelength from 300 to 450 nm at a speed of 72 nm/min. Temperature induced conformational measurements were performed on aliquots of purified hibernator PKAc; emission spectra were recorded and corrected for the corresponding blank activity (minus

enzyme) by subtraction. Spectral titrations were carried out by adding increasing amounts of 8 M guanidine hydrochloride to the same enzyme solution. The solution was incubated for 30 min with stirring at 37 or 5°C before recording the spectrum after each addition. The volume increase due to the addition of the denaturant solution was held constant for all trials at both temperatures investigated. PKAc activity loss with increasing concentration of guanidine hydrochloride was measured by sampling a small aliquot (2 μ L) of the incubations and measuring enzyme activity as described above.

RESULTS

PKA activity, the percent PKAc, and cAMP levels in *S. richardsonii* tissues

Initial studies characterized the changes in the activity of PKA in control and hibernating tissues. Table 4.1 shows that the percentage of total PKA that was present during hibernation as PKAc decreased significantly in heart, but was unchanged in all other tissues examined. The percentage of PKAc declined from 28 ± 1 % in euthermic heart to 21 ± 1 % in hibernating heart ($P < 0.05$; Student's t-test). The highest % PKAc was 70% in euthermic and hibernating ground squirrel liver and skeletal muscle. Brain exhibited the lowest total PKA activities with euthermic and hibernating activities of about 40 mU per gram wet weight. The highest euthermic PKA activities were found in BAT and heart tissues with activities of 125 mU/gww. Total PKA activity was altered by hibernation in liver and brown adipose tissues. Total activity of PKA in BAT of euthermic squirrels was 126 ± 10 mU/gww with a very low percentage present as the free catalytic subunit, just 1.0 ± 0.2 %. In BAT from hibernating specimens, total activity was

22 % higher at 154 ± 5 mU/gww ($P < 0.05$; Student's t-test) but the % PKAc was still low at 2.7 ± 0.8 %. Total activity of PKA in heart of euthermic squirrels was 80 ± 2 mU/gww, and total activity was 26 % higher at 101 ± 3 mU/gww ($P < 0.05$; Student's t-test) in heart from hibernating specimens.

Table 4.1 also shows the effect of hibernation on tissue cAMP concentrations. A slight decline in cAMP concentrations was observed in the heart from hibernating individuals. More substantial decreases were observed in brain and kidney of hibernators; euthermic cAMP levels were 4076 ± 357 and 908 ± 21 pmol/gww, respectively. Levels of cAMP in hibernating brain and kidney were 58 and 34 % lower at 1710 ± 379 and 603 ± 56 pmol/gww. Hibernation had no effect on cAMP levels in liver, brown adipose, or skeletal muscle of *S. richardsonii*.

***In vitro* phosphorylation of BAT proteins by endogenous PKAc**

To assess the patterns of PKA-mediated phosphorylation of BAT proteins and the influence of temperature on this, extracts of interscapular BAT from both euthermic and hibernating animals were incubated *in vitro* under four different conditions and at temperatures representing euthermic (37°C) and hibernating (5°C) body temperatures. ^{32}P -Labeled phosphoproteins were then separated by one-dimensional SDS-PAGE, followed by autoradiography and densitometry scanning to reveal the relative intensity of radiolabeled bands. Extracts incubated under Incubation I conditions (+ EDTA to chelate the Mg^{2+} needed for kinase function) showed very few bands of very low intensity labeling on autoradiograms from either hibernating or euthermic animals at either

incubation temperature; hence, very little protein phosphorylation occurred under these conditions. Autoradiograms of gel lanes containing samples from Incubation II conditions (representing endogenous PKAc activity) also showed very little ^{32}P incorporation into proteins. Extracts incubated under Incubation III conditions (+ cAMP) showed pronounced labeling of multiple bands, whereas when the specific inhibitor, PKA-I, was added (Incubation IV, representing non-PKA mediated phosphorylation) fewer bands were labeled in a manner similar to the incubation II band intensity. Figure 4.1 also shows the net result when densitometry scans from Incubation IV autoradiograms were subtracted electronically from the scans of Incubation III autoradiograms. This provides the net pattern of cAMP-stimulated, PKA-mediated protein phosphorylation in BAT extracts. Several peaks representing phosphoproteins were seen, generally centered at 210, 110, 66, 45, 30, and 20 kDa. Comparisons of extracts from euthermic versus hibernating animals revealed several instances of differential labeling. A small amount of ^{32}P incorporation was observed at 210 kDa in BAT extracts from euthermic animals incubated at either temperature. However, this peak was absent in extracts prepared from BAT of hibernating individuals suggesting that the protein involved may not be present while the animals are hibernating. In incubations done at 5°C, a marked difference in ^{32}P -labeling pattern was seen for proteins of 40-50 kDa in extracts from euthermic versus hibernating animals. Pronounced labeling of this peak was found in extracts from hibernating individuals whereas in the euthermic situation only two small peaks were seen in this molecular weight range. At 37°C, however, both hibernating and euthermic extracts showed the same overlapping peak at 41 kDa but a small peak at 50 kDa was present in BAT extracts from euthermic but not hibernating animals. Incubations done at

both temperatures also showed small differences in the labeling patterns of 60-70 kDa proteins with proportionally greater labeling of the peaks in this region at 37°C for euthermic extracts and at 5°C for extracts from hibernating individuals; the relatively greater labeling in this peak when body temperature and incubation temperature were matched may suggest that there are hibernation-related changes in either the properties of PKA or of its protein targets. Finally, a large smear of ^{32}P incorporation was present at low molecular weights (<20 kDa) in the gel lanes containing extracts from 37°C incubations; this may represent protein degradation products.

PKA activity *in vivo* and holoenzyme dissociation studies

Total activity of PKA in brown adipose tissue of euthermic squirrels was 1.2 ± 0.05 mU per mg soluble protein (mean $\pm$ SEM, $n=4$) with a very low percentage present as the free catalytic subunit, just 1.0 ± 0.2 %. In BAT from hibernating specimens, total activity was 33 % higher at 1.5 ± 0.06 mU/mg protein ($P < 0.05$; Student's t-test) but the % PKAc was still low at 2.7 ± 0.8 %. Calculated per gram wet weight of tissue, these values were 126 ± 10 and 154 ± 5 mU/gww ($P < 0.05$; Student's t-test), for tissue from euthermic and hibernating animals, respectively.

The PKA holoenzyme consists of two regulatory and two catalytic subunits that dissociate *in vivo* upon cAMP binding to the regulatory subunits. The effect of temperature (5 versus 37°C incubation) on the dissociation of the holoenzyme type II from euthermic BAT was investigated by determining the cAMP concentration at which 50% of the holoenzyme was dissociated. At 37°C, the K_d value for cAMP was calculated

to be $0.582 \pm 0.029 \mu\text{M}$ cAMP and the Hill coefficient was 1.1 ± 0.2 , a near hyperbolic relationship. A similar K_s value was determined at 5°C ($0.591 \pm 0.022 \mu\text{M}$) but the Hill coefficient in this case was 2.2 ± 0.1 and significantly different from the value at 37°C ($P < 0.01$, 2-tailed Student's t-test).

Isoforms

The initial step in the purification procedure, DE-52 chromatography with salt gradient elution, is commonly used to separate the two PKA holoenzyme isoforms (Reimann et al., 1971; Corbin et al., 1975). However, DE-52 chromatography of BAT PKA produced only a single peak of enzyme activity eluting at about 0.25 M KCl indicating the presence of a single holoenzyme type in the tissue (Figure 4.2). Isoforms of the catalytic subunit have also been detected and these are separable by CM-52 chromatography (Kinzel et al., 1987). Partially pure BAT PKAc was prepared by DE-52 chromatography with elution by a cAMP gradient and then subjected to CM chromatography. BAT PKAc eluted as a single peak at 130 mM KCl with only a very minor shoulder seen at 195 mM (Figure 4.3a). By contrast, when a sample of PKAc was prepared from a mixture of ground squirrel organs (samples of equal tissue mass from liver, kidney, brain, muscle, BAT, heart), the presence of two major types of catalytic subunit in other tissues was clear (Figure. 4.3b). One peak was centered at 130 mM KCl and the second at 170-180 mM; a third, minor peak was also seen at 245 mM.

The same result was confirmed by column isoelectrofocusing. Partially purified BAT PKAc (prepared as above for CM chromatography) showed only a single peak of

activity after isofocusing. Furthermore, the isoelectric point was the same in samples from either euthermic (Figure 4.4a) or hibernating animals (7.8 ± 0.08 , $n=2$, 1 euthermic, 1 hibernating sample) indicating that no change in isoform occurred while hibernating. By contrast, isofocusing of a comparably prepared liver sample from euthermic ground squirrels showed two distinct peaks with pI values of 6.5 and 7.2 (Figure 4.4b).

Purification of ground squirrel PKAc

Overall, the purification scheme developed for BAT PKAc from control ground squirrels resulted in a 197-fold purification with a yield of 16% (Table 4.2); the scheme was highly reproducible with an approximately 200-fold purification occurring in all trials ($n = 3$). The final specific activity of purified PKAc when assayed with Kemptide as the substrate (without cAMP) was 279 mU/per mg protein at 22°C. SDS-PAGE of the purified catalytic subunit showed a single protein band with a molecular weight of 41 ± 4 kDa ($n = 3$) (Figure 4.5). Specific protein kinase inhibitors were tested to confirm the identification of the ground squirrel enzyme as a cAMP-dependent protein kinase catalytic subunit. PKA-I (Cheng et al. 1986) and H-89 (Chijiwa et al. 1990) potently decreased enzyme activity with I_{50} values of 26.0 ± 2.4 nM and 45.8 ± 3.2 nM, respectively. The specific inhibitor of cGMP-dependent protein kinase, KT-5823 (Kase et al. 1987) and the inhibitor of protein kinase C, PKC-I (House and Kemp 1977) were much less effective, however, with I_{50} values of greater than 80 μ M and 50 μ M, respectively. Purified ground squirrel PKAc lost activity rapidly over time during storage at 5°C and was not stabilized by the addition of either glycerol (up to 50% v/v) or Triton

X-100 (0.1% v/v). However, in the presence of high phosphate concentration (200 mM) the enzyme retained stability for at least 3 weeks.

Kinetic characterization of ground squirrel PKAc

The substrate preference of purified PKAc was assessed by monitoring incorporation of ^{32}P onto different PKAc substrates (Kemptide, histone IIA, histone IIIS, myelin basic protein, and histone VIS); enzyme activity with Kemptide proved to be the greatest so this was used as the preferred substrate for subsequent kinetic studies. The specific activity of the purified ground squirrel enzyme was 71.9 mU/ mg protein at 5°C and 470 mU/ mg protein at 37° with kemptide as the substrate. Michaelis constants for substrates of ground squirrel PKAc all decreased dramatically with a decrease in assay temperature (Table 4.3). K_m values measured at 5°C were only 20% for Kemptide, 49% for histone IIA, and 47% for Mg.ATP of the corresponding values at 37°C. In contrast, rabbit muscle PKAc showed K_m values that were temperature-independent for Mg.ATP and Kemptide or decreased by one-third for histone IIA when assayed at 5°C. K_m values also differed between species and were higher in all cases for the ground squirrel enzyme when compared with the rabbit enzyme at 37°C. At 5°C, however, K_m for histone IIA of the ground squirrel enzyme was still greater than the rabbit value (by 2.5-fold) but K_m for Kemptide was only about one-third of the rabbit value and K_m ATP was similar for the two enzymes. The effects of salts on PKAc activity at the two assay temperatures are also presented in Table 4.3 as I_{50} values. In general, the ground squirrel enzyme was inhibited at both temperatures by salts in the order: NaF>NH₄Cl>KCl~NaCl. Fluoride was also the strongest inhibitor of rabbit PKAc whereas the other salts had similar effects at 37°C;

however, the order of inhibition was reversed at 5°C (KCl>NaCl>NH₄Cl). A decrease in assay temperature to 5°C reduced the inhibition of both enzymes by NaCl and NH₄Cl (*I*₅₀ values increased) but only significantly raised the *I*₅₀ KCl of the ground squirrel enzyme. Inhibition by fluoride was insensitive to assay temperature. Ground squirrel PKAc had higher *I*₅₀ values than the rabbit enzyme for all salts investigated at 5°C. The *I*₅₀ values for NaCl and NaF were also higher for the ground squirrel enzyme when assayed at 37°C.

The effect of assay temperature on the maximum velocity of purified ground squirrel and rabbit PKAc is shown as Arrhenius plots in Figure 4.6. Both plots showed two distinct linear segments but with different break points. The break in the plot occurred at 9°C for ground squirrel PKAc with activation energies of 29 ± 1 kJ/mol for $T > 9^\circ\text{C}$ and 60 ± 2 kJ/mol for $T < 9^\circ\text{C}$, a rise of 2.1-fold at the lower temperatures (Figure 4.6a). For the rabbit enzyme, the break occurred at 17°C with activation energies of 24 ± 0.4 kJ/mol at $T > 17^\circ\text{C}$ and 87 ± 1 kJ/mol at $T < 17^\circ\text{C}$ (Figure 4.6b), a rise of 3.7-fold at the lower temperatures. PKAc from both animals displayed similar velocity changes with temperature over the higher temperature range; however, the activation energy of rabbit PKAc at low temperatures was significantly higher than that determined for the ground squirrel enzyme.

The pH optimum of ground squirrel PKAc changed dramatically with a change in assay temperature. Figure 4.7a shows that the optimum was about pH 6.8 at 37°C but increased by about 2 full pH units to 8.7 at 5°C. However, at 5°C high activity was retained over a broad range (85-90 % of maximum between pH 6.5-8). By contrast, rabbit PKAc showed very little change in pH optimum with temperature with an optimum of 6.8 to 7.5 at both assay temperatures (Figure 4.7b).

Fluorescence measurements of *S. richardsonii* PKAc

Temperature induced conformational changes in the protein were monitored by following intrinsic tryptophan fluorescence intensity. Fluorescence is a sensitive probe of localized protein motion and has been used extensively to monitor conformational changes induced by various structural modifiers such as temperature and chemical denaturants (Eftink 1994). The wavelength of maximum fluorescence (λ_{max}) for ground squirrel PKAc shifted to lower values as temperature increased. The change was linear with a calculated slope $-0.047 \text{ nm}/^{\circ}\text{C}$ increase in temperature ($r = 0.94$) (Figure 4.8a). Fluorescence emission intensity measured at the λ_{max} also changed with temperature (Figure 4.8b), decreasing linearly with an increase in temperature; the change was $-0.347 \text{ fluorescence units}/^{\circ}\text{C}$ ($r = 0.96$).

Protein unfolding induced by guanidine-HCl was also monitored by fluorescence emission spectrum. The λ_{max} of purified ground squirrel PKAc increased with increasing guanidine-HCl concentration at both 5 and 37°C incubation temperatures with no discernable differences between the results at the two temperatures (Figure 4.9a). In both cases, the λ_{max} at 0 M guanidine-HCl was 338 nm and this rose to a denatured value, in the presence of 4 M guanidine-HCl, of 350 nm. The effect of guanidine-HCl on fluorescence intensity was also examined at 5 and 37°C. A wavelength of 335 nm was selected for this study because the change in fluorescence intensity between native and denatured states was the greatest at this wavelength. In this case, incubation temperature did influence protein unfolding (Figure 4.9b). At 37°C, there was an initial rapid decrease in fluorescence intensity as guanidine-HCl concentration increased up to 0.5 M whereas

Table 4.1. Total protein kinase A activities, percentage of the enzyme present as the free catalytic subunit and cAMP concentrations in tissues of euthermic and hibernating Richardson's ground squirrels.

	Euthermic			Hibernating		
	Total Activity (mU/gww)	% PKAc	[cAMP] pmol/gww	Total Activity (mU/gww)	% PKAc	[cAMP] pmol/gww
Liver	79.7 ± 2.0	65.2 ± 3.5	1043 ± 163	100.8 ± 2.9 ^a	71.6 ± 7.9	995 ± 90
Skeletal Muscle	55.8 ± 6.6	72.0 ± 4.4	909 ± 54	63.3 ± 3.4	64.6 ± 3.3	1136 ± 151
Heart	125.5 ± 8.2	27.7 ± 1.1	1070 ± 30	120.0 ± 3.2	20.9 ± 0.7 ^a	938 ± 18 ^a
Brown Adipose	126.1 ± 10.1	1.0 ± 0.2	1590 ± 87	154.1 ± 5.0 ^a	2.7 ± 0.8	1387 ± 236
Kidney	110.5 ± 1.3	14.8 ± 0.6	908 ± 21	115.1 ± 6.9	12.1 ± 1.9	603 ± 56 ^a
Brain	43.0 ± 6.1	26.3 ± 1.8	4076 ± 357	32.7 ± 7.0	27.8 ± 3.7	1710 ± 379 ^a

Data for total activities are in milliunits per gram wet weight, means ± SEM (n=4) with statistical testing using the Student's t-test (two-tailed). ^a - Significantly different from corresponding euthermic value, P < 0.05.

Table 4.2. Purification of cAMP-dependent protein kinase catalytic subunit from brown adipose tissue of euthermic ground squirrels.

Step	Total Protein (mg)	Total Activity (mU)	Yield (%)	Purification (fold)	Specific Activity (mU/mg)
Crude extract	109.4	155	100	1	1.41
DE-52 (KCl)	1.79	116	75	46	65.1
Gel Filtration	0.26	66.0	43	180	255
DE-52 (cAMP)	0.09	25.1	16	197	279

Table 4.3. The effect of temperature on euthermic ground squirrel brown adipose and rabbit muscle PKAc kinetic properties: substrate affinities and inhibition by salt.

	Ground Squirrel		Rabbit	
	37°C	5°C	37°C	5°C
Substrates:				
K_m Mg.ATP (μ M)	49.2 $\pm$ 3.4	23.3 $\pm$ 2.0 ^a	27.1 $\pm$ 0.8 ^b	28.6 $\pm$ 2
K_m Kemptide (μ M)	50.0 $\pm$ 4.5	10.1 $\pm$ 1.1 ^a	34.4 $\pm$ 1.6 ^b	34.8 $\pm$ 6 ^b
V_{max} (mU/mg protein)	6.9 $\pm$ 0.1	1.9 $\pm$ 0.1	4.82 $\pm$ 0.2	0.57 $\pm$ 0.03
K_m Histone IIA (mg/mL)	0.4 $\pm$ 0.1	0.2 $\pm$ 0.04 ^a	0.12 $\pm$ 0.01 ^b	0.08 $\pm$ 0.01 ^{a,b}
V_{max} (mU/mg protein)	1.6 $\pm$ 0.1	0.4 $\pm$ 0.01	1.3 $\pm$ 0.10	0.2 $\pm$ 0.01
Salt I_{50} values:				
KCl (mM)	181 $\pm$ 9	438 $\pm$ 23 ^a	158 $\pm$ 21	198 $\pm$ 20 ^b
NaCl (mM)	210 $\pm$ 9	378 $\pm$ 30 ^a	133 $\pm$ 22 ^b	253 $\pm$ 17 ^{a,b}
NH ₄ Cl (mM)	121 $\pm$ 5	154 $\pm$ 6 ^a	129 $\pm$ 17	302 $\pm$ 7 ^{a,b}
NaF (mM)	65 $\pm$ 8	52 $\pm$ 2	41 $\pm$ 7 ^b	35 $\pm$ 2 ^b

Data are means $\pm$ S.E.M., n=3 separate determinations. ^a-Significantly different from the corresponding value at 37°C using the Student's t-test (2-tailed), $P < 0.05$; ^b- significantly different from the corresponding value for the ground squirrel enzyme at the same temperature, $P < 0.05$.

Figure 4.1. Densitometry scanning of autoradiograms showing ^{32}P incorporation into cytoplasmic proteins *in vitro* in extracts of ground squirrel BAT catalyzed by endogenous protein kinases at (A) 5°C and (B) 37°C. Phosphoproteins in extracts of euthermic (broken line) and hibernating (solid line) animals are presented. Samples from *in vitro* phosphorylation incubations were run on SDS-PAGE, autoradiographed, and scanned; relative signal intensity of bands is shown. Scans of autoradiograms representing Incubation IV (+ PKA-I inhibitor) were subtracted electronically from those of Incubation III (+ cAMP) to correct for protein phosphorylation that was not PKA-mediated. The data shown represent a single trial but essentially the same results were achieved with duplicate incubations. Protein standards shown on the x-axis are: rabbit muscle myosin (210 kDa), *E. coli* β -galactosidase (116 kDa), rabbit muscle phosphorylase β (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), and carbonic anhydrase (29 kDa).

Figure 4.2. Elution profile of euthermic ground squirrel BAT PKA holoenzyme from DE-52. The column was eluted with a 0-500 mM KCl gradient in PEM buffer pH 6.8. Activity was measured in the presence of 10 μ M cAMP (●) and in the absence of cAMP (○) in 20 μ L aliquots of column fractions (1 mL each).

Figure 4.3. Elution profiles of euthermic ground squirrel PKA catalytic subunit from CM-52 Cellulose. (A) brown adipose PKAc; (B) a multi-tissue extract with equal amounts of liver, kidney, brain, heart, muscle and BAT tissues. The column was eluted with a 0-300 mM KCl gradient in PEM buffer pH 7.5. Activity was measured in 20 μ L aliquots of column fractions (1 mL each) and is plotted relative to the maximum activity in the peak tube which was 90-95,000 dpm/20 μ L in both cases.

Figure 4.4. Isoelectrofocusing of brown adipose (A) and liver (B) PKA catalytic subunit in a pH 3.5-10 gradient of ampholines. Activity was measured in 20 μ L aliquots of column fractions (2 mL). The pH gradient is shown by the dashed line.

Figure 4.5. SDS-PAGE of purified cAMP-dependent protein kinase catalytic subunit from brown adipose of *S. richardsonii*. Lane A, MW standards; Lane B, brown adipose crude supernatant; Lane C, pooled peak after DE-52 (KCl gradient); Lane D, pooled peak after gel filtration, Lanes E1-E2, peak activity fractions after DE-52 (cAMP bump) showing the final purified enzyme. MW standards are as in Figure 4.2.

Figure 4.6. Arrhenius plots for (A) *S. richardsonii* and (B) rabbit PKAc. Reaction mixtures were pre-incubated for 10 min to allow temperature equilibration before initiating the reaction with the addition of PKAc. Optimal assay conditions were used. Data points shown are for assay temperatures from 1-40°C for ground squirrel PKAc with the break seen at 9°C and from 2-35°C for rabbit PKAc with the break at 17°C. Data are means $\pm$ SEM, n=4.

Figure 4.7. Effect of assay pH on purified ground squirrel (A) and rabbit (B) PKAc at two assay temperatures: 5°C (■) and 37°C (●). Activities are plotted relative to the maximal activity at the optimum pH. The pH of the assay was changed by adding 20 mM potassium phosphate pre-adjusted to the appropriate pH value. The pH of 'dummy' reactions containing all components except ^{32}P -ATP was determined at $22 \pm 1^\circ\text{C}$. Total Mg^{2+} and ATP concentrations were adjusted to maintain constant concentrations of $\text{Mg}\cdot\text{ATP}$ and free Mg^{2+} at each temperature and pH (Brooks and Storey, 1992a). Data are means $\pm$ SEM, $n=4$.

Figure 4.8. The effect of changing temperature on (A) the wavelength of maximum fluorescence emission (λ_{max}) and (B) fluorescence emission intensity at λ_{max} for *S. richardsonii* PKAc (8 $\mu\text{g/mL}$) in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. The solution was equilibrated with stirring at each temperature for 30 min prior to fluorescence measurement. Data are means $\pm$ SEM, $n=3$.

Figure 4.9. The effect of guanidine-HCl concentration at 37°C (■) and 5°C (●) on (A) the wavelength of maximal fluorescence emission (λ_{max}) and (B) the fluorescence emission intensity at 335 nm. Successive aliquots of 8 M guanidine-HCl were added to *S. richardsonii* PKAc (8 $\mu\text{g/mL}$ initial concentration) in 10 mM potassium phosphate buffer (pH 7.0) containing 10 mM 2-mercaptoethanol. The solution was equilibrated with stirring at each denaturant concentration for 30 min prior to fluorescence measurement. Data are means $\pm$ SEM, n=3.

Figure 4.10. The effect of guanidine-HCl on the activity of purified euthermic ground squirrel PKAc at 37°C (■) and 5°C (●). After each 30 min incubation of samples described in Fig. 5, a 2 μ L aliquot was removed for activity assay under optimal conditions at 23°C. Measured activities were adjusted for the volume dilution of adding successive aliquots of guanidine-HCl. Data are means $\pm$ SEM, n =3.

the subsequent addition of more denaturant up to 4 M resulted in a more gradual decline in fluorescence intensity. At 5°C, however, there was no change in fluorescence intensity up to 1.0 M guanidine-HCl but above this amount of denaturant, a gradual decrease in fluorescence intensity was observed. The changes in PKAc fluorescence characteristics with added guanidine-HCl were also reflected in a loss of enzyme activity with increasing concentrations of guanidine-HCl (Figure 4.10). Denaturation occurred at lower guanidine-HCl levels at 37°C with 90% of enzyme activity lost by 0.3 M guanidine-HCl whereas 0.9 M guanidine-HCl elicited the same activity loss at 5°C. The initial rate of enzyme activity loss was 2.4-fold greater at 37°C than the rate of loss at 5°C. The concentration of guanidine-HCl that resulted in 50 % loss of activity was 274 ± 16 mM at 5°C and 107 ± 8 mM at 37°C.

DISCUSSION

In hibernating mammals, β -adrenergic signals that are mediated intracellularly via PKA are key to the thermogenic response of BAT (Horwitz 1989; Nedergaard and Cannon 1990). Hence, the regulation and function of PKA at both low and high body temperatures is critical to signal transduction in BAT. The present study was undertaken, therefore, to determine whether adaptive changes were made to the kinetic or physical properties of the ground squirrel BAT PKAc to support enzyme function at euthermic versus hibernating body temperatures. One factor that could be important to enzyme function at low temperature was an increase (by 33%) during hibernation in the total activity of PKA present in BAT which raises the scope for enzyme activation during arousal. Both euthermic and hibernating animals also had very low steady state

percentages of PKAc (1.0-2.7 %) which shows that the potential activation of the enzyme during arousal can be as much as 30-100 fold. This large potential for PKA activation would be highly beneficial during arousal for it could elicit very rapid and similarly large increases in the activities of enzymes and functional proteins that are targets of PKAc phosphorylation. It is also of interest that of six major organs tested, total PKA activity in BAT was exceeded only in brain; this again emphasizes the importance of this signaling system in the activation of thermogenesis in BAT.

The *in vitro* incubation studies indicated that a variety of cellular proteins in ground squirrel BAT were targets of PKAc-mediated phosphorylation. Proteins of 40-50 kDa were particularly interesting for these showed a high level of ^{32}P -incorporation in extracts from hibernating animals during incubation at 5°C whereas phosphorylation of target proteins in this size range was much lower in extracts from euthermic animals. This suggests that target proteins become available in BAT of hibernating animals that are lacking in non-hibernating individuals. Whether these proteins were induced by cold exposure when ground squirrels were transferred to 4°C or by the transition into the torpid state cannot be determined from our present data but clearly significant differences exist in the suite of PKA target proteins present in BAT of warm, euthermic animals compared with cold, torpid ones. Furthermore, phosphorylation of proteins in the 45-50 kDa peak (and to a lesser extent the 60-70 kDa peaks) was relatively more intense after 5°C incubations compared with those at 37°C suggesting that low temperature can alter either PKAc action towards selected protein targets or protein susceptibility to PKAc-mediated phosphorylation. Hence, these might represent proteins that are important targets of PKAc during arousal from low body temperature. If PKAc action towards

target proteins changes at low body temperatures, then the properties of ground squirrel BAT PKAc, and in particular the effects of temperature change on the enzyme, are of great interest.

Two isoforms of PKA holoenzyme are routinely found in mammalian tissues, Type I and II, and their differences have been attributed to two distinct types of regulatory subunits (Taylor and Radzio-Andzelm, 1994). The tetrameric holoenzymes can be separated on DE-52 when eluted with a salt gradient and tissue specific patterns are found (Reimann et al., 1971; Corbin et al., 1975). Most tissues show varying amounts of both holoenzyme forms but white adipose tissue (rat epididymal fat pad) showed only the Type II enzyme which elutes at a higher KCl concentration than Type I (Corbin et al., 1975). The single peak of PKA holoenzyme detected in ground squirrel BAT is, therefore, consistent with expectations for an adipose tissue. For a number of years it was believed that there was only one form of catalytic subunit until Kinzel et al. (1987) documented the presence of two catalytic subunit types in rat and rabbit skeletal muscle with different pI values and sensitivities to protease degradation. This has been confirmed by others (Olsen and Uhler 1989) and a third form was reported in one instance (Beebe et al., 1990). The functional significance of catalytic subunit isoforms, however, is still unknown for not only do both types I and II holoenzyme consist of mixtures of both catalytic subunit forms but, except for small differences in amino acid sequence which define the pI and protease susceptibility differences, most other properties of the two forms are the same. Thus, there are no significant differences in subunit migration in SDS gels, K_m values for ATP, kemptide and other phosphate-accepting substrates, pH dependence, and inhibition by ions, regulatory subunits of holoenzyme I or II, or specific

heat and acid-stable inhibitors (Kinzel et al., 1987; Olsen and Uhler, 1989). Kinzel et al. (1987) separated the two catalytic subunit forms on CM-cellulose but comparable trials of CM-cellulose chromatography with the PKAc preparation from ground squirrel BAT revealed only one peak of enzyme activity, eluting at 130 mM in a 0-300 mM KCl gradient (with a very minute shoulder at 195 mM). By contrast, CM-cellulose chromatography of catalytic subunit derived from a homogenate of multiple tissues gave two strong peaks of activity, one eluting at 130 mM and the other at 170-180 mM and a minor peak at 240-250 mM. Thus, some ground squirrel tissues clearly contain both holoenzyme types and 2 or 3 forms of catalytic subunit but brown adipose tissue contains, virtually exclusively, only one holoenzyme and one catalytic subunit type. The presence of a single PKAc in BAT was also supported by the very sharp, single peak of activity on IEF ($pI = 7.8$) whereas a comparable analysis of liver showed 2 isoforms (pI 6.5 and 7.2) (Figure 4.4). Two forms of catalytic subunit were also found in bovine cardiac muscle Type II PKA (pI values approximately 7.5 for C_A and 8.0 for C_B) (Kinzel et al. 1987). Thus, we are confident that the purified BAT PKAc studied here represents a single isoform.

The purification procedure developed for PKAc from euthermic ground squirrel BAT was highly reproducible. Generally an approximately 200-fold purification was achieved resulting in a final specific activity of 268 nmol phosphate transferred per min⁻¹.mg protein⁻¹ at 22°C. The enzyme was judged to be homogeneous by SDS-PAGE. The subunit molecular weight of *S. richardsonii* BAT PKAc, 41 kDa, was similar to the molecular weight of the enzyme isolated from other mammalian sources (Taylor et al. 1990; Olsen and Uhler 1989; Kinzel et al., 1987).

Differential temperature effects on the properties of ground squirrel PKAc may benefit enzyme function in activating thermogenesis. One such effect was on cAMP-mediated dissociation of the holoenzyme. Although the K_d for cAMP dissociation of the ground squirrel holoenzyme was unaltered by temperature change between 37 and 5°C, the relationship between [cAMP] and holoenzyme dissociation shifted from near-hyperbolic at 37°C ($n_H = 1.1$) to sigmoidal at 5°C ($n_H = 2.2$). This would increase the sensitivity of enzyme dissociation to small changes in cAMP concentration and would permit a rapid increase from low % PKAc (1-3% in the steady state) to maximum PKAc over a very small range of cAMP concentration. Thus, when arousal is initiated by β -adrenergic signals, which in turn activate adenylyl cyclase to enhance cAMP production, the sigmoidal response of PKA to cAMP at low body temperature would result in a very rapid increase in PKAc content in BAT that could thereby facilitate an equally rapid increase in the phosphorylation of PKAc target proteins.

The kinetic constants determined for *S. richardsonii* BAT PKAc were similar to those reported for the PKA catalytic subunit from other mammalian sources although some minor differences were apparent that could facilitate enzyme function in the hibernator. The apparent K_m values for both Mg.ATP and Kemptide of the ground squirrel enzyme were 2-10 times higher than those reported for other mammalian sources (Whitehouse et al. 1983; Kinzel et al. 1987). However, the K_m values for all three substrates (Mg.ATP, Kemptide, histone IIA) decreased at 5°C which could maintain or enhance responsiveness of the enzyme at the low body temperature of the hibernating state. The total adenylate pool in tissues of hibernating animals frequently falls during

torpor to only about 50 % of the values in the euthermic animal (Storey 1997). Hence, the low temperature effect in reducing K_m ATP of PKAc would effectively "self-adjust" the ratio between K_m and *in vivo* ATP concentration. This is not uncommon for enzymes in ectothermic animals (Hochachka and Somero 1984) and, in the hibernators, could allow a similar relative activity of the enzyme to be maintained at both high and low body temperatures. The greater affinity for both model phosphate acceptors (Kemptide and histone) at low temperature also suggests that the enzyme might have greater affinity for various protein substrates *in vivo* at low body temperature and this could be another mechanism that would aid the rapid initiation of thermogenesis when arousal begins. The rabbit enzyme, by contrast, showed K_m values that were largely temperature insensitive.

Protein kinases in general are highly flexible enzymes. Large rotational movements of the conserved kinase lobes as well as movements of flexible loops and domains accompany binding of different protein substrates, cofactors, auto-inhibitory domains or interacting proteins either during catalysis or for regulatory purposes (Scott 1993; Taylor and Radzio-Andzelm 1994). The break in the Arrhenius plot at 9°C implies that a conformational change also occurred in *S. richardsonii* BAT PKAc below this temperature (Figure 4.6a) that resulted in a 2.3-fold increase in the activation energy of the enzyme over the lower temperature range. The Q_{10} of the enzyme reaction at temperatures below this break were slightly higher than the Q_{10} value above the break, 2.4 versus 1.8 respectively. The break in the Arrhenius plot (Figure 4.6b) for the rabbit enzyme appeared at a considerably higher temperature (17°C), and also distinguished two sections of the plot. Velocities of the rabbit enzyme above this temperature increased with a Q_{10} value (1.7) that approximated that of the ground squirrel enzyme; however, the Q_{10}

value (4.9) for enzyme velocities below this point was significantly larger than that determined for the ground squirrel enzyme. The differences in the temperature sensitivities of the two enzymes suggests a difference in their primary structures that is also consistent with the different kinetic properties of the two enzymes. Body temperatures below 10°C frequently occur during hibernation and the smaller Q_{10} value at low temperatures for the reaction catalyzed by the hibernator enzyme would maintain a good potential for enzyme activity at lower body temperatures. This could enhance the ability of the hibernator kinase to propagate the signal for the initiation of brown fat NST during arousal from the torpid state. However, the implied changes in ground squirrel PKAc conformation that are suggested by the Arrhenius data were not mirrored by the fluorescence data. Both the emission intensity and the wavelength of maximum fluorescence responded to changing temperature in a linear manner throughout the temperature range examined (3-55°C). It should be noted, however, that the fluorescence data were obtained from the protein alone in solution whereas the Arrhenius data come from measurements of enzyme activity in the presence of substrates that may change protein conformation. Since the two methods revealed different trends in conformational change with changing temperature, those different behaviors are likely to be substrate-related. It would appear then that temperature effects on the conformation of the hibernator protein are minimal in the absence of substrates and slightly more substantial when substrates are present.

The influence of temperature on the pH optimum of ground squirrel PKAc was dramatic. The optimum shifted from about 6.8 at 37°C to 8.6 at 5°C. This change cannot be accounted for by a temperature effect on buffer pH for the assays were done in

phosphate buffer which shows only a very minor change in pH with temperature (0.0016 pH unit increase per 1°C decrease in temperature or 0.05 pH unit for the 32°C change in assay temperature). Furthermore, the pH optimum of rabbit PKAc was virtually unaffected by temperature change being 6.8-7.5 at both assay temperatures. Therefore, another specific difference between the enzymes of the hibernating and nonhibernating species could be indicated. The two enzymes also differed in the shape of the pH versus activity curve with the hibernator enzyme showing high activity (> 75% of maximum) over a broad range of pH values from pH 5-9 whereas the rabbit enzyme retained less than 40 % of maximum activity at pH 6 and fell to only 60-70% of $V_{\max}$ at pH 8.2. The broad optimum range of the hibernator enzyme could allow enzyme function over a wide range of intracellular pH values and this could also benefit enzyme function as intracellular pH changes over cycles of hibernation and arousal, typically by 0.2-0.4 pH units (Malan 1988; McArthur et al. 1990). Hence, the basic shift in pH optimum at hibernating body temperatures as well as the generally broad pH range over which high activity is retained could facilitate PKAc activity and allow for expression of high enzyme activity no matter what the body temperature. The specific changes to the structure and kinetic properties of the catalytic subunit of protein kinase A in ground squirrel brown adipose tissue facilitate enzyme function at both high and low body temperatures and prime the enzyme for a key role in signal transduction during the activation of thermogenesis that initiates arousal from torpor.

CHAPTER 5

Differential activation of ERK, JNK/SAPK and p38 MAP kinase family members in the hibernating ground squirrel, *Spermophilus richardsonii*

INTRODUCTION

Mitogen-activated protein (MAP) kinases, which include the extracellular signal-regulated protein kinase (ERK), c-jun NH₂-terminal kinase (JNK/SAPK), and the recently identified mammalian homologue of the yeast HOG1 kinase (p38 MAP kinase) (Galcheva-Gargova et al., 1994), comprise a family of serine/threonine kinases that appear to play a central role in the transduction of the mitogenic responses to epidermal growth factor (EGF) and several other growth factors, heat shock (Kyriakis et al, 1994), osmotic stress, and ultraviolet light (Kyriakis et al, 1994). The protein kinases are important regulatory proteins through which extracellular signals are transduced into intracellular events. All three kinases are activated through complex phosphorylation cascades and their activation leads, in turn, to the phosphorylation of a variety of proteins including downstream kinases and transcription factors. Although the pathways involved in activating ERK, JNK and p38 share some cellular signaling machinery, they also contain distinct regulatory members (Figure 1.3). The well studied ERK group is typically activated by growth factors via a p21^{ras}-dependent signal transduction pathway (Seger and Krebs, 1995). Interaction of growth factors with their receptors leads to the activation of membrane associated receptor tyrosine kinases followed by the sequential activation of Ras and Raf. Raf then phosphorylates a MAPK/ERK kinase (MEK), which in turn activates ERKs via dual phosphorylation on both tyrosine and threonine residues. Activated ERKs can activate other kinases, such as MAPKAPK-1 (p90^{RSK}), MAPKAPK-2, and p70^{S6K} (Seger and Krebs, 1995), or regulate the transcription of mitogen or stress-activated genes through the action of transcription factors such as c-jun, c-fos, and p62^{TCF}

(or Elk-1) (Davis, 1994). These transcription factors bind to the promoter regions of DNA and induce gene expression of their target genes.

The JNK pathway is homologous to the ERK pathway in its overall form but appears to be activated mainly by distinct stimuli. JNKs, unlike ERKs, are only weakly activated by growth factors or phorbol esters, but respond strongly to proinflammatory cytokines and environmental stresses such as UV radiation, heat shock, ischemia, reperfusion injury, or protein synthesis inhibition (Kyriakis et al., 1994; Hibi et al, 1993; Sluss et al, 1994; Pombo et al, 1994). SEK1, the upstream activator of JNK homologous to MEK but is not activated by Raf (Yan et al, 1994). Although ERKs will phosphorylate c-jun at Ser-63 and Ser-73 in its N-terminal transactivation domain, JNK phosphorylates these residues with higher efficiency (Pulverer et al, 1991). The mechanisms involved in p38 activation are poorly understood, and it is unclear to what extent the initiating signals and the pathways leading to p38 activation overlap with those for JNK and ERK. Since SEK has been shown to phosphorylate and activate both p38 and JNK, it may serve as a branch point to activate both pathways (Force et al, 1996). Activated p38 MAP kinase has been shown to phosphorylate and activate MAPKAP kinase-2 (Rouse, 1994) and to phosphorylate and activate the transcription factors, Elk-1 (Whitmarsh, 1995) and ATF-2 (Gupta, 1995). Unlike JNK and ERK, p38 does not phosphorylate c-jun.

Hibernation requires a variety of metabolic adaptations including those that regulate and coordinate a profound metabolic depression as well as those that re-organize metabolism for long term survival at a reduced body temperature. Although the effects of oxidative (including hypoxia and reperfusion insults), heat shock, osmotic, and inflammatory stresses on the ERK, JNK and p38 signal transduction pathways have been

characterized in mammalian cell cultures using stresses applied *in vitro*, their role in mediating adaptive responses to environmental stress in stress-tolerant mammals is largely unknown. We have been interested in hibernation as a natural state that confers tolerance of ischemia and hypothermia onto cells. In this light, I have explored the role of the ERK, p38 and JNK members of the MAP kinase family during hibernation.

MATERIALS AND METHODS

Animals and Chemicals

Adult Richardson's ground squirrels, *Spermophilus richardsonii*, were obtained from the Calgary area and experimentally treated as described in Chapter 4. A rabbit polyclonal antibody recognizing the amino terminus of human c-fos and a goat anti-rabbit antibody coupled to horseradish peroxidase were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antibodies specifically recognizing the phosphorylated (tyrosine 182) and unphosphorylated form of p38, the phosphorylated (serine 133) and unphosphorylated form of CREB (cAMP response element binding protein), the phosphorylated (serine 383) form of Elk1, and the phosphorylated (threonine 71) and unphosphorylated forms of ATF-2 (activating transcription factor-2) were from New England Biolabs Inc. (Mississauga, ON). The antibody recognizing mouse ERK1/2, the antibody recognizing amino acids 26-175 of mouse c-jun, and a rabbit anti-mouse antibody coupled to horseradish peroxidase were from Transduction Laboratories (Lexington, KY). The ECL kit and Reflection autoradiography film were from Mandel Scientific (Guelph, ON). The peptides used for assaying MAPKAPK-1, MAPKAPK-2, and p70^{S6K} activities were synthesized at the Protein Facility at Queen's University

(Kingston, ON). Bovine brain myelin basic protein (MBP) and glutathione (GSH)-agarose were from Sigma Chemical Co. (St. Louis, MO). Protein kinase C inhibitor peptide (PKC-I; 19-31) was from Boehringer Mannheim (Montreal, PQ), and protein kinase A inhibitor peptide (PKA-I; 5-24) was purchased from Peninsula Labs Inc. (Belmont, CA). Phosphocellulose paper (P81) was obtained from Whatman (VWR, Mississauga, ON). [γ - ^{32}P] ATP (3000 Ci/mmol) was obtained from New England Nuclear (Montreal, PQ). Plasmid DNA for human GST(glutathione S-transferase)-c-jun (5-89) was a kind gift of Dr. J. Woodgett (Ontario Cancer Institute, Toronto, ON). Production of GST-jun fusion protein and preparation of GST-jun-GSH agarose conjugates were carried out as described previously (Smith and Johnson, 1988). All other chemicals were purchased either from Sigma Chemical Co. or Boehringer Mannheim.

Protein kinase assays

Frozen tissues were rapidly weighed and then homogenized 1:10 (w/v) in ice-cold 25 mM MOPS (pH 7.2), 50 mM β -glycerophosphate (β -GP), 2 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_4 , 2 mM dithiothreitol (DTT) containing 5 $\mu\text{g}/\text{mL}$ aprotinin, 1 $\mu\text{g}/\text{mL}$ leupeptin, 1 $\mu\text{g}/\text{mL}$ pepstatin A, and a few crystal of phenylmethylsulfonyl fluoride (PMSF). Homogenates were centrifuged at 18,000 g for 25 min at 4°C. Supernatants were removed, kept on ice, and assayed immediately for enzyme activity.

In a final volume of 50 μL , 5 μL of tissue extract was incubated with 27.5 mM MOPS (pH 7.2), 35 mM β -GP, 1.7 mM EGTA, 0.2 mM EDTA, 0.2 mM Na_3VO_4 , 1.2 mM DTT, 2 mM MgCl_2 , 100 μM ATP, 12 μCi [γ - ^{32}P] ATP, 1 μM PKC-I, 0.5 μM PKA-I

and appropriate substrates and inhibitors (as follows). The peptide RRRLSSLRA, corresponding to amino acids 231-239 of human 40S ribosomal protein S6, was used as a substrate for p70^{S6K} and is non-specifically phosphorylated by both MAPKAPK-1 and p70^{S6K}. To determine the relative contribution of these kinases to the phosphorylation of this peptide 0.1 mM of the potent MAPKAPK-1 inhibitor H7 was included in the assay solution (Stokoe *et al.*, 1992). The peptide KKKNRTLSVA is specific for MAPKAPK-1 and is not phosphorylated by either MAPKAPK-2, CaM kinase-II, or p70^{S6K} when used at a concentration of 0.2 μ M (Stokoe *et al.* 1993). To check that the phosphorylation of this peptide was due to the activity of MAPKAPK-1, 0.1 mM of the potent MAPKAPK-1 inhibitor H7 was included in blank assays run in parallel. The peptide KKLNRRTLSVA is efficiently phosphorylated by MAPKAPK-2 (Stokoe *et al.*, 1993). However, since this peptide is also phosphorylated by MAPKAPK-1, 0.1 mM of the inhibitor H7 was included in the assay solution to inhibit MAPKAPK-1 activity (Stokoe *et al.*, 1992).

All assays were initiated by adding the radioactive ATP plus Mg²⁺ and terminated after 10 min at 22 $\pm$ 1°C by spotting 35 μ l aliquots of assay mixture onto 2 x 2 cm pieces of Whatman P81 filter paper, followed by four 5 min washes with 75 mM phosphoric acid. The assays were linear under these conditions as determined by preliminary experiments (data not shown). Two controls were included: 1) a zero time control, assays were stopped immediately by spotting onto the P81 paper and 2) a minus substrate control, 10 μ L of dH₂O was used in substitution of substrate. All assays were performed in triplicate, and the activity of simultaneous assays carried out in the absence of kinase substrate were subtracted as blanks. Radioactivity was counted in a LKB 1900CA liquid

scintillation counter using Cerenkov methodology. One unit is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of phosphate onto substrate per minute at $23 \pm 1^\circ\text{C}$.

In-gel kinase assays for ERKs

This method was adapted from Kameshita and Fujisawa (1989). Ground squirrel tissues were homogenized 1:10 (w/v) in homogenization buffer and centrifuged at 14,000 rpm in a Biofuge 15 microcentrifuge for 30 min. SDS gel-loading buffer was added to the samples, 1:1 (v/v). The samples were boiled for 5 min and loaded onto a 10% w/v acrylamide gel. Care was taken to ensure equal protein loading between corresponding euthermic and hibernating samples. Protein kinase substrate (MBP) was mixed into SDS-PAGE gel solution before gel-polymerization. This allows an even distribution of kinase substrate throughout the gel. The SDS-PAGE gels were run at 5°C at 150 V. Following PAGE, SDS was removed with 2 washes of 100 mL each 20% v/v propanol in 50 mM Tris-HCl (pH 8.0) for 1 h each. The gel was then washed with 250 mL freshly prepared buffer A (50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol) for 1 h; all washes, except where specified, were at room temperature with constant agitation. Kinases within the gel were denatured with 2 changes of 100 ml each 6 M guanidine-HCl in buffer A; each wash was for 1 h. The kinases were renatured within the gel by incubating with a renaturing buffer (Buffer A containing 0.04% (v/v) Tween-20); five 250 mL changes of renaturing buffer at 4°C over 16 hours (overnight) were used. The gel was pre-incubated with 20 mL kinase buffer minus ATP (both “hot” and “cold”) for 1 hour prior to

incubation with 10 mL of kinase buffer containing 10 $\mu\text{Ci/mL}$ $\gamma\text{-}^{32}\text{P}\text{-ATP}$ for 1 hour at 30°C. The gel was washed with five exchanges of 500 mL of wash buffer containing 2% (w/v) sodium pyrophosphate and 10% (w/v) TCA for 1 hour each until radioactivity of the solution was negligible. The gel was dried and exposed to X-ray film at -80°C with an intensifying screen. The phosphorylated substrate can then be then visualized by autoradiography; the duration of exposure depended on the amount of ^{32}P incorporated into the substrate. This assay allows one to see which kinases (ERK1/2 are identifiable by molecular weight) are activated (increased rate of substrate phosphorylation) or deactivated during stress.

Solid phase JNK assays

For JNK activities, frozen tissue samples were homogenized 1:10 (w/v) in 20 mM HEPES (pH 7.5), 25 mM $\beta\text{-GP}$, 2 mM EDTA, 2 mM EGTA, 0.1 mM Na_3VO_4 , 1 mM DTT (containing 5 $\mu\text{g/mL}$ aprotinin, 1 $\mu\text{g/mL}$ leupeptin, 1 $\mu\text{g/mL}$ pepstatin A, and a few crystal of PMSF) and then centrifuged as described above. The phosphorylation of GST-c-jun(5-89)/glutathione-Sepharose was performed essentially as described by Bogoyevitch et al. (1995). Phosphorylated GST-c-jun(5-89) was eluted with boiling SDS-PAGE sample buffer and separated on 10% SDS-PAGE gels. Following staining with Coomassie Blue to detect GST-c-jun (5-89) and autoradiography, the ^{32}P incorporation in GST-c-jun (5-89) gel band was determined by Cerenkov counting.

Immunoblotting

Tissue extracts were prepared as outlined above for kinase assays and protein concentration of the supernatants was determined using the Bio-Rad dye reagent (Bradford, 1976). Extracts were diluted 1:1 (v/v) with SDS-PAGE sample buffer, boiled for 5 min, loaded onto 12% w/v SDS-PAGE mini-gels, and then electrophoresed at 150V. Care was taken to ensure equal protein loading between the corresponding euthermic and hibernating samples. Proteins were electroblotted to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Montreal, PQ) by wet transfer in 25 mM Tris (pH 8.3), 192 mM glycine and 10% (v/v) methanol for 50 min at 100V. Western blotting was carried out as recommended by the antibody supplier and results were visualized by ECL.

RESULTS

ERK and ERK-activated kinase activities.

The effect of hibernation on the activity levels of members of the ERK family of protein kinases was investigated in the hibernating ground squirrel, *S. richardsonii* (Figure 5.1). In control animals, ERK activity levels were highest in kidney and brain, somewhat lower in liver, and lowest in heart and skeletal muscle. ERK was always the dominant activity measured, being anywhere from 2-fold (muscle) to 10-fold (brain) greater than the measurable activities of MAPKAPK-1, p70^{S6K}, or MAPKAPK-2. A significant change in ERK activity was observed during hibernation in kidney (a 74 % decrease), brain (30% increase) and muscle (2.6-fold increase).

Since the ERK activities shown in Figure 5.1 were determined using MBP as the substrate, the values represent a sum of both ERK1 (p44) and ERK2 (p42) activities. To separately assess the two activities, the technique of in-gel assay was used to determine

the relative activities of ERK1 and ERK2 whereas antibody staining assessed relative amounts of ERK proteins (Figure 5.2). Brain appeared to contain equal proportions of both ERK1 and ERK2 (Figure 5.2b), both of which increased in activity in the hibernating condition (Figure 5.2a). ERK2 appeared to be the dominant form in liver. Both ERK1 and ERK2 protein levels decreased in hibernating liver and their activities were also reduced. Kidney appeared to contain slightly more ERK2 than ERK1, and the protein levels of ERK1 and ERK2 were unchanged by hibernation. There was no activity associated with ERK in either states and very little change in activity was detected in hibernation. In heart, only ERK2 exhibited any activity in either state although crossreaction with the polyclonal antibodies showed that both ERK1 and ERK2 could be detected with slightly more ERK2 protein but no difference between euthermic and hibernating states. It appears then that the changes in ERK2 in hibernation were a result of changes in the phosphorylation state of the enzyme and not an increase in the quantity of enzyme protein. In muscle, only ERK2 was present in euthermic and hibernating conditions with no difference in the quantity of protein present in either state. ERK2 activity in muscle increased in the hibernating condition.

The activities of other ERK family kinases are also shown in Figure 5.1. The peptide KKKNRTLSVA is specific for MAPKAPK-1 and is not phosphorylated by either MAPKAPK-2, CaM kinase-II, or p70^{S6K} when used at a concentration of 0.2 μ M (Stokoe et al., 1993). Hibernation resulted in an increase in MAPKAPK-1 activity in both muscle (3.2 fold) and brain (1.7-fold) but enzyme activity was unchanged in the other tissues examined. MAPKAPK-2 efficiently phosphorylates the peptide KKLNRTLSVA. This

peptide is also phosphorylated by MAPKAPK-1 and, therefore, 0.1 mM H7 was included in the MAPKAPK-2 assay to inhibit MAPKAPK-1 (Stokoe et al., 1992). The activity of MAPKAPK-2 changed in all tissues examined during hibernation. Activity increased by 2.2-fold in muscle, 1.6-fold in heart and 3.5-fold in brain but decreased in liver and kidney to levels that were just 50% and 32% of euthermic values, respectively. The activity of p70^{S6K} kinase was determined using a peptide (KKKLSSLRA) that corresponds to the phosphorylation site of the S6 ribosomal subunit. The euthermic activity of this kinase was highest in kidney (461 ± 78 U/g wet weight, n=6), followed by heart (198 ± 22 U/gww), brain (176 ± 15 U/gww), liver (131 ± 18 U/gww), and skeletal muscle (56 ± 20 U/gww). However, the only tissue which exhibited changes in activity during hibernation was kidney where activities declined by 48%.

Effect of hibernation on JNK and p38 activity levels.

The ERK and JNK subfamilies of MAPKs phosphorylate serine-63 and serine-73 of the N-terminal transactivation domain of c-jun *in vitro* (Pulverer et al, 1991); however, ERKs do not bind c-jun with high affinity so JNKs appear to be responsible for phosphorylation of c-jun *in vivo*. We used a solid-phase kinase assay to measure the activity of protein kinases that bind to and phosphorylate the N-terminal domain of c-jun; this assay therefore solely determines JNK mediated phosphorylation of c-jun. The effect of hibernation on JNK activity in ground squirrel tissues is shown in Figure 5.3. Activities of JNK in tissues of euthermic animals were highest in kidney and brain; liver, heart, and skeletal muscle exhibited lower activities of JNK. Brain activity decreased to levels 70% of control activity. In all other tissues examined, JNK activity increased

during hibernation by amounts that were 1.5-fold in heart and liver, 1.4-fold in kidney, and 1.8-fold in muscle.

An antibody specific for the phosphorylated (Tyr 182) form of p38 MAPK was used to determine the phosphorylation level, and hence the activity, of the kinase by immunoblotting (Figure 5.4). The phosphorylation state of p38 was unchanged by hibernation in all tissues except muscle and heart. The amount of phosphorylated p38 increased slightly in hibernating heart and rose in hibernating skeletal muscle. An antibody to the unphosphorylated form of p38 was used to determine whether there were changes in the total amount of the enzyme present in hibernation but no differences were observed between euthermic and hibernating conditions in any organ. The kinase was present in all tissues examined and the levels were highest in skeletal muscle and lowest in brain.

In vivo levels of transcription factors

The transcription factor c-jun was detected by immunoblotting in all tissues examined (Figure 5.5). Levels appeared to increase in hibernation in liver, muscle, and kidney but decreased in brain and heart. The effects of hibernation on levels of the immediate-early genes c-myc, c-fos and egr-1 were assessed by Western blotting. Control levels of egr-1 were highest in liver, followed by kidney and were undetectable in heart, skeletal muscle and brain (Figure 5.6). The concentration of egr-1 appeared to increase in hibernating heart, liver, brain, and muscle while the levels in kidney remained constant. Immunoblots showed that c-myc was present in all *S. richardsonii* tissues

Figure 5.1. Maximal activities (U/g wet weight) of the ERK and ERK activated kinases in extracts from kidney and brain (A) and skeletal muscle, liver and heart (B) of euthermic (open bars) and hibernating (filled bars) Richardson's ground squirrels. Data are means $\pm$ S.E.M. (n=5-8). * - Significantly different from the corresponding euthermic values using the Student's t-test (2-tailed), * $P < 0.05$.

Figure 5.2. A. ERK1 and ERK2 activities in euthermic and hibernating ground squirrel tissues. Extracts were assayed by in-gel kinase assays using 0.5 mg/mL MBP polymerized in 10% SDS-PAGE gels as described under experimental procedures. The molecular masses (kDa) of marker proteins are indicated by the numbers to the left of the panel. The arrows indicate the positions of the ERK1 (p44) and ERK2 (p42) protein kinase activities. B. Representative western blot of tissue extracts from euthermic and hibernating *S. richardsonii* probed with an anti-MAPK antibody recognizing both ERK1 and ERK2. Equal amounts of protein were loaded for each pair of samples. Lanes are: euthermic and hibernating liver (1 & 2), kidney (3 & 4), brain (5 & 6), muscle (7 & 8), and heart (9 & 10), respectively.

Figure 5.3. Effect of hibernation on JNK activity. **A.** Representative autoradiogram showing GST-jun phosphorylation from extracts of heart (1 & 2), kidney (3 & 4), liver (5 & 6), muscle (7 & 8), and brain (9 & 10). The GST-jun glutathione-Sepharose pellets from the 'solid-phase' JNK assay were resuspended in SDS-PAGE buffer and run on 10% SDS-PAGE gels. Radioactive phosphate incorporation onto GST-jun was visualized by autoradiography. **B.** JNK activity was assayed in euthermic (open bars) and hibernating (closed bars) tissues, and the results of multiple experiments were quantified as described in the experimental procedures. Values are means $\pm$ SEM (n=3) and were analyzed using Student's t-test (2-tailed). * $P < 0.05$.

A.

Figure 5.4. Representative results of Western blotting of extracts from *S. richardsonii* tissues sampled from euthermic and hibernating conditions probed using an antibody specific for the unphosphorylated and phosphorylated (tyr 182) forms of p38. For each tissue, blots were standardized by loading equal amounts of protein for each pair of samples. Lanes are: euthermic and hibernating brain (1 & 2), liver (3 & 4), kidney (5 & 6), muscle (7 & 8), and heart (9 & 10), respectively.

Figure 5.5. Representative results of Western blot analyses of *S. richardsonii* tissue extracts sampled from euthermic and hibernating conditions probed for the presence of c-jun. For each tissue, blots were standardized by loading equal amounts of protein for each pair of samples. Lanes are: euthermic and hibernating heart (1 & 2), brain (3 & 4), liver (5 & 6), muscle (7 & 8), and kidney (9 & 10), respectively. A sample of c-jun (39 kDa) was also run as a positive control (+ve).

Figure 5.6. Representative results of Western blot analyses of *S. richardsonii* tissue extracts sampled from euthermic and hibernating conditions probed for the presence of *egr-1* and *c-myc*. For each tissue, blots were standardized by loading equal amounts of protein for each pair of samples. Lanes are: hibernating and euthermic muscle (1 & 2), liver (3 & 4), brain (5 & 6), heart (7 & 8), and kidney (9 & 10), respectively.

Figure 5.7. Representative results of Western blotting of extracts from *S. richardsonii* tissues sampled from euthermic and hibernating conditions probed using an antibody specific for the unphosphorylated and phosphorylated (Ser 133) forms of CREB. For each tissue, blots were standardized by loading equal amounts of protein for each pair of samples. Lanes are: euthermic and hibernating brain (1 & 2), liver (3 & 4), kidney (5 & 6), muscle (7 & 8), and heart (9 & 10), respectively. A sample of either phospho-CREB or CREB was also run as a positive control (+ve).

except brain; no differences were observed in the levels of c-myc present in samples taken from hibernating or euthermic conditions for any tissue. Western blots using antibodies to c-fos were unable to detect its presence in any tissue of *S. richardsonii*. The amounts of phosphorylated-CREB were assessed by immunoblotting with an antibody specific to the phosphorylated (Ser 133) form of the protein (Figure 5.7). The amount of phosphorylated CREB increased significantly during hibernation in kidney, brain, heart and liver but was unchanged in muscle. However, the level of CREB protein, as assessed with an antibody to the unphosphorylated form, was unaffected by hibernation in all tissues examined. The amounts of phosphorylated-Elk1 and ATF-2 were assessed by immunoblotting with an antibodies specific to the phosphorylated forms of the two transcription factors; blots were unable to detect the presence of either phosphorylated protein in any tissue of the ground squirrel.

DISCUSSION

In this study, we have shown that the tissue activities of ERK, JNK and p38 MAPK sub-families respond to hibernation in distinct and individual manners. Hibernating muscle and heart exhibited increased activity for kinases from all three sub-families. Liver demonstrated an increase in JNK activity in the hibernating state. Increases in ERK and decreases in JNK activities were seen in hibernating brain whereas kidney showed increases in JNK but decreases in ERK activities.

Current evidence suggests that considerable similarity exists in the patterns of activation of JNK and p38 in response to various stimuli which are distinguishable from ERK. It is clear that diverse signals, including inflammatory cytokines (Bird et al, 1995),

protein synthesis inhibitors (Cano et al., 1994), ischemic reperfusion (Pombo et al., 1994; Knight and Buxton, 1996) and osmotic stress (Bogoyevitch et al., 1996; Galcheva-Gargova et al., 1994) activate the JNK and p38 subfamilies of MAPKs. However, ERK is highly activated by mitogenic stimuli, and only modestly activated by most other stresses (Kyriakis, 1994), so that the upstream mediators of ERK, JNK and p38 subfamilies are distinct (Sanchez et al., 1994; Yan et al., 1994). JNK and p38 may share a common upstream regulator although the pathways of regulation appear to be relatively insulated from one another. MAP-kinase activity during hibernation also exhibits this secular nature; ERK activity changes (Figure 5.1) appeared to be independent of JNK (Figure 5.3) activation. Thus, there were no consistent correlations between the direction or magnitude of changes in JNK and ERK tissue activities during hibernation.

It is important to understand the extent of crosstalk between the JNK and ERK pathways since an increase in the transactivating activity of c-jun may be an end response of activation of both pathways. The two pathways have been proposed to operate in parallel with c-jun as a common substrate (Davis, 1994); however, the activity profiles presented here suggest that the two pathways may have different terminal substrates in hibernating mammals. For example, there was an increase in ERK activity in brain but a decrease in JNK activity. A difference in pathway response was also evident in kidney; ERK activity decreased while JNK activity increased. Differential activation of the JNKs and ERKs by cellular stresses has been previously reported in cultured cardiac myocytes (Bogoyevitch et al., 1995), and in the perfused rat heart (Knight and Buxton, 1996). Ischemic stress was a more potent activator of JNK while phorbol 12-myristate 13-acetate, a hypertrophic agonist, equally activated both ERK and JNK, and the

mechanisms of activation were determined to be different. Both the ERK and JNK pathways were involved in hibernation and clearly demonstrate that each tissue responds uniquely to initiate its own genetic survival program.

Recent evidence has suggested that Elk-1, a ternary complex factor (TCF), is the primary *in vivo* substrate of ERKs and may also be a substrate of p38 (Whitmarsh et al., 1995). Elk-1, together with the serum response factor, controls transcription from the serum response element (SRE). The SRE mediates the expression of many immediate-early genes in response to serum and growth factors. We were unable to detect phosphorylated Elk-1 in either hibernating or euthermic tissues of the ground squirrel. The basal amount of Elk-1 protein is normally (ie. in other species and cell lines) maintained at an extremely low level so our failure to detect its presence does not preclude the possibility that it is phosphorylated in hibernation. SAPKs may also phosphorylate and activate Elk-1, which together with the serum response factor control the induction of c-fos. Interestingly, we were also unable to detect c-fos in any *S. richardsonii* tissues with an antibody purchased from Santa Cruz Biotechnology. Again this may have a number of interpretations; c-fos may not be present in this animal, the antibody may fail to recognize ground squirrel c-fos, or the level of c-fos protein may be below the detection limits of the immunoblot). As a transcription factor, c-fos functions primarily as a heterodimer with c-jun and is targeted to AP-1 promoter elements (Karin, 1995).

Induction of the proto-oncogene c-jun has been shown to occur in response to a wide variety of stresses and tissues. This transcription factor belongs to the bZip family of transcription factors and functions primarily as a heterodimer with c-fos or ATF-2.

When complexed with c-fos, the dimer is targeted to promoters containing AP-1 elements such as the tetradecanoyl-phorbol ester response element (TRE). However, when complexed with ATF-2, the dimer appears to prefer cAMP response element (CRE) sequences (Force et al., 1996). Since phosphorylation at the N-terminal transactivation domain is critical for the transcriptional activating activity of c-jun and JNK readily phosphorylates c-jun at a rate 10 times faster than ERKs (Pulverer et al, 1991), changes in c-jun phosphorylation and activation in the hibernating state are likely the responsibility of JNK. The significant increase in c-jun protein levels in four organs during hibernation, in the absence of c-fos, may indicate that genes induced during hibernation are primarily responsive to transcription factors that act on the CRE sequences.

The significant activation of CREB, as determined by increases in its level of phosphorylation in several tissues during in hibernation also suggests an increase in transcriptional activation at the cAMP response element. CREB has been shown to be activated by increases in cAMP and PKA activation, resulting in translocation of PKAc to the nucleus and phosphorylation of CREB (Hagiwara et al., 1993). Increased intracellular levels of Ca^{2+} also stimulate CREB phosphorylation via the calmodulin-dependent protein kinases CamK II and CamK IV; in addition, MAP kinase stimulation of CREB phosphorylation may also occur via MAPKAPK-1 (Lev et al., 1995). The cAMP levels did not appear to change during hibernation, nor did the amount of free PKAc (Chapter 4); phosphorylation of CREB may therefore occur by one, or a combination, of the other two activating pathways. MAPKAPK-1 activity and phospho-CREB levels were both increased in brain; indicating that this kinase may be responsible for CREB phosphorylation during hibernation in this tissue.

MAPKAPK-1 and $p70^{S6K}$ are broad specificity ERK activated Ser/Thr kinases that phosphorylate a variety of substrates including the glycogen binding subunit of PP-1 and the 40S ribosomal subunit protein S6 (Kyriakis and Avruch, 1994). Muscle and brain showed MAPKAPK-1 activation, and kidney was the only tissue to exhibit any change (a decrease) in the activity of $p70^{S6K}$ (Figure 5.1). One response controlled in part by the Rsk kinases is the insulin regulation of glycogen synthesis in skeletal muscle. Rsk phosphorylation of the glycogen binding subunit of PP-1 increases the catalytic efficiency of the phosphatase and promotes glycogen storage (Dent et al., 1990). Rsk activation in muscle during hibernation could re-enforce the inhibition of glycogenolysis and aid in the conservation of carbohydrate reserves.

The phosphorylation level of p38 in ground squirrel skeletal muscle and heart increased during hibernation. Nuclear substrates of p38 that have been identified include the transcription factors Elk-1, CREB, and ATF-2. Thus activation of either JNK, p38 or ERK signal transduction pathways may stimulate transcription of hibernation specific genes. Indeed, preliminary studies in our lab using differential display PCR and differentially screened cDNA libraries have found hibernation-induced genes in skeletal muscle (Cai and Storey, unpublished) and heart (Fahlman and Storey, unpublished). p38 also phosphorylates and activates MAPKAPK-2 (Rouse et al, 1994), a kinase that phosphorylates and thereby regulates the activity of the small heat shock protein Hsp27. Hsp27 has chaperone-like properties and confers resistance to heat shock and osmotic stress (Knauf et al, 1992). Though there is no information regarding the activation of Hsp27 in hibernation, MAPKAPK-2 activation may be important in conferring cold tolerance in brain, muscle, and heart tissues.

Kyriakis and Avruch (1994) questioned whether the function of 'mitogen' activated protein kinases was to promote the mitogenic response or to execute the specialized, tissue specific programs that characterize differentiated tissues. In the hibernating mammal, the purpose of the MAPK cascade may have expanded to include the execution of specialized, tissue specific programs that enable cell survival in the hypometabolic state. The complex regulation of ERK, SAPK and p38 kinase activities observed in the hibernating ground squirrel, *S. richardsonii*, suggests a dominant cellular role of MAP kinase signal transduction in the control of hibernation.

CHAPTER 6

The effects of hibernation on protein phosphatases from ground squirrel organs

INTRODUCTION

Reversible phosphorylation of structural and regulatory proteins in the cell is a widespread phenomenon and a critical mechanism for coordinating metabolic responses by multiple cellular processes to extracellular stimuli. The phosphorylation state of a protein is dynamic and modulated by the action of protein kinases and protein phosphatases. Protein phosphatase-1 (PP-1) is a widely distributed enzyme capable of dephosphorylating a variety of key regulatory enzymes (Hubbard and Cohen, 1989a; Alemany et al., 1986). PP-1 is the major activity that dephosphorylates and inactivates glycogen phosphorylase and phosphorylase kinase, and dephosphorylates and activates glycogen synthase *in vivo* (Alemany et al., 1986). This implies that it has an important role in coordinating and regulating glycogenolysis and glycogen synthesis. Several holoenzyme forms of PP-1 exist; these are composed of a catalytic subunit which is complexed to different regulatory subunits. The ATP/Mg-dependent enzyme is a complex of PP-1 with inhibitor-2 (Ballou et al., 1985), the glycogen bound enzyme consists of PP-1 complexed to a glycogen binding (G) subunit (Hubbard and Cohen, 1991), a myofibril associated enzyme consists of PP-1 bound to myosin subunits (Chen et al., 1994), and a nuclear enzyme consists of PP-1 associated with NIPP-1 (nuclear inhibitor of protein phosphatase-1) inhibitor protein (Beullens et al., 1993). The role of each is to target the catalytic subunit toward specific subcellular locations and to increase activity towards particular substrates. For example, phosphorylation of the mammalian PP-1 glycogen binding regulatory subunit by PKA leads to dissociation of the catalytic subunit, resulting in an inhibition of the dephosphorylation of glycogen bound enzymes (Hubbards and Cohen, 1989b; 1989c).

The members of the type-2 phosphatases are subdivided on the basis of their metal ion requirements: spontaneously active (PP-2A), Ca^{2+} /calmodulin-dependent (PP-2B) and Mg^{2+} -dependent (PP-2C) (Wera and Hemmings, 1995). Several trimeric holoenzyme forms of PP-2A exist and have been extensively characterized. These structures consists of a 36 kDa catalytic subunit complexed with regulatory subunit A; the core dimer is able to associate with a variety of regulatory subunits, the roles of which have not been fully resolved. Preliminary studies have indicated that there are two major isoforms of the 43-48 kDa PP-2C catalytic subunit; however, little is known about the physiological role of PP-2C or its regulation.

The present study examines the tissue specific changes in Ser-/Thr-phosphatase activities during hibernation in ground squirrels to determine whether the regulation of protein phosphatases is a factor influencing hibernation-induced metabolic rate depression.

MATERIALS AND METHODS

Animals and Chemicals

Adult Richardson's ground squirrels, *Spermophilus richardsonii*, were obtained in the Calgary area and treated as described previously (Chapter 4). $[\gamma\text{-}^{32}\text{P}]$ ATP (3000 Ci/mmol) was obtained from New England Nuclear (Montreal, PQ). Okadaic acid was from Calbiochem (La Jolla, CA). Microcystin-agarose, 75 μg of microcystin per 0.5 mL of matrix, was from Upstate Biotechnology Inc. (Lake Placid, NY). All other chemicals, column materials, and enzymes were purchased either from Sigma or Boehringer

Mannheim. Rabbit polyclonal antibodies recognizing the carboxy-terminus of rat α , δ , and γ 1 phosphatase type-1 isozymes were a kind gift of Dr. Emma Villa-Moruzzi (University of Pisa, Italy). Goat anti-rabbit antibody coupled to horseradish peroxidase was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The ECL kit and Reflection autoradiography film were from Mandel Scientific (Guelph, ON). ^{32}P -labeled rabbit skeletal muscle glycogen phosphorylase α (10^6 cpm/nmol) was prepared as described previously (Mehrani and Storey, 1995).

Enzyme Assay and Determination of “Active” and “Total” PP-1 Activities

Glycogen phosphorylase phosphatase activity was assayed by monitoring the release of ^{32}P from ^{32}P -labeled glycogen phosphorylase α (Cohen et al, 1988), one unit (U) of activity being the amount of enzyme that catalyzes the release of 1 μmol phosphate per min at 25°C. Unless otherwise indicated, the standard assay mixture contained in a 50 μL volume: 20 mM Tris, pH 7.0, 0.1 mM EGTA, 10 mM β -mercaptoethanol, 5 mM theophylline, and 2.5 nM okadaic acid. Assays were initiated by the addition of 20 μL of ^{32}P -labeled glycogen phosphorylase α (10^8 cpm/ μmol) and terminated by the addition of 100 μL ice-cold 25% (w/v) trichloroacetic acid/10 mM phosphoric acid. After incubating in an ice bath for 5 min, all samples were centrifuged and then 100 μL of each supernatant was removed and counted on an LKB 1900CA liquid scintillation counter using Cerenkov methodology. Two control assays were included and ^{32}P released under these conditions was subtracted as a blank: (1) zero time control, assays were stopped

immediately by the addition of TCA/phosphoric acid solution; and (2) minus enzyme control, buffer replaced the enzyme extract. Both controls gave similar results.

Activities were measured both in concentrated tissue extracts (1:3 w/v homogenization) and after pre-treatment of tissue extracts with trypsin (Drake and Palmer, 1995). Measurement of PP-1 activity in concentrated extracts provides an approximate measure of the "active" phosphatase activity at physiological concentrations of modulatory proteins and other factors (Toth et al., 1991). However, whereas the PP-1 catalytic subunit is relatively resistant to trypsinolysis, pre-treatment with trypsin removes modulatory proteins that would otherwise inhibit phosphatase activity and so gives a measure of "total" phosphatase present (Bollen, et al., 1988). In this procedure, frozen tissue samples were homogenized with 2-3 volumes of 50 mM imidazole-HCl, pH 7.4, containing 0.25 M sucrose, 4 mM EDTA and 0.5 mM DTT. Homogenates were centrifuged at 1,000g for 3 min. The supernatants were removed and immediately assayed for "spontaneously active" PP-1 activity. In addition, a 100 μ L aliquot of supernatant was incubated with 1 mg/mL trypsin and 1 mM MnCl_2 for 10 min (25°C) and then digestion was stopped by the addition of soybean trypsin inhibitor to a concentration of 10 mg/mL. Dilution buffer (50 mM Tris, pH 7.0, 0.1 mM EGTA, 15 mM β -mercaptoethanol, 2 mg/mL BSA) was then added to prepare dilutions of 1:50 v/v for liver and 1:10 v/v for all other tissues. The diluted sample was then assayed for "total" PP-1 activity.

Assay of type-2 protein phosphatase activities.

Frozen tissue samples were homogenized 1:10 w/v in ice-cold homogenization buffer (50 mM Tris, pH 7.0, 0.1 mM EGTA, 15 mM β -mercaptoethanol) with 1 mM phenylmethylsulfonyl fluoride, 1 μ g/mL leupeptin, and 1 μ g/mL aprotinin. Homogenates were centrifuged in a BioTek microfuge at 20,000 g for 20 min. Supernatants were removed and layered onto small columns (5 mL; 0.25 mL supernatant per column) of Sephadex G-25 equilibrated in homogenization buffer at room temperature in order to remove low molecular weight metabolites and endogenous inorganic phosphate that might interfere with protein phosphatase assays. The columns were centrifuged in an IEC bench-top centrifuge for 1 min and the desalted enzyme filtrate was collected and centrifuged through a second fresh G-25 column. The enzyme filtrate was collected and stored on ice until assayed.

The activities of the type-2 protein phosphatases were measured using the Promega Serine/Threonine Phosphatase Assay system. This system determines the amount of free phosphate generated in a reaction by measuring the absorbance of a molybdate:malachite green:phosphate complex (Ekman and Jager, 1993) in a 96 well microplate (1/2 area, flat-bottom, 190 μ L volume). PP-2A assay mixture contained in a 50 μ L volume: 50 mM imidazole-HCl, pH 7.2, 0.2 mM EGTA, 100 μ M substrate hexapeptide (RRApTVA) and 0.02% v/v β -mercaptoethanol. PP-2C activity was measured under the same conditions but in the presence of 1 μ M okadaic acid and 10 mM $MgCl_2$. Reactions were initiated by the addition of up to 10 μ L of enzyme preparation, incubated for 10 min at 22°C, and terminated by addition of 50 μ L ice cold malachite green dye reagent. After incubating at room temperature for 30 min to allow for full color development, absorbance at 600 nm

of the individual microplate wells were measured with a Dynatech MR5000 Microplate reader and Biolynx Data Capture software. Two control reactions were included and phosphate released under these conditions was subtracted as the blanks: 1) zero time control - reaction stopped immediately by addition of the malachite green dye reagent, and 2) minus substrate control - 10 μ L of buffer A was added in place of substrate peptide. Initial trials determined the optimum amount of tissue extract to be used in each assay and demonstrated that reaction rates were linear over the reaction time. One unit of activity is defined as the amount of enzyme that catalyzes the release of 1 nmol phosphate per min at $22 \pm 1^\circ\text{C}$.

Purification of Protein Phosphatase Type-1 from Ground Squirrel Liver

Frozen liver from euthermic ground squirrels was homogenized 1:5 (w/v) in Buffer A (20 mM Tris, pH 7.2, 20 mM β -glycerophosphate, 2 mM EDTA, 2 mM EGTA, 15 mM β -mercaptoethanol, and 0.1 mM sodium orthovanadate), containing 0.6 M KCl, 0.5 % v/v Triton X-1000, and protease inhibitors: 1 mM benzamidine, 20 μ g/mL PMSF, 20 μ g/mL aprotinin, 10 μ g/mL TPCK, 5 μ g/mL leupeptin, and 10 μ g/mL soybean trypsin inhibitor and then centrifuged at 15,000g for 15 min. Supernatant was removed and PEG 8000 was added to 12% (w/v). After resting on ice for 20 min, the suspension was centrifuged at 15,000g for 15 min, and then the supernatant was discarded. The pellet was resuspended by homogenization in the original volume of buffer A containing protease inhibitors. All purification steps were performed at room temperature unless otherwise noted.

DEAE-cellulose chromatography. The resuspended extract was loaded onto a DEAE cellulose (DE-52) column (2.5 x 6 cm) equilibrated in buffer B (50 mM Tris, pH 7.2, 0.1 mM EDTA, 10 mM β -mercaptoethanol, 10% v/v glycerol). The column was washed with 10 vol of buffer B, and then the enzyme was eluted using 100 mL of a linear gradient of 0-750 mM NaCl in buffer B. Fractions (2 mL) were collected and assayed for phosphorylase phosphatase activity. Peak fractions (10-14 mL) were pooled, placed in dialysis tubing, and concentrated on solid PEG 8000 to a volume of 5 mL.

Microcystin-agarose affinity chromatography. The concentrated peak fractions were then mixed with 1 mL of microcystin-agarose equilibrated in buffer B. The slurry was allowed to incubate for 5 h at 5°C with constant mixing. The microcystin-agarose resin was transferred to a column and was then drained. The column was washed with 10 vol of buffer B containing 500 mM NaCl. One volume of 3 M NaSCN in buffer B was passed through the column; the column was capped and incubated at room temperature for 30 min. Enzyme was eluted with 7 mL of 3 M NaSCN in buffer B and dialyzed overnight at 5°C against buffer B with 2 changes of buffer.

Blue dextran chromatography. The enzyme was then applied to a blue dextran column (1 x 4 cm) equilibrated in buffer B. The column was washed with buffer B and eluted with 50 mL of a linear gradient of 0-750 mM NaCl in buffer B. Peak fractions were pooled and concentrated on solid PEG 8000 to a 0.25 mL volume.

Sephacryl S-200 gel filtration. The concentrated sample was loaded onto a Sephacryl S-200 column (1 x 30 cm) equilibrated with buffer C (buffer B but with 20% v/v glycerol and 100 mM NaCl). One mL fractions were collected and assayed for

phosphatase activity. Fractions containing enzyme were dialyzed against buffer D (buffer B with 40% v/v glycerol), and then used for kinetic analysis.

SDS-PAGE, Protein Assay, and Molecular Weight Determination

SDS-polyacrylamide electrophoresis was performed by the method of Laemmli (1970) with 12% w/v acrylamide separating gels and 5% w/v stacking gels. Proteins on the gels were stained by Coomassie brilliant blue R-250 or by silver staining (Wray et al., 1981). Protein concentrations were determined by the method of Bradford (1976) with BSA as a standard. Enzyme native molecular weights were determined by Sephacryl S-200 gel filtration with pyruvate kinase, aldolase, bovine serum albumin, carbonic anhydrase, and cytochrome c as standards.

Enzyme Kinetics

Kinetics of purified PP-1 were conducted at 37°C and 5°C. Temperature was manipulated using a Lab-Line Multi-bloc thermal controller and a cold-water bath. The assay mixtures were pre-incubated for 10 min to allow thermal equilibration before initiating the reaction with the addition of purified phosphatase. All assays were completed in 20 mM imidazole-HCl, 0.1 mM EGTA, with 15 mM β -mercaptoethanol plus various additions. The pH of imidazole-HCl buffers were fixed at room temperature and allowed to vary with temperature so that the pH at 37°C was 7.2 and the pH at 5°C was 7.5 (as explained in Chapter 2). Experimental pH values were selected to match

intracellular pH values measured in skeletal muscle of euthermic and hibernating animals as determined by *in vivo* ^{31}P NMR spectroscopy (McArthur et al., 1990).

Immunoblot Analysis

PP-1 was isolated from heart, liver, kidney, muscle and brain of euthermic ground squirrels. Frozen tissue was homogenized in 4 volumes of buffer A containing 0.1 % (v/v) Triton X-100 and protease inhibitors and was centrifuged at 10,000g for 10 min. The supernatants were applied to microcystin-agarose columns and phosphatase activity was eluted as described above. Cytosolic and glycogen fractions were prepared from euthermic and hibernating ground squirrel liver. Tissue samples were homogenized in buffer A containing protease inhibitors. The homogenate was centrifuged at 10,000g for 10 min to remove cellular debris. The supernatant, comprising cytosolic proteins and glycogen particles, was centrifuged at 100,000g for 60 min to isolate the glycogen fraction. The 100,000g supernatant (cytosolic fraction) and pellet (glycogen particles) were applied to microcystin-agarose columns and phosphatase activity was eluted as previously described.

Equal amounts of protein sample for each euthermic/hibernator pairing were diluted 1:1 (v/v) with SDS-PAGE sample buffer, boiled for 5 min, loaded onto 12% SDS-polyacrylamide gels, and then electrophoresed at 150V. Proteins were electroblotted to Immobilon polyvinylidene difluoride (PVDF) membranes (Millipore, Montreal, PQ) by wet transfer in 25 mM Tris (pH 8.3), 192 mM glycine and 10% (v/v) methanol for 50 min at 100V. Western blotting and probing of PVDF membranes was completed using a

1:1000 dilution of primary antisera. Immunoblots were developed using the ECL chemiluminescence system with HRP-conjugated goat anti-rabbit secondary antibody.

Data and Statistics

Data on organ activities of phosphatase were assessed via analysis of variance followed by the Dunnett's test, two-tailed. Statistical testing of kinetic differences between assay temperatures for purified liver PP-1 used the Student's t-test.

RESULTS

The glycogen phosphorylase phosphatase (PP-1) activity in five organs from euthermic and hibernating ground squirrels is shown in Table 6.1. Maximal total phosphatase activity was found in liver, values 4.6-fold higher than the activities found in the tissues with the lowest activity, brain and heart. During hibernation, total PP-1 activities decreased in liver and kidney by 23 and 24 %, respectively. An increase in total activity occurred in muscle (by 44%) and brain (by 93%), and no change was observed in heart. The spontaneously active component of PP-1 dropped significantly during hibernation in heart and kidney (by 75 and 68 %, respectively), increased in brain (by 82%), and was unchanged in muscle and liver. In combination, the percentage of the enzyme present in the "active" form changed significantly in 4 out of 5 tissues during hibernation, rising in liver but decreasing in the other 3 organs. Hibernating muscle and liver "active" activities were not significantly different from the corresponding euthermic values, but the % active PP-1 levels do change in muscle (decrease) and liver (increase) with respect to those found in the hibernating state.

Immunoblot analysis was used to characterize the microcystin-agarose purified PP-1 isozymes of different ground squirrel organs (Figure 6.1). All tissues except liver contained significant amounts of PP-1 γ 1. Muscle, brain, and kidney had significant amounts of PP-1 δ ; heart and liver had very low amounts. The level of PP-1 α in brain was barely detectable, but significant levels were present in heart, kidney, muscle, and liver with kidney having the highest levels.

The subcellular distribution of PP-1 α , as the predominant form of the enzyme, was assessed in liver of euthermic and hibernating squirrels; enzyme was separated into cytosolic and glycogen-associated fractions by microcystin-agarose affinity chromatography. A doublet band was observed for liver PP-1 α isolated by microcystin-agarose affinity chromatography from cytosol of both euthermic and hibernating animals (Figure 6.2). A slight amount of PP-1 α was found associated with the glycogen fraction of euthermic liver but no enzyme was detected in association with the glycogen fraction of hibernating liver.

Type-2 protein phosphatase activities

The effect of hibernation on type-2 protein phosphatase activities in ground squirrel organs is shown in Table 6.2. PP-2A activities in euthermic animals were highest in brain and heart at 6.6 mU/mg protein and were lowest in liver at 0.74 mU/mg protein. Activities in kidney, brown adipose and brain were significantly lower in hibernating specimens, 62-70 % of the values found in the euthermic condition. Hibernation had no effect on PP-2A activities in liver, muscle and heart. PP-2C activity in euthermic animals

was highest in brain and kidney at 0.6 mU/mg protein and lowest in heart (0.2 mU/mg protein); activity was undetectable in skeletal muscle. Activities in brain and liver increased during hibernation by 80 and 70 %, respectively.

Subcellular distribution of skeletal muscle PP-1

Subcellular fractionation via differential centrifugation was used to localize PP-1 activities in skeletal muscle (Figure 6.3). There was no difference in the total Triton X-100 extractable PP-1 activity found in either euthermic or hibernating skeletal muscle. About 20 % of the PP-1 activity was found in the glycogen fraction (100,000 g pellet), and this percentage was unchanged by hibernation. Seventy-six percent of the total PP-1 activity in euthermic samples was localized with the myofibril fraction (1,500 g pellet) with an additional small amount, 3 %, in the cytosolic fraction (100,000 g supernatant). Hibernation had a significant effect enzyme distribution in these latter two fractions; the percentage of PP-1 found in the cytosol increased to 12 % whereas the percentage of enzyme found with the myofibrils decreased to 67 %.

Purification of protein phosphatase type 1 from ground squirrel liver

PP-1 from euthermic ground squirrel liver was purified ~3700-fold with a final yield of 14% (Table 6.3). Phosphatase activity eluted off the DEAE-cellulose and blue dextran columns in single peaks at 285 mM and 235 mM NaCl, respectively. The final specific activity of the purified enzyme was 39.9 U/mg protein and an apparently homogeneous preparation was confirmed by a single protein staining band seen on SDS-PAGE (Figure 6.4). SDS-PAGE of the final purified enzyme indicated a subunit

molecular weight of 37 kDa, and Sephacryl S-200 gel filtration (Step 5) showed a native molecular weight of 37 ± 1 kDa ($n=3$), indicating that the enzyme was a monomer. The eluate from the microcystin column was chromatographed on a DE-52 ion exchange column (Figure 6.5) and the fractions were assayed for phosphorylase phosphatase activity. One peak of activity was resolved at 280 mM NaCl. This peak eluted from Sephacryl S-200 with an apparent molecular weight of 37 kDa, demonstrating that this protein was the catalytic subunit of PP-1.

Kinetic properties of purified ground squirrel liver protein phosphatase

The effects of various divalent cations on PP-1 activity were analyzed. Neither crude nor purified samples of PP-1 were activated by divalent cations including CaCl_2 , MnCl_2 , and MgCl_2 over a concentration range from 0 to 10 mM. Ground squirrel liver extract and the purified enzyme exhibited identical behavior with respect to inhibition by okadaic acid; crude and purified samples of PP-1 had I_{50} values of 66 ± 8 and 65 ± 7 nM, ($n=3$) respectively (Figure 6.8).

The purified enzyme was characterized with respect to inhibition by nucleotides at both hibernating and euthermic body temperatures (Table 6.4). All inhibition constants were significantly higher at the lower assay temperature. Purified PP-1 was potently inhibited by ATP and ADP at 37°C, with I_{50} values of 0.32 mM and 0.44 mM, respectively. The two adenylates were also inhibitory at 5°C but I_{50} values were over 20-fold higher. IMP was also inhibitory but at extremely high, non-physiological levels ($I_{50} = 10.3$ mM at 37°C and 7.5 mM at 5°C). Ground squirrel PP-1 was only very weakly inhibited by AMP at both temperatures examined. Cohen et al. (1988) reported that

Table 6.1. Glycogen phosphorylase phosphatase activities in ground squirrel organs

Tissue & Condition	Total Activity	"Active" Activity	% Active
Liver - Euthermic	73.8 $\pm$ 4.3	49.3 $\pm$ 2.8	67 $\pm$ 7
- Hibernating	56.7 $\pm$ 2.4*	48.1 $\pm$ 3.1	85 $\pm$ 6*
Muscle - Euthermic	23.9 $\pm$ 0.6	24.3 $\pm$ 3.7	100 $\pm$ 15
- Hibernating	34.4 $\pm$ 3.2*	21.1 $\pm$ 1.3	61 $\pm$ 9*
Heart - Euthermic	16.0 $\pm$ 1.3	17.5 $\pm$ 0.8	100 $\pm$ 13
- Hibernating	12.5 $\pm$ 2.0	4.3 $\pm$ 0.6*	34 $\pm$ 10*
Kidney - Euthermic	35.4 $\pm$ 3.8	27.2 $\pm$ 2.7	77 $\pm$ 16
- Hibernating	26.8 $\pm$ 2.2*	8.6 $\pm$ 1.0*	32 $\pm$ 6*
Brain - Euthermic	16.7 $\pm$ 2.1	10.5 $\pm$ 0.6	63 $\pm$ 12
- Hibernating	32.2 $\pm$ 2.5*	18.7 $\pm$ 1.1*	58 $\pm$ 8

Activities are expressed as mU/g wet weight tissue at 23°C. Data are means $\pm$ S.E.M. for n=4-6 separate determinations. Values are significantly different from those in corresponding euthermic tissues (Student's t-test, two-tailed): *- P<0.05.

Table 6.2. Protein phosphatase type 2A and 2C activities in ground squirrel organs

	Tissue	Euthermic	Hibernator
PP-2A	Liver	0.74 ± 0.13	0.73 ± 0.058
	Muscle	0.91 ± 0.16	1.09 ± 0.19
	Kidney	1.53 ± 0.13	$0.95 \pm 0.093^*$
	Brown Adipose	1.60 ± 0.14	$0.99 \pm 0.023^*$
	Brain	6.53 ± 0.15	$4.60 \pm 0.031^*$
	Heart	6.60 ± 0.93	5.92 ± 0.047
PP-2C	Liver	0.29 ± 0.058	$0.48 \pm 0.028^*$
	Kidney	0.60 ± 0.040	0.66 ± 0.047
	Brown Adipose	0.28 ± 0.036	0.36 ± 0.055
	Brain	0.56 ± 0.068	$1.02 \pm 0.11^*$
	Heart	0.20 ± 0.049	0.19 ± 0.021

Activities are expressed as mU/mg protein. Data are means $\pm$ S. E. M., n=4-6 separate determinations. *- Values are significantly different from the corresponding euthermic value (Student's t-test, two-tailed), $P < 0.05$. PP-2C activity was not detected in skeletal muscle.

Table 6.3. Purification of PP1 α from euthermic ground squirrel liver

Step	Total	Total	Specific	Fold-	% Yield
	Protein	Activity	Activity	Purification	
	(mg)	(mU)	(mU/mg)		
Crude	4677	50.7	0.01	1	100
12% polyethylene glycol	3487	81.5	0.02	2	161
DEAE-cellulose (DE-52)	429	34.2	0.08	7	67
Microcystin-agarose	0.99	21.5	21.7	1998	42
Blue dextran	0.63	17.0	27.1	2498	33
Sephacryl S-200	0.17	6.9	39.9	3682	14

Table 6.4. Temperature effects on nucleotide inhibition of purified PP-1 α catalytic subunit activity

I ₅₀ (mM)	Assay Temperature	
	5°C	37°C
ATP	6.3 $\pm$ 0.3	0.32 $\pm$ 0.002*
ADP	12.1 $\pm$ 1.6	0.44 $\pm$ 0.04*
AMP	>25	8.2 $\pm$ 0.5*
IMP	7.5 $\pm$ 0.5	10.3 $\pm$ 0.1*

Data are means $\pm$ SEM for n=3 separate enzyme preparations. *Significantly different from the corresponding 5°C value, P < 0.01.

Figure 6.1. Expression of PP-1 α , PP-1 δ and PP-1 γ 1 proteins in various organs of euthermic ground squirrels. Microcystin-agarose was used to affinity purify PP-1 in extracts from heart (1), muscle (2), liver (3), brain (4), and kidney (5). A sample of recombinant bovine PP-1 γ 1 was used as a control (+ve). Samples of eluates containing 40 μ g protein were subjected to electrophoresis on 12% w/v SDS-PAGE gels. This was followed by immunoblotting using the indicated antibodies and detection of a 37-39 kDa immunoreactive band by ECL chemiluminescence system with HRP-conjugated goat anti-rabbit secondary antibody. Anti-PP-1 γ 1 (A), anti-PP-1 α (B), or anti-PP-1 δ (C) antibody was used as the primary antibody.

Figure 6.2. PP-1 α isozyme levels in glycogen-particle (G) and cytosolic (C) fractions of euthermic and hibernating ground squirrel liver. Microcystin-agarose was used to affinity purify PP-1 from the glycogen and cytosolic fractions as described in “Materials and Methods.” Care was taken to ensure equal protein loading between the euthermic and hibernating samples. Immunoblotting with the anti- PP-1 α antibody and detection were as described in Figure 6.1. Bio-Rad "Kaleidoscope" pre-stained standards, from top to bottom, were bovine serum albumin (87 kDa), carbonic anhydrase (44 kDa), and soybean trypsin inhibitor (32 kDa).

Figure 6.3. Distribution of glycogen phosphorylase phosphatase (PP-1) activity in subcellular fractions of euthermic and hibernating ground squirrel muscle. Enzyme activity is expressed as percentages relative to activity of the euthermic enzyme in the crude homogenate. Subcellular fractions are (W) Triton X-100 extracted crude homogenate, (M) Triton X-100 washed myofibrils, (G) glycogen-particles, and (C) cytosolic supernatant. Data are means $\pm$ S.E.M. of 4 separate determinations. * - significantly different from corresponding euthermic value, $P < 0.05$.

Figure 6.4. Purification of PP-1 α catalytic subunit from ground squirrel liver. SDS-PAGE with silver staining of samples taken from different steps in the purification; lanes are (A) PEG precipitation, (B) microcystin-agarose, and (C) Sephacryl S-200 gel filtration. Standards, from top to bottom, were myosin (210 kDa), β -galactosidase (116 kDa), glycogen phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa).

Figure 6.5. Elution profile of ground squirrel liver phosphorylase phosphatase activity from DE-52 ion exchange chromatography. Pooled dialyzed eluate from the microcystin-agarose column was applied to the column (1 x 1 cm). Enzyme was eluted with a 35 mL of a linear gradient of NaCl (0-0.5M) in buffer B, and fractions of 1.0 mL were collected and assayed for PP-1 (●) activity. The dotted line denotes the NaCl gradient.

Figure 6.6. The effect of pH on purified ground squirrel liver PP-1 α activity at 5°C (o) and 37°C (●). Data are means $\pm$ SEM for n=3 separate enzyme preparations and are relative to the maximum activity at the optimal pH. PP-1 activity was measured in 20 mM imidazole-HCl, 0.1 mM EGTA, 15 mM β -mercaptoethanol.

Figure 6.7. Arrhenius plot of ground squirrel liver PP-1 α activity versus temperature. Activity was measured in 20 mM imidazole-HCl, 0.1 mM EGTA, 15 mM β -mercaptoethanol. pH was defined to be 7.2 at 23°C and then allowed to self-adjust with changing temperature. The assay mixtures were pre-incubated for 15 min to allow temperature equilibration before initiating the reaction with the addition of purified PP-1 α . Data are means $\pm$ SEM for n=3 separate enzyme preparations. SEM values are contained within the symbol.

Figure 6.8. Effect of okadaic acid on ground squirrel liver protein phosphatase activity. Purified PP-1 α (○) and crude liver (●) extracts (1:25 dilution) were assayed with increasing concentrations of okadaic acid in the absence of divalent cations and in the presence of 0.1 mM EGTA. Activities are means of three determinations and are relative to enzyme activity in the absence of okadaic acid. SEM values are contained within the symbols.

caffeine blocked the inhibitory effect of AMP and also activated the mammalian enzyme. However, the addition of 7.5 mM caffeine or theophylline in the assay was of little significance; neither crude nor purified ground squirrel liver PP-1 activity was affected by caffeine or theophylline in the absence or presence of AMP at either of the temperatures examined.

The pH curves for ground squirrel PP-1 assayed at euthermic and hibernating body temperatures are shown in Figure 6.6. The pH optimum at 37°C was quite narrow and centred at pH 6.9; the optimum pH range at 5°C was much broader, stretching from pH 5.8 to 7.5. The effect of temperature on ground squirrel PP-1 activity is shown as an Arrhenius plot in Figure 6.7. A distinct break in the plot was seen at 9°C. The E_a for the reaction above 9°C was 59 ± 2 kJ/mol and below 9°C was 144 ± 5 kJ/mol.

DISCUSSION

The studies reported in the previous two chapters documented the importance of protein kinases and protein phosphorylation in the regulation of metabolism during hibernation in ground squirrels. Given that the patterns of protein kinase activities changed during hibernation, it was predicted that protein phosphatase activities should also be modified to support the metabolic rearrangement that accompanies hibernation. The results show that tissue specific changes in phosphatase activities occurred in hibernation. Furthermore, specific changes in the subcellular localization of phosphatase activities, eg. PP-1 (this study) and PTPase (MacDonald and Storey, unpublished data) also occurred in a number of tissues.

The phosphatase purified from ground squirrel liver showed the characteristics of a protein phosphatase type-1 and lacked properties that are typical of type-2 phosphatases (e.g. activation by cations, very strong inhibition by okadaic acid in the 0.5 nM range). Like the mammalian enzyme from other sources, the ground squirrel PP-1 α catalytic subunit showed high activity with glycogen phosphorylase a as its substrate and was moderately inhibited by okadaic acid. The I_{50} value (65 nM) for purified PP-1 α was close to the range (10-20 nM) reported for the mammalian enzyme from other sources (Cohen, 1991). Shima et al. (1993) revealed differences by immunoblot in the proportions of the different PP-1 catalytic subunit isozymes found in various rat tissues. They found that PP-1 γ 1 was predominant in the brain and was low in liver and muscle. PP-1 δ was predominant in the brain, lung, and small intestine but was low in the liver and muscle. PP-1 α was not a predominant isotype in any tissue examined. These immunoblot results seem strange since the authors show significant levels of PP-1 α , PP-1 γ 1, and PP-1 δ mRNA in liver within the same paper. Villa-Moruzzi et al. (1996) report high levels of PP-1 δ , moderate levels of PP-1 α and low levels of PP-1 γ 1 in mouse muscle. Different isozyme levels have also been detected in HeLa and NIH-3T3 cell extracts (Villa-Moruzzi et al., 1996). These results may reflect real tissue and species differences in the amounts of the different isozymes or alternatively, differences in the affinities of the three antibodies.

The importance of covalent modification in the regulation of glycogen phosphorylase and the control of glycogenolytic rate during hibernation in mammals has been previously illustrated (Brooks and Storey, 1992). These authors found a significant

decrease in both total and active glycogen phosphorylase in the hibernating condition.

Decreased GP_a activity will limit glucose export to the blood and is consistent with the primary dependence on fatty acid oxidation during hibernation. Substances that interact with PP-1, PP-2A and PP-2C directly alter the rates of dephosphorylation of many proteins. Selective effects can be achieved by the interaction of metabolites with substrates. For example, the binding of glucose to hepatic phosphorylase a stimulates its dephosphorylation by PP-1 (Hers, 1976).

AMP, an allosteric activator of phosphorylase b, selectively inhibits the dephosphorylation of GP_a by making it a worse substrate for PP-1 (Pelech et al., 1984). Substances that accelerate the conversion of phosphorylase a to b (i.e. glucose and caffeine) abolish the AMP inhibition of PP-1 GP_a dephosphorylation. However, ground squirrel PP-1 was only affected by high non-physiological levels of AMP. AMP levels remain constant or rise slightly in liver during hibernation (Storey and Kelly, 1995), yet temperature effects drastically decrease the ability of AMP to inactivate PP-1. Also the I_{50} values for AMP for ground squirrel PP-1 catalytic subunit are totally outside any relevant physiological range of AMP. AMP inhibition of GP_a dephosphorylation is key to regulation in typical mammalian model systems (Gratecos et al., 1975), and there are very dramatic differences for I_{50} AMP values in other species. For example, the typical mammalian enzyme shows a 85% reduction in phosphatase activity toward GP_a in the presence of 200 μ M AMP (Pelech et al., 1984), and turtle liver PP-1 had I_{50} values for AMP, ADP and IMP (23 μ M, 99 μ M, and 389 μ M respectively) that were very different to the values determined for the ground squirrel enzyme. The turtle PP-1 I_{50} value for ATP (525 μ M) was similar to the value determined for ground squirrel PP-1 (Mehrani

and Storey, 1995). This unique observation appears to result from a unique interaction between two proteins, the catalytic PP-1 subunit and GP α . AMP normally acts as an allosteric activator of GP β ; converting the enzyme to its active form while simultaneously preventing dephosphorylation of the active form by PP-1. Removing AMP effects on GP α dephosphorylation should therefore aid in the conservation of liver glycogen stores during hibernation. Hibernating liver PP-1 might also be composed of PP1 catalytic subunit complexed to a glycogen binding (G_L) subunit.

The active forms of PP-1 are believed to be largely particulate, and their association with the subcellular structures is mediated by targeting subunits that direct PP-1 to particular locations, enhance its activity toward certain substrates and confer important regulatory properties upon it. This concept is best established for the glycogen-bound enzymes in skeletal muscle (PP-1 G_M) and liver (PP-1 G_L) and the myofibrillar form (PP-1 M) in skeletal muscle (for review, Cohen, 1989). Glycogen-bound PP-1 (i.e. PP-1 G_L) in liver is composed of PP-1 catalytic subunit complexed to a G_L subunit. Hepatic PP-1 G_L has greatly enhanced activity towards glycogen synthase while activity towards phosphorylase is strongly suppressed. Hepatic PP-1 G_L also has a remarkable sensitivity to phosphorylase α . The I_{50} for phosphorylase α is in the nanomolar range, more than 1000-fold lower than its K_M as a substrate, suggesting that inhibition occurs by an allosteric mechanism. Inhibition of PP-1 G_L by phosphorylase α is a mechanism for inhibiting glycogen synthesis when glycogenolysis is activated and vice versa.

Inhibition of PP-1 G_L by phosphorylase α only occurs in the fed state (Mvumbi and Stalmans, 1987). In fasted animals, glycogen synthase is largely dephosphorylated despite a high level of phosphorylase α . This is explained by the very low levels of

glycogen in fasted animals. Hepatic PP-1_{GL} is inhibited by GP_a much more potently in the presence of glycogen. But, during hibernation, even though animals stop eating, glycogen phosphorylase activity is suppressed to spare carbohydrate reserves as opposed to starvation where glycogen is rapidly depleted (Hochachka and Somero, 1984).

Decreases in liver %GP_a observed in hibernation could occur via three mechanisms: 1) by actively increasing the dephosphorylation potential (PP-1 activity) relative to the phosphorylation potential (% PKAc), 2) by actively suppressing the phosphorylation potential relative to unchanging dephosphorylation potential, or 3) by altering the levels of allosteric substances that accelerate the conversion of phosphorylase a to b (caffeine, glucose). The purpose of glycogen degradation in liver is to form glucose for export to other tissues when the blood glucose level is low. Liver glycogen phosphorylase is essentially regulated by phosphorylation via cAMP and PKA and by allosteric effectors; the enzyme is responsive to glucose and less so to AMP, an indicator of its own metabolic status (Gratecos et al., 1973). Liver cAMP levels were unchanged in hibernation as was the percentage of PKA present as the free catalytic form (Chapter 4). The % active of PP-1 in liver increased in the hibernating condition as a result of an overall decrease in the total measurable activity (Table 6.1). The catalytic PP-1 α subunit was not associated with the glycogen fraction in liver during hibernation (Figure 6.2) which indicates a decrease in glycogen use and an increase in glycogen storage. The association of the PP-1 catalytic subunit with the liver regulatory subunit GL targets the catalytic subunit to the glycogen particles and decreases the dephosphorylation of GS

relative to the dephosphorylation of GP_a by 10-fold. Also, since blood glucose levels are not affected by hibernation, allosteric inactivation of GP_a by glucose should remain high.

Protein phosphatases 2A and 2C exhibited differences in maximal activities in several organs between the hibernating and euthermic states. PP-2A activity was significantly reduced in 3 organs whereas PP-2C rose in both brain and liver during hibernation. The activities of PP-2A and PP-2C can be modulated by extracellular signals and can thereby modulate signal transduction pathways via the MAP-kinase cascade (Wera and Hemmings, 1995) and the AMP-activated protein kinase (AMPK) cascade (Corton et al., 1994). PP-2C can also account for a significant proportion of the glycogen synthase phosphatase activity; however, PP-2C is not specific for glycogen synthase and strong evidence has been presented for dephosphorylation of both GS and GP by a single form of PP-1 (Alemany et al., 1986). Recent analysis of yeast PP-2C isoforms has provided clues about the *in vivo* targets of PP-2C. The enzyme has been associated with signal transduction processes involving an osmosensing MAP kinase cascade comprising the high osmolarity glycerol response 1 (HOG1) kinase (Shiozaki and Russell, 1995).

In cell free assays, PP-2A and PP-2C also inactivate the AMPK cascade; however, in isolated hepatocytes okadaic acid had no effect on the dephosphorylation of Acetyl CoA carboxylase which implies that PP-2C may be responsible for dephosphorylation of AMPK in liver (Hardie and Carling, 1997). AMPK exerts the major control over fatty acid synthesis, and potential suppression of the cascade by increasing PP-2C activity during hibernation is not unexpected since the animals are not eating and, hence, not synthesizing fatty acids. Indeed, fatty acid oxidation dominates as the primary source of

metabolic fuel during torpor. A complete study of the AMPK cascade in hibernation has not been initiated, though such a study would likely provide useful information regarding metabolic control during hibernation. This protein kinase cascade represents a sensitive system which is activated by cellular stresses that deplete ATP, and thus acts like a cellular fuel gauge. By detecting low fuel situations it protects the cell by switching off ATP-consuming pathways (e.g. fatty acid synthesis and sterol synthesis) and switching on alternative pathways for ATP generation (e.g. fatty acid oxidation) (Hardie and Carling, 1997). The present study demonstrates that alterations in activities of protein phosphatases PP-1, as well as PP-2A and PP-2C, occur in response to hibernation in a tissue specific manner. Changes in their activity levels, in concert with changes in protein kinase activities, suggests the relative importance of these modules in controlling metabolic function during hibernation. The mechanisms of "cross-talk" between kinases and phosphatases during hibernation continues to be investigated.

CHAPTER 7

General Discussion

Transitions to and from the hypometabolic state in hibernating mammals require: 1) coordinated control for the suppression of all cellular functions and metabolic flux, and 2) adaptations enabling enzyme function over wide temperature ranges. One of the most powerful mechanisms for producing coordinated changes in the activity states of proteins and enzymes in cells is post-translational modification via protein phosphorylation and dephosphorylation reactions. Indeed, the reversible phosphorylation of proteins regulates most aspects of cell life. A dynamic balance exists between phosphorylation and dephosphorylation, resulting from interplay between protein kinases and protein phosphatases. As a consequence, modification of either component is likely to have an equally important impact on signal transduction. The occurrence of controlled arousal from hibernation also indicates that cellular senses are maintained at hibernating body temperatures, and mechanisms for the rapid increase in basal metabolic rate are functional at low temperature. Mammalian hibernators, which endure prolonged hypothermia, provide an excellent model for studying the kinase/phosphatase control of cellular function in a depressed metabolic state.

Control of energy utilization in hibernation

With respect to energy utilization during hibernation, fat metabolism supports nearly all the energy needs (Nedergaard and Cannon, 1984), and glucose oxidation is profoundly inhibited due to the depression of glycolysis. The preferential utilization of fat results in increases in plasma ketone bodies, and changes in the mitochondrial oxidative pathway favor the utilization of fat. The consequence of these changes observed during hibernation is glucose sparing. Since hibernating ground squirrels are typically in a

fasting state (these species do not feed between hibernation bouts), glucose sparing ultimately leads to conservation of tissue protein. Carbohydrate reserves are quickly exhaustible. The precursors of glucose are scarce, since most available carbon is stored as fat, which in animals (lacking the glyoxylate cycle) cannot be converted into glucose because acetyl CoA cannot be transformed into pyruvate. The only other potential source of glucose is amino acids derived from the breakdown of proteins. Muscle is the largest source of protein, but a compromise must be struck between the demands for glucose and the preservation of muscle for locomotion. The result is a dependence on ketone bodies derived from fat stores as a primary fuel source.

Previous studies argued that the suppression of PFK activity, the key regulatory enzyme of glycolysis could be attributed to a combination of low intracellular pH and Q_{10} effects on enzyme rate as the result of the low body temperature during hibernation (Hand and Somero, 1983). Brooks and Storey (1992) further showed that glycolytic flux was also gated at glycogen phosphorylase and pyruvate dehydrogenase, in these instances by reversible phosphorylation to interconvert active and inactive enzyme forms. Furthermore, additional regulatory control of PFK appeared to be a result of changes in the cellular concentrations of allosteric molecules, most noticeably fructose 2, 6,-bisphosphate. Proposing that the PFK suppression attributed to low pH was, in fact, an artifact of the use of dilute protein solutions for *in vitro* assays, I began my analysis of the regulatory control of enzymes during hibernation with a re-examination of the effects pH and cold on the inactivation of PFK under conditions that would mimic the crowded conditions of the intracellular environment. When measured under such conditions, produced via the use of high PEG concentrations, cold inactivation of PFK was found to

be insignificant. As well, the optimum pH for enzyme function shifted with temperature so that PFK appeared to be always fully functional with respect to intracellular pH *in vivo*. Thus, the regulation of PFK activity during hibernation seems to stem from other mechanisms; the temperature-induced inversion of allosteric behaviour observed for inorganic phosphate, and/or from temperature influences on the catalytic, activating and inhibitory sites. These properties serve to link the concentrations of allosteric metabolites with temperature and enzyme activity to permit carbohydrate sparing during hibernation.

Hypothermia disrupts energy metabolism in non-hibernators (Hochachka, 1986). Under many environmental stresses, including ischemia, hypoxia, heat shock, and arsenite treatment, the imbalances created by differential effects of stress on the ATP-producing versus ATP-utilizing reactions in a cell result in a net depletion of ATP with a concomitant rise in AMP concentration (Corton et al., 1994). Concentrations of AMP are normally very low and rising AMP is a key signal that activates catabolic pathways (e.g. glycolysis via AMP activation of PFK and glycogenolysis via AMP activation of GPb) to increase ATP synthesis. This situation would clearly be disadvantageous for a hibernator during torpor. And, indeed, although ATP falls during hibernation, a corresponding rise in AMP did not occur. This, plus the maintenance of a constant energy charge during hibernation, indicated that reduction in net ATP turnover that defines the hypometabolic state must be the result of controlled suppression of the rates of all metabolic processes in cells. This must include all the major consumers of cellular ATP in mammalian cells, such as the Na⁺K⁺-ATPase, which continuously hydrolyzes ATP to maintain the transmembrane gradients of sodium and potassium ions. Cells from hibernating

mammals are more proficient at maintaining ion gradients in the cold than non-hibernator cells (Willis, 1986), and one way to facilitate hypometabolism may be to suppress the rate of ATP consumption devoted to the pumping of ions. My results showed that the Na/K pump activity was depressed in a number of ground squirrel tissues during hibernation. This decrease in maximal activity could be accounted for by reversible phosphorylation of the protein since incubations in the presence of protein kinases or their activators caused a decrease in activity that could be recovered by incubation in the presence of alkaline phosphatase or phosphatase activators.

A kinase cascade has been identified that protects cells during periods of stress when the cellular fuel status is compromised (Hardie, 1994). The AMP-activated protein kinase (AMPK) acts as a metabolic sensor which monitors the levels of the key metabolites AMP and ATP, and is activated by increases in the AMP:ATP ratio. It then switches off ATP-consuming anabolic pathways and switches on ATP-producing catabolic pathways such as fatty acid oxidation. Thus a system which protects against glucose starvation in lower eukaryotes (Woods et al., 1994) appears to have been adapted to protect against environmental stress in vertebrates (Corton et al., 1994). AMPK function in hibernation remains to be explored. Yet, if the AMPK cascade is integrated with other kinase cascades, changing energy status during hibernation could control the coordination of all factors produces a balancing of anabolic and catabolic pathways.

Protein kinase and phosphatase action in hibernation

The amount of PKA that was present as the active catalytic subunit was not greatly effected by hibernation. The levels of second messengers, cAMP and cGMP were also

relatively unaffected by hibernation; cAMP decreased in brain and heart, and cGMP levels were unchanged in all tissues except kidney (Appendix 2). The specific changes to the structure and kinetic properties of the catalytic subunit of PKA in ground squirrel BAT facilitate enzyme function at both high and low body temperatures and enable the activation of thermogenesis by facilitating a rapid increase in the phosphorylation of PKAc target proteins. The amount of PKC activity found in the membrane-associated, or active state, was affected by hibernation in liver (Appendix 1). The members of the PKC family respond differently to various combinations of lipids including several phospholipids, particularly PS, DAG, fatty acids, and probably many other lipid metabolites in membranes; therefore, the patterns of activation of PKC isoforms may vary in extent, duration, and intracellular localization (Nishizuka, 1995). An examination of the temperature effects on ground squirrel PKC isozymes and phospholipid activation remains to be completed.

Eukaryotic cells use a variety of intracellular signaling pathways to respond to changes in their external environment. Among these pathways, those that activate kinases belonging to the mitogen-activated protein kinase family have received particular attention because many of these enzymes translocate to the cell nucleus to regulate transcription factor activity and gene expression (Su and Karin, 1996). Novel changes in gene expression and gene products that support survival in hibernation can be identified from cDNA library construction and differential screening. Indeed, application of this methodology has resulted in the identification of unique genes activated by hibernation (Cai and Storey; Fahlman and Storey, unpublished data). Whether the expression of these genes is regulated by MAPK activation is unknown, but activation of the MAPK

cascade offers the ability to regulate genetic as well as metabolic aspects of cell function in hibernation. Increases or decreases in specific MAPK activities were tissue specific; changes in ERK activity were independent of changes in JNK or p38 activity. The results of Chapter 5 suggest an increase in transcriptional activation at the cAMP response element during hibernation since a significant activation of CREB, as determined by increases in its level of phosphorylation, was present in several tissues. CREB phosphorylation in hibernating brain may be a result of increased MAPKAPK-1 activity; however, the kinase(s) responsible for increases in phospho-CREB levels in other tissues remains unidentified.

Individual kinases and phosphatases are involved in the regulation of multiple processes. How do these enzymes select the correct substrates at the correct time to coordinate the complex organization of cellular events? One factor involved in substrate selection is substrate structure (Kemp et al., 1994; Pinna and Donella-Deana, 1994). The other major factor determining target specificity may be restricting the subcellular distribution of kinase and phosphatase activities via subcellular compartmentation of the enzymes or their activators. Recent studies have shown that protein kinases and phosphatases are targeted to specific compartments of cells and are translocated dynamically during cellular events (Inagaki et al., 1994). For example, at least 15 distinct PP-1 regulatory subunits have been identified in mammals (Egloff et al., 1997), and these allow PP-1 to regulate functions as disparate as glycogen metabolism, muscle contraction and exit from mitosis. Differences in subcellular localization of PP-1 activity were evident in muscle and liver during hibernation, but further investigation is required to determine if protein kinase activities are localized to specific subcellular areas during

hibernation. It is known that type II PKA is targeted by association of its regulatory subunit (RII) with an A kinase anchoring protein, AKAPs (Coghlan et al., 1993). Several PKC-binding proteins, that act as receptors for the activated form of PKC, have been isolated from mammalian tissues (Mochly-Rosen, et al., 1991). These proteins have been termed RACKs (receptors of activated C-kinase), and their phospholipid-dependent binding to PKC could serve to stabilize the association of the active enzyme to specific areas of the membrane (Liao et al., 1994).

Thermal adaptation of enzyme function

By purifying and kinetically characterizing enzymes that are key to cellular regulation, we can determine whether enzymes are adapted to cope with both the temperature changes and metabolite re-adjustments associated with hibernation. PKA and PP1 were selected as key members of the kinase and phosphatase families that have key regulatory functions in glycogenolysis, and phosphofructokinase was chosen as the key regulatory enzyme of glycolysis.

The inhibition of PFK activity by inorganic phosphate at low temperatures is a unique finding. Although PFK isolated from microbial sources demonstrated unique changes in ADP allostery with temperature change (Braxton et al., 1994), this is the first example of thermal changes in allostery on the mammalian enzyme. The ground squirrel enzyme is less likely to be activated and more likely to be inhibited in the torpid muscle; hence, muscle glycolysis is less sensitive to changing energetic state when torpid. These unique kinetic properties are only induced at the low body temperatures that distinguish

hibernation. The enzyme from a non-hibernator (rabbit) does not exhibit these low temperature effects on allostery (MacDonald and Storey, unpublished).

The functional and structural adaptations of regulatory enzymes in the hibernator were further explored with PKA. Characterization of the catalytic subunit purified from BAT revealed that it was different from the typical non-hibernator enzyme. The thermal effects on substrate affinity constants were distinctive for the hibernating species when compared with those from the non-hibernator. Changes in the $V_{\max}$ with temperature indicated hibernator PKAc was less temperature sensitive than the enzyme from a non-hibernating mammal. Temperature also appeared to have an effect on protein phosphorylation *in vitro*; the number and identity of BAT proteins phosphorylated by PKA *in vitro* differed when temperature was changed. The number and identity of protein kinase substrates may change *in vivo* because 1) hibernation is known to induce novel protein expression in BAT (Cannon et al., 1996), some of which may be PKA substrates, and 2) temperature effects on substrate protein conformation may change the amount or rate of *in situ* phosphorylation.

Appendix 1: Effect of hibernation on total protein kinase C activity and the percentage of the enzyme associated with the plasma membrane in selected Richardson's ground squirrel tissues

Tissue & Condition		Cytosolic (mU/gww)	Membrane Bound (mU/gww)	Total Activity (mU/gww)	% Membrane Bound
Brain					
	Euthermic	50.4 ± 5.6	19.5 ± 1.7	69.9 ± 7.3	28.1 ± 1.8
	Hibernating	51.2 ± 4.4	20.3 ± 4.3	71.5 ± 8.7	25.0 ± 6.9
Kidney					
	Euthermic	0.55 ± 0.08	0.92 ± 0.10	1.77 ± 0.12	62.5 ± 5.0
	Hibernating	0.54 ± 0.03	0.67 ± 0.07	1.33 ± 0.16	50.1 ± 4.1
Liver					
	Euthermic	0.26 ± 0.02	0.41 ± 0.08	0.84 ± 0.06	59.3 ± 5.6
	Hibernating	0.19 ± 0.01	0.67 ± 0.05*	0.90 ± 0.05	76.9 ± 1.9*

Values are means ± SEM, n=4 animals in each condition for assays with MARCKS peptide as the substrate. *- Values are significantly different from the euthermic value at $p < 0.05$. Student's t-test, two-tailed.

Protein kinase C assay

Protein kinase C was monitored by a radioactive assay that measured incorporation of radiolabeled ^{32}P -ATP onto the phosphate accepting artificial substrate "Durkin peptide" (Chakravarthy et al., 1991). The desired amounts of phosphatidylserine and 1,2-diacylglycerol were dissolved in chloroform/methanol (19:1, v/v) and stored at -20°C until use. Working solutions containing lipids were prepared fresh daily. Phospholipid and diacylglycerol were placed in a 1.5 mL microcentrifuge tube on ice, then the mixture

was dried under a stream of nitrogen gas, and solubilized in 25 mM HEPES buffer by vortexing, sonicating 2x 30 sec, and incubating at 30°C for 10 min. The lipid preparation was kept at room temperature away from a light source. The standard assay contained 30 mM HEPES, pH 7.4, 5 mM MgCl₂, 5 mM β-mercaptoethanol, 0.5 mM CaCl₂, 20 μM ATP, 6.5 μg/mL phosphatidylserine (PS), 1 μg/mL diacylglycerol (DAG), and 100 μM Durkin peptide (FKKSFKL-NH₂). The reaction was started with the addition of suitable amounts of enzyme (proved to be optimal for time and temperature by preliminary analysis). Assays were terminated by spotting samples (35 μL) onto 2 x 2 cm pieces of Whatman P81 paper which were then washed four times with 75 mM phosphoric acid and counted using Cerenkov methodology. PKC activity was determined in the presence of calcium plus phospholipids; non-specific enzymatic activity was measured in the absence of Ca²⁺ and phospholipids, and values were subtracted as background. One unit of PKC is defined as the amount of enzyme that catalyzes the incorporation of 1 μmol of phosphate onto a phosphate acceptor per minute.

Preparation of extracts for the determination of % membrane associated PKC

Samples of frozen tissue from euthermic or hibernating ground squirrels were homogenized 1:10 (w/v) in ice-cold 0.25 M sucrose, 10 mM mercaptoethanol, 5 mM EGTA, 5 mM EDTA, 1 mM PMSF, 10 μg/mL leupeptin, 2 μg/mL aprotinin, 10 μg/mL soybean trypsin inhibitor and 20 mM 4-(2-hydroxyethyl)-1-piperazine ethansulfonic acid (HEPES) pH 7.4 (buffer A), for 30 s using an Ultra-turrax (Tekmar) tissue homogenizer. After centrifugation at 1800 g for 10 min, the supernatant was removed and centrifuged at

100,000g for 30 min to yield a pellet (membrane fraction) and a supernatant (cytosol fraction). The supernatant was immediately loaded onto a Whatman DE-52 ion-exchange column (1.0 mL of bed volume for 0.1 g tissue) equilibrated in buffer A and washed with 5 vol of buffer B (20 mM HEPES pH 7.4, 10 mM β -mercaptoethanol, 1 mM EGTA, 1 mM EDTA, 10% v/v glycerol) and 5 vol of buffer B containing 50 mM NaCl. The wash did not contain detectable PKC activity. Enzyme was eluted by washing the column with 4 mL of 350 mM NaCl in buffer B; this salt concentration was determined to be optimal from the elution position of the enzyme in preliminary experiments using a NaCl gradient. Soluble PKC was then immediately assayed. The pellet containing the membrane fraction was rehomogenized in one-half the original volume of buffer A + 0.2% v/v Triton X-100, incubated for 30 min on ice and then centrifuged at 100,000g for 30 min. The resulting supernatant (membrane-associated fraction) was loaded onto a fresh DE-52 column and treated in an identical fashion as the first supernatant.

Appendix 2: cGMP levels in tissues of euthermic and hibernating *S. richardsonii*

Tissue	Euthermic [cGMP] (pmol/ g wet weight)	Hibernating [cGMP] (pmol/g wet weight)
Liver	63.6 ± 21.2	88.2 ± 21.2
Kidney	113.4 ± 10.6	64.9 ± 3.5*
Skeletal muscle	54.0 ± 7.7	65.1 ± 5.1
Heart	47.7 ± 2.6	56.3 ± 11.9
Brain	89.1 ± 11.5	106.8 ± 18.7

Values are means ± SEM, n=4 animals in each condition. *- Values are significantly different from the euthermic value at $P < 0.05$, Student's t-test (two-tailed).

For the determination of cGMP levels, perchloric acid extracts of frozen ground squirrel tissues were prepared as in Chapter 4. cGMP concentrations were determined from competitive protein binding assays using the commercial RIA kit from Amersham.

PUBLICATION LIST

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