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**Enzymes of adenylate metabolism from the skeletal muscle of the
hibernating prairie dog, *Cynomys leucurus***

by

Tamara Erica English, B.Sc. (honours)

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

Master of Science

**Ottawa-Carleton Institute of Biology
Carleton University
Ottawa, Ontario
November, 1995**

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**Enzymes of AMP metabolism from the skeletal muscle of
the hibernating prairie dog, *Cynomys leucurus***

submitted by
Tamara Erica English, B.Sc. (hons)

in partial fulfillment of the requirements for
the degree of Master of Science

Chair, Department of Biology
Thesis Supervisor

Carleton University
1995

Abstract

A varied group of mammals have evolved the ability to hibernate in response to limited energy resources in winter. In many hibernating mammals, including *Cynomys leucurus* (the subject of this study) adenylate levels drop over the course of hibernation but the relative amounts of ATP, ADP, and AMP (also referred to as the energy charge) remain stable. Two enzymes, AMP deaminase (AMPD) and adenylate kinase (AK) are known to be integral to the regulation of adenylates, were purified from skeletal muscle and examined at warm and cold temperatures to (1) assess functional ability at cold temperature and (2) elucidate a potential role in the stabilization of energy charge during hibernation. Hibernator AMPD had a higher activation energy and was more sensitive to inhibition by ADP, ATP, GTP, and salts than rabbit AMPD at cold temperatures. Though AMPD was still functional at cold temperatures, its activity was not enhanced with respect to a non-hibernator enzyme. AK showed a similar trend; the hibernator and rabbit enzymes shared many kinetic and functional parameters including the inhibitory effects of salts, activation by magnesium, pH optima, E_a values and effect of urea on enzyme stability. Only sensitivity to high temperature incubations showed the two enzymes to be distinct, with the hibernator enzyme being more sensitive to high temperature over time. It was proposed that this may be the result of a more flexible structure for the *C. leucurus* enzyme. Because kinetic and metabolite data do not support a role for these enzymes in energy charge stabilization, other possible research avenues were proposed.

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List of Abbreviations

ADP - adenosine diphosphate

AK - adenylate kinase

AMP - adenosine monophosphate

AMPD - AMP deaminase

ATP - adenosine triphosphate

E_a - activation energy

EC - energy charge

EDTA -ethylenediamine tetraacetate

G-6-PDH - (NAD^+ -dependent) glucose-6-phosphate dehydrogenase

G-6-P - glucose-6-phosphate

GTP - guanidine triphosphate

HK - hexokinase

HPRT - hypoxanthine phosphoribosyl transferase

I_{50} - concentration of inhibitor that lowers enzyme velocity by 50%

IEF - isoelectric focusing

IMP - inosine monophosphate

K_a -concentration of activator that produces half maximal activation of the enzyme

K_m - Michaelis-Menten constant (substrate affinity constant)

LDH - lactate dehydrogenase

NAD^+ - nicotinamide adenine dinucleotide, oxidized form

NADH - nicotinamide adenine dinucleotide, reduced form

NP - nucleoside phosphorylase

NST - non-shivering thermogenesis

5'NT - 5'nucleotidase

PDH - pyruvate dehydrogenase

PEG - polyethyleneglycol

PEP - phosphoenolpyruvate

PFK - phosphofructokinase

6-PG - 6-phosphogluconate

pI - isoelectric point

Pi - inorganic phosphate

PK - pyruvate kinase

PKC - protein kinase C

PMSF - phenylmethylsulfonyl fluoride

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

T_b- body temperature

V_{max} - maximal enzyme velocity

XO - xanthine oxidase

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Chapter 1

General Introduction

Environments where climatic conditions change drastically with the seasons present thermoregulatory challenges to small mammals. The maintenance of endothermy is metabolically expensive and requires sustained levels of energy utilization and food consumption to generate heat and maintain a relatively high, constant body temperature. Cold winter temperatures demand an increased metabolic output to sustain body temperature, but at the same time food supplies decline when the mammal needs them most. While birds have the option of migrating to warmer climates with richer resources, terrestrial mammals have little choice but to adjust to the cold environment. To avoid this energy challenge, some mammals have evolved the ability to hibernate.

Hibernation

Hypothermia is a lethal stress for most mammals, inducing a severe hypoglycemia (Hochachka, 1986) and a rapid dissipation of membrane potential difference (Wang and Wolowyk, 1988). However, hibernators do not show these effects and have developed the ability to sharply reduce metabolic rate and body temperature in order to sustain life on only a fraction of the fuel reserves that would otherwise be required. These animals survive long periods of time with body temperatures (T_b) lowered to near ambient, often as much as 35°C lower than the euthermic T_b . They are cold to the touch, immobile, and exhibit very low breathing and heart rates. By abandoning homeothermy and allowing T_b to fall to within a few degrees of freezing, hibernators can reduce the total energy expenditures required for overwintering by as much as 88 percent (Wang, 1989). During torpor, hibernators rely on endogenous fuel reserves that are deposited prior to the hibernation season. During a period of autumn hyperphagia, hibernators increase their body weight dramatically; the smallest of these frequently double their body weight in 4 to 6 weeks (Wang, 1989). The additional weight is primarily deposited as two types of

fat stores which can be mobilized as needed over the winter: white adipose tissue (reserves to fuel basal metabolism) and brown adipose tissue (reserves to fuel the non-shivering thermogenesis that is important for rewarming the body during arousal) (Dark and Ruby, 1993).

Although at least five orders of the Mammalia (Insectivora, Rodentia, Chiroptera, Monotremata, and Marsupialia) contain species that are capable of hibernation (Lyman, 1982), not all mammals can hibernate. Even among species that do hibernate, variation exists; some species experience obligatory bouts of winter hibernation whereas others hibernate only if temperature and food conditions necessitate. The duration of individual torpor bouts also varies within and between species and varies over the winter season for individuals. This indicates that hibernation is a specialized winter survival strategy, the employment of which depends upon the animal and its environment.

In terms of energetic expenditure, there is a point at which the returns of hibernation are outweighed by the costs. A limiting factor for hibernation is animal body mass. For example, to maintain a euthermic body temperature for 100 days under thermoneutral conditions without food, a mammal must amass a large amount of stored fuel; the amount is relatively much higher for a small mammal than a large one. A small mammal, such as a 10 gram mouse, has a weight-to-surface area ratio that favors the dissipation of heat; the mouse would have to increase its body weight 320% to store the required fuel. A black bear, however, though it requires a larger total amount of energy than the mouse, would only need to increase its body weight by 17% to survive the same conditions (Nedergaard et al., 1990). It is, therefore, more efficient for a small mammal to hibernate and decrease the amount of energy that it requires to survive the winter. Not surprisingly, then, the profound hibernators (those which hibernate on a regular, seasonal basis) are small mammals (Wang, 1989; Marchand, 1991). Larger mammals such as the black bear experience metabolic rate suppression to 30 % of resting euthermic rate

(Watts, 1981) but experience only a small drop in body temperature during torpor because the large mass impedes heat dissipation (Nedergaard et al., 1990).

In addition to the size restriction of hibernation, the development of a winter survival strategy may be related to the environment of the animal. For example, the white-tailed prairie dog, found in mountainous areas of Colorado, Utah, Wyoming and Montana, is exposed to very harsh winters (Bakko et al., 1986). These animals are profound hibernators and are not active above ground after August when they retreat underground to begin hibernation as conditions warrant (Tileston et al., 1966). By contrast, the closely related black-tailed prairie dog, which is found at lower elevations in the somewhat gentler climate on the Great Plains, is frequently observed above ground year round (Tileston et al., 1966). The white-tailed prairie dog must rely on hibernation to deal with the harsh environment whereas the black-tailed prairie dog hibernates only when conditions are severe enough to demand it and never for a predictable or prolonged period of time (Wang, 1989; Marchand. 1991).

Physiological manifestations of a hibernation bout

To lessen the impact of temperature fluctuations during the winter, all hibernating mammals retreat to sheltered sites (burrows, caves) that are dry and insulated; most often, sites are chosen where temperatures will not fall below 0°C. Within this hibernaculum, the animals cycle in and out of torpor over the winter season. A hibernation bout consists of entry into, maintenance of, and arousal from hibernation. The lower panel of Figure 1.1 illustrates the changes in body temperature over the course of the winter season (Wang, 1989). Following complete arousal there is a period of euthermia lasting from a few hours to a few days before the animal reenters another hibernation bout. To date, all heterothermic endotherms are known to follow this pattern (Wang 1989).

Entry into hibernation

Entry into hibernation is characterized by a marked decrease of heart rate, a progressive increase of vasoconstriction to maintain blood pressure while heart rate decreases, a decrease in respiratory rate with irregular periods of apnea, and a significant decrease in oxygen consumption (Lyman, 1984). Body temperature falls following the decrease in heat production and shivering occurs periodically to temporarily raise or stabilize the body temperature against too rapid a decrease. These deliberate controls, as well as the observation that it takes an average of three to five times longer to cover the same body temperature change during entry into hibernation as during arousal, indicate that entry is an actively regulated process rather than a passive abandonment of euthermia (Hudson, 1973). This is illustrated in the upper panel of Figure 1.1 (from Heldmaier et al., 1993). An induction trigger for the onset of hibernation, if there is one, has yet to be determined. In the past, brown adipose tissue was thought to possess endocrine functions that triggered hibernation, but it is now known to be strictly a thermogenic tissue (Horwitz, 1992). There have also been attempts to identify a brain peptide as the "torpor trigger" (Swan, 1982). While these studies have not been successful as of yet, there most likely is an endocrine or nervous system signal(s) that is triggered by environmental cues such as shortened photoperiod, lowered ambient temperature and food availability, to induce hibernation.

The hibernating state

A hibernation bout may last anywhere from a few hours to a few weeks; the longest observed bout was recorded in bats and lasted 76 days (Twente 1985). During a hibernation bout all physiological functions operate at a minimum level. Oxygen consumption is reduced to 1/100 or less of the euthermic level, heart rate may be reduced to less than 1/30, and the overall metabolic rate can drop to as low as 1 to 8% of the resting euthermic level (Wang, 1989). Depending on the species, long periods of apnea

can occur. In golden-mantled ground squirrels, apnea may last as long as 40 minutes (Steffen et al., 1982) and up to 150 minutes in the hedgehog (Malan, 1982).

The tremendous decrease in body temperature is the most dramatic characteristic of hibernation. Body temperature typically falls to within 2°C of the ambient temperature at the hibernation site. All species appear to have a critical minimum temperature below which a further decrease in body temperature elicits one of three responses. Firstly, the body temperature may be stabilized at the "critical" level at the expense of an increase in metabolic rate. Secondly, the animal can initiate arousal. Or thirdly, the animal could die if its body temperature falls below the "critical" level and it is incapable of arousing from hibernation (Hudson, 1973). The lower critical level is species dependent but ranges from 1-5°C for most hibernating mammals (Wang, 1989). The critical limiting factors that govern the low temperature tolerance are generally unknown, but it has been proposed that the cold tolerance of membrane-mediated functions such as bioenergetics and ionic transport could be a limiting factor (Wang, 1989).

The mechanisms that control the decrease in body temperature during entry into hibernation are still not fully understood. The rates of most chemical processes change two- to three-fold with a 10°C change in temperature (giving Q_{10} values of 2 to 3). Because calculations of Q_{10} values for oxygen consumption (an indication of metabolic rate) are often found to be 2-3 in many hibernator species, several researchers have proposed that the metabolic rate depression during hibernation is simply the effect of the drop in body temperature (Geiser, 1988; Snapp and Heller, 1981). However metabolic rate suppression for many species, particularly those with small body mass, is often more profound than would be predicted from the effect of temperature on reaction rates alone. Therefore, other 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). For example, for the genus *Zapus*

(jumping mice), Q_{10} values for oxygen consumption between the euthermic and hibernating states range from 3 to 4 (Geiser, 1988). Therefore, hypothermia appears to be the result, not the cause of reduced metabolic rates in hibernators. This is also illustrated in Figure 1.1, which shows that body temperature does not decline as quickly as the metabolic rate during entry into a hibernation bout. However the mechanism of the active suppression of metabolism has not yet been determined.

Malan (1982) has suggested that since the pH of neutrality is increased at lower temperatures the active suppression of metabolic rate during the hibernating phase of a torpor bout could result from "acid inhibition" of metabolism. Covalent modification of key regulatory enzymes has also been implicated in the coordinated depression of metabolic rate. Hibernating mammals primarily rely on lipid oxidation during hibernation (Hochachka and Guppy, 1987) and a strong inhibition of carbohydrate metabolism is expected during the euthermic to hibernating transition (Storey and Storey, 1990). For example pyruvate dehydrogenase (PDH), an enzyme that gates carbohydrate oxidation by the mitochondria, is closely regulated by phosphorylation in hibernating ground squirrels (Brooks and Storey, 1989). The percentage of inactive, phosphorylated PDH increased during hibernation to the point where only 1 to 6 percent of the total enzyme remained in the active, dephosphorylated form. Consequently, covalent modification of PDH effectively blocked aerobic carbohydrate oxidation and shut off the catabolism of carbohydrates during hibernation (Storey and Storey, 1990).

Arousal from a hibernation bout

Throughout the winter the hibernator repeatedly rewarms itself inside its hibernaculum. In small mammals such as pocket mice (Wang and Hudson, 1970) and bats (Hayward, 1968) arousal from a body temperature of 5°C is complete within 20 minutes. Larger mammals such as marmots and squirrels require a few hours to arouse from the same body temperature (Wang, 1979). Rewarming is accomplished by two

mechanisms: shivering and non-shivering thermogenesis (NST). Shivering is involuntary and is characterized by rhythmic, random, uncoordinated but simultaneous contractions of the flexor and extensor muscles. NST is a heat-producing mechanism that does not require muscle contraction. The main location of NST in mammals is the brown adipose tissue where the oxidation of lipid substrates is uncoupled from the production of ATP in mitochondria; the resulting electrochemical energy that would otherwise be used to produce ATP is dissipated as heat, warming the animal (Wang, 1989). The initial heat production during arousal is accomplished by NST in the brown adipose followed somewhat later by the addition of shivering thermogenesis in skeletal muscles supported by glycogen oxidation (Davenport, 1992).

Arousal is energetically expensive for the hibernator, accounting for 90% of the energy expenditure during the hibernation season. The reasons for arousal are not known, but several hypotheses have been proposed. It was originally thought that hibernators terminated a hibernation period to eliminate metabolic wastes and replenish fuel sources (Mrosovsky, 1971) but this hypothesis has since been discarded. It has recently been proposed that arousal occurs to reestablish cellular ionic balance (Willis, 1982).

Experimental Animal

The white-tailed prairie dog, *Cynomys leucurus*, is the subject of this study. *C. leucurus* is found in parts of Colorado, Utah, Wyoming and Montana (Clark et al., 1971). This small mammal is a profound hibernator and its hibernation patterns, which are similar to other hibernating mammals, have been documented in a laboratory setting by Bakko et al. (1986). In this study, the animals first entered torpor an average of 172 hours after initial exposure to an ambient temperature of 5°C and the average length of a torpor bout was 121 hours with the longest stretching to 186.8 hours (more than 8 days). Over a sixty-day study, the animals spent 85.5 percent of their time in hibernation. Body temperature dropped from a euthermic T_b of 35.5°C to 7°C during hibernation.

Energy charge

Because a hibernator must undergo arousal in a very short period of time, metabolism must still be regulated at the low body temperature of torpor. The adenylates (ATP, ADP, AMP) exert regulatory effects on a wide range of enzymes and metabolic systems. Atkinson (1968) proposed that in terms of metabolic control, the actual concentrations of individual adenine nucleotides were not as important as their relative amounts. To simplify this concept he proposed the idea of "energy charge", as related by this equation:

$$\text{Energy Charge} = ([\text{ATP}] + 0.5[\text{ADP}]) / ([\text{ATP}] + [\text{ADP}] + [\text{AMP}])$$

The energy charge system is fully charged (a value of 1.0) when only ATP is present and fully discharged (a value of 0) when only AMP is present. In unstressed conditions the E.C. is normally around 0.90 - 0.95. Many regulatory enzymes are modulated by energy charge but the way in which they react to changes in the adenylate pool depends on the metabolic pathway in which they function. For example, many catabolic enzymes of glycolysis or the citric acid cycle (such as phosphofructokinase, pyruvate dehydrogenase and pyruvate kinase) (Boyer, 1968) are inhibited by a high energy charge (approaching 1.0) and stimulated by a falling energy charge. Anabolic enzymes, by contrast, such as citrate lyase, phosphoribosyl ATP synthetase and aspartokinase (Boyer, 1968) are affected in the opposite manner - activated by a high energy charge and inhibited by a falling energy charge. Therefore, the energy charge of the adenylates system provides a means of metabolic control.

Adenine nucleotide metabolism

Degradation of ATP occurs by the pathway illustrated in Figure 1.2. The numerous processes involved in the production and utilization of ATP result in a regular turnover of adenylates. In general, ATP is converted to ADP or AMP, and ADP is converted to AMP; both steps occur via numerous enzymatic reactions. Adenylates are also needed as substrates for synthesis. For example, adenosine, a regulatory metabolite, is produced from AMP.

Muscle concentrations of AMP are normally very low (0.05 mM or less) and the catabolism of adenine nucleotides occurs very slowly. Under conditions of hypoxia or ischemia, however, oxidative phosphorylation is impaired. This provokes catabolism of ATP at a rate faster than it can be replenished, resulting in an accumulation of AMP and an decreased energy charge. The rate of AMP degradation then increases as a mechanism to minimize the accumulation of AMP and, thereby, maximize energy charge. Under such conditions, higher levels of adenine nucleotide degradation products can be expected, and increases in plasma levels of these such as hypoxanthine and uric acid (Figure 1.2) have been reported. Similarly, as shown in Appendix 2, the energy charge was maintained in euthermic and hibernating prairie dogs, despite a significant decrease in ATP concentration. The fate of adenylates during hibernation is therefore worth investigation.

The Study

Metabolic control over the course of hibernation is poorly understood. Because hibernation is not a continuous state, the mechanisms by which the animal enters a hypometabolic state must be fast and readily reversible. In addition, since the hibernator's metabolism is maintained at the hibernation set point and periodic arousals from torpor require contributions from a variety of tissues, including muscle (Wang, 1988), the functional capacity of muscle must be maintained despite large changes in

body temperatures (Musacchia et al., 1989). It follows then, that enzymes in hibernator tissues must still be functional at low temperatures, either inherently so (i.e. the same for all mammals) or modified by evolution to create enzyme proteins unique to hibernators that can retain their function over a wide range of temperatures from euthermic (about 37°C) to the near freezing temperatures that can occur in deep torpor. To date, the kinetic parameters of adenylate degradation enzymes from mammalian hibernators have not been examined to determine how they function at low temperatures.

So, are there enzyme adaptations unique to hibernating species that allow them to maintain enzyme control? This study will explore the impact of drastic temperature changes on the kinetic and thermodynamic parameters of mammalian hibernator enzymes. As illustrated, the enzymes involved in cellular adenylate degradation have important homeostatic roles. Their activities regulate cellular energy charge and protect organs against nucleotide depletion, which may be critical during the hibernation season when no food is consumed to replenish depleted energy stores. Two enzymes will be studied in detail with respect to AMP metabolism. AMP deaminase and adenylate kinase were purified from *C. leucurus* skeletal muscle and their kinetic parameters were analyzed at 5°C and 37°C, and compared with the comparable enzymes from the rabbit, a non-hibernating mammal, of similar body mass as the prairie dog.

Figure 1.1: Body temperature changes in hibernating ground squirrels within a hibernation bout and over the hibernation season. The upper panel illustrates the general pattern of change in body temperature and metabolic rate within a single hibernation bout for *Spermophilus lateralis* (taken from Heldmaier et al., 1993). The lower panel illustrates body temperature changes over a hibernation season for *Spermophilus richardsonii*; note the increasing length and depth of torpor bouts as the season progresses (taken from Wang, 1989).

Figure 1.2: Adenine nucleotide degradation pathway. Enzymes are abbreviated as follows: AK=adenylate kinase, 5'NT=5'nucleotidase, AMPD=AMP-deaminase, NP=nucleoside phosphorylase, XO=xanthine oxidase, HPRT=hypoxanthine phosphoribosyl transferase.

Chapter 2

Effect of temperature on the kinetic and thermodynamic properties of skeletal muscle AMP deaminase from the hibernating prairie dog, *Cynomys leucurus*

Introduction

AMP deaminase (5'-AMP aminohydrolase, E.C. 3.5.4.6) is an enzyme that catalyzes the irreversible hydrolytic cleavage of AMP to IMP and ammonia. The enzyme is widely distributed in animal tissues but the highest activities by far are found in skeletal muscle (Lowenstein, 1972). In mammalian skeletal muscle under an energy stress (for example, during exercise), there is an inverse correlation between ATP depletion and IMP production. Therefore, a primary role for AMP-deaminase appears to be in the removal of AMP from the adenylate pool when the rate of ATP production is outstripped by the rate of ATP utilization. The most important consequence of this function appears to be the stabilization of cellular energy charge. By removing AMP from the energy charge equation (see chapter 1), the overall energy charge can be stabilized, even though the total adenylate pool size decreases. Additional consequences of this function include: (1) to help push the adenylate kinase reaction towards ATP generation, (2) to supply allosteric effectors (such as NH_4^+), (3) to remove some of the protons that accumulate from anaerobic glycolysis via the production of ammonium ions ($\text{AMP} + \text{H}_3\text{O}^+ \rightarrow \text{IMP} + \text{NH}_4^+$) (Mommensen and Hochachka, 1988) and (4) modulate the regulation of AMP-sensitive metabolic pathways (Lee et al., 1968).

There are several proposed mechanisms of AMP deaminase (AMPD) regulation *in vivo*. AMPD is an allosteric enzyme. Allosteric effectors such as GTP, ATP, ADP and inorganic phosphate (P_i) are all known to modify AMPD activity (Smiley and Suelter, 1967; Raffin, 1983; Makarwicz, 1969). Inorganic phosphate is considered the primary inhibitor of AMPD *in vivo*, with a K_i of 1-2 mM (Wheeler and Lowenstein, 1978). AMPD is activated by ADP (a modulator whose concentration increases in active muscle) and inhibited by GTP.

Ashby and Frieden (1978) demonstrated that AMPD from rabbit muscle has three types of binding sites: an active site, an inhibitory site that is relatively specific for

nucleoside triphosphates (in particular GTP), and an activator site that is non-specific and can bind nucleoside mono-, di- and tri- phosphates. AMPD from rat muscle behaves in a similar manner; binding of ADP at an activator site enhances enzyme activity and weakens triphosphate nucleotide (such as GTP) binding at the inhibitory site (Wheeler and Lowenstein, 1979). Allosteric behavior has also been demonstrated for the enzyme from other sources; in trout gill, for example, ADP and GTP are competitive inhibitors (Raffin, 1984).

AMPD is also regulated by pH change (Stakiewich and Spychala, 1979). Purified AMPD is enhanced by acidosis *in vitro*, with an optimum pH of 6.2 to 6.5 (Raggi et al., 1987), values similar to those found in myocytes during severe contraction (Dudley and Terjung, 1985). In addition, ADP is more effective at relieving Pi inhibition under acidic conditions (Wheeler et al., 1979).

The regulation of AMPD *in vivo* also involves the binding of AMPD to the contractile elements of skeletal muscle, but the degree to which enzyme activity is regulated by this binding is unclear. Shiraki et al. (1979) found a 50-70% increase in maximal enzyme velocity was induced by myosin binding, whereas Ashby and Frieden (1978) and Barshop and Frieden (1984) reported only slight activation. Binding to myosin also affects the kinetic parameters of AMPD: binding can decrease GTP inhibition (Ashby and Frieden, 1978), enhance activation by ADP (Barshop and Frieden, 1984), and lower K_m AMP (Rundell et al., 1992b). Rundell et al. (1992b) also found that inhibition by orthophosphate can be reversed by 50 μ M ADP (which also rises during exercise) when the bound enzyme was examined but not the free enzyme. Of physiological relevance, the proportion of total AMPD that is bound to myosin increases significantly in intensely contracting muscle (Rundell et al., 1992a).

Recent studies have shown that AMPD can also be regulated via reversible protein phosphorylation by Ca^{2+} and phospholipid-dependent protein kinase C (PKC) (Hu et al., 1991; Tovmasian et al., 1990; Thakkar et al., 1993). PKC-mediated

phosphorylation enhances rabbit cardiac AMPD activity by increasing the apparent affinity for substrate, but does not change the maximal enzyme activity (Thakkar et al., 1993). In addition, phosphorylation does not alter the effects of allosteric modulators; GTP still inhibits, ADP and ATP still activate (Thakkar et al., 1993). Rat skeletal muscle AMPD has also been reported to be a PKC substrate (Tovmasian et al., 1990). As with the rabbit cardiac AMPD, phosphorylation results in a 3-fold decrease in K_m , but maximal velocity is unchanged (Tovmasian et al., 1990).

AMP deaminase has been purified to apparent electrophoretic homogeneity from various vertebrate skeletal muscle sources including rat (Ogasawara et al., 1974; Stankiewicz et al., 1979), rabbit (Smiley et al., 1967; Stankiewicz et al., 1979), man (Kaletha et al., 1988), hen, frog and pikeperch (Stankiewicz et al., 1979). The subunit molecular weight of these skeletal muscle enzymes range from 68.0 -71.6 kDa. Stankiewicz et al. (1979) also reported that each AMPD is a tetramer with a molecular weight of approximately 280 kDa (range from 272 - 287 kDa).

To date, AMPD has not been isolated from the skeletal muscle of a hibernating mammal, nor has the importance of temperature to AMPD regulation been assessed for its influence on muscle adenylate metabolism in the hibernating state. The present study reports the purification and characterization of AMPD from the skeletal muscle of the mammalian hibernator, *C. leucurus*. The kinetic parameters of the enzyme from *C. leucurus* and rabbit muscle were assessed and compared at assay temperatures representative of the euthermic and hibernating states. Presented are temperature effects on substrate affinity coefficients, K_a values for activators, I_{50} values for inhibitors, and pH optima along with an analysis of temperature effects on enzyme activity (including Arrhenius plots) and a determination of enzyme subunit molecular weight.

Materials and Methods

Animals and Biochemicals

Adult white-tailed prairie dogs, *Cynomys leucurus*, were captured in south-eastern Wyoming during July 1993. The animals were individually housed in standard rat cages at the animal care facility of the University of Wyoming. Holding conditions included an ambient temperature of 22°C and natural (autumn) photoperiod. Animals were fed a modified version of Purina 5001 rodent diet which contained additional fat (fat content = 15 %) for 5 weeks. At the end of this period (October 1993) half of the animals were killed by decapitation. The remaining animals were allowed to continue feeding and fattening for an additional 5 weeks and were then moved (in their cages) on November 7 into an environmental chamber at 5°C to induce hibernation. Within 7 days all began hibernating and they continued to hibernate over the next 10 weeks. At the end of this time the animals were sacrificed by decapitation while still torpid. For both sets of animals, selected tissues were rapidly dissected out and immediately frozen in liquid nitrogen. Tissues were then shipped to Carleton University on dry ice where they were stored at -80°C until use.

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

AMP-Deaminase (E.C. 3.5.4.6) Assay

AMP-deaminase (AMPD) activity was assayed using a Gilford recording spectrophotometer with an attached circulating water bath for temperature control of cuvettes. Cuvette temperature was measured using a YSI telethermometer with a thermistor placed inside the cuvette. Optimal activity was measured in the presence of 20 mM imidazole-HCl (pH 7.0) and 2.0 mM AMP in a reaction volume of 1.0 mL. One unit of enzyme activity is defined as the amount required to produce 1 μ mol of IMP per

minute at room temperature of approximately 22°C, under optimal conditions. This was equal to the change in optical density per minute at 285 nm divided by 0.3, because the difference in molar absorptivity of the two nucleotides at 285 nm was 300 (Smiley et al., 1967).

Determination of Percent Bound and Free

The proportion of AMPD found free in the cytosol versus bound to myofibrils in *C. leucurus* skeletal muscle was determined according to the method of Rundell et al. (1992) with modifications. Frozen tissue samples were homogenized 1:10 (wt:vol) in a solution of 150 mM KCl, 5 mM potassium phosphate, 2 mM EDTA, 10 mM β -mercaptoethanol and a few crystals of PMSF at pH 7.0. The homogenate was centrifuged in a Sorvall refrigerated centrifuge at 23,400 x g for 20 minutes at 4°C. Myofilaments are not soluble in 150 mM KCl and sediment rapidly; myosin-bound AMPD is carried along because it does not dissociate at this low salt concentration (Ashby et al., 1977). The supernatant was removed and assayed for AMPD activity; this represents the fraction of AMPD that is free in the cytosol. The pellets were then homogenized in 10 volumes (wt:vol, based on original tissue weight) of 500 mM KCl, 50 mM potassium phosphate, pH 7.5; (the higher salt concentration removes the enzyme from myofibrils). This second homogenate was then centrifuged, its supernatant removed and assayed for AMPD activity; this represented the fraction of AMPD that was bound to myofibrils in the muscle.

Enzyme Purification

AMP-deaminase was purified from euthermic *C. leucurus* muscle according to the method of Smiley et al. (1967) with modifications. Frozen tissue samples were homogenized in 5 vol (wt:vol) of Buffer A (180 mM KCl, 54 mM KH_2PO_4 , 36 mM K_2HPO_4 , 10 mM β -mercaptoethanol, pH 6.5) with a few crystals of PMSF, using a

Polytron homogenizer at 50% full power. The homogenate was centrifuged at $23,400 \times g$ at 4°C for 20 minutes in a Sorvall RC-5B Refrigerated Superspeed Centrifuge. The supernatant was removed and the pellet discarded. Phosphocellulose (previously equilibrated in Buffer A) was added to the supernatant and the suspension stirred for 1 hour on ice. The slurry was centrifuged at $23,400 \times g$ in the Sorvall centrifuge for 10 minutes and the supernatant was discarded. The resulting phosphocellulose pellet was washed twice with Buffer A (resuspended and then centrifuged at $23,400 \times g$ for 10 minutes) and twice with a solution of Buffer B (0.45 M KCl, 10 mM β -mercaptoethanol, adjusted to pH 7.0 with KH_2PO_4). The final pellet was then resuspended in 8.0 mL of Buffer B. The slurry was poured into a plastic 1.5 x 10 cm column and washed with Buffer B until the absorbance at 280 nm reached a low value that was constant over several wash fractions. AMPD was then eluted from the column with a KCl gradient from 0.45 M to 1.5 M (solutions were adjusted to pH 7.0 with 1.0 M KH_2PO_4) and 1 mL fractions were collected. Fractions were assayed for enzyme activity and peak activity fractions were combined and then concentrated using either a Centricon-30 microconcentrator or dialysis against solid polyethylene glycol 8000. The concentrated sample was then diluted 2-fold with 100% glycerol and stored at -20°C until use.

Purified rabbit muscle AMPD (55 U/mg protein, in 66 % glycerol, 0.33 M KCl and 1 mM β -mercaptoethanol at pH 7.0) was purchased from Sigma Chemical Co.. Protein concentrations were determined with the Coomassie blue G-250 dye-binding method using the Bio-Rad prepared reagent and bovine serum albumin as the standard (Bradford, 1976).

Kinetic Parameters

All kinetic parameters were determined for rabbit and *C. leucurus* skeletal muscle AMPD at both 5°C and 37°C . Substrate affinity constants (K_m) were determined for AMP using Hill plots. I_{50} values (the concentration of inhibitor that results in a 50%

reduction of enzyme activity) were determined for GTP, Pi, MgCl_2 , Mg_2SO_4 , CaCl_2 , NH_4Cl , $(\text{NH}_4)_2\text{SO}_4$. Activator constants (K_a) were determined for ATP and ADP using double reciprocal plots of $(1/V - 1/V_0)$ vs. $1/[\text{activator}]$. To determine pH optima, enzymes were assayed at several pH values under optimal substrate conditions. The pH of the 20 mM imidazole assay buffer was adjusted to the required pH at room temperature and then pH was allowed to change with temperature following the $\Delta\text{pH}/\Delta T$ for imidazole which is -0.018 pH unit per 1°C . This mimics the change in pH with temperature that naturally occurs *in vivo* in most animals cells due to their reliance on intracellular histidine buffers. Arrhenius plots were constructed for *C. leucurus* and rabbit AMPD. Assays were run at optimal conditions over the range $1\text{--}35^\circ\text{C}$, in increments of 2 or 3°C .

SDS-Polyacrylamide gel electrophoresis

Purity and subunit molecular weight of the purified enzyme were determined using SDS-PAGE. The running gel was 8% acrylamide and the stacking gel was 5% acrylamide. The running buffer was 24.8 mM Tris, 192 mM glycine, pH 8.8 containing 0.1% w/v SDS. Before application to the gel, protein samples were mixed 1:1 v/v with a solution of 46.8 mM Tris buffer, pH 6.8, containing 7.5% v/v glycerol, 2.25% SDS w/v and 3.75% v/v 2-mercaptoethanol. Samples were incubated in a boiling water bath for 2 min and then loaded. The gel was run at 125 volts until the bromophenol blue dye reached the bottom of the gel (approximately an hour) and gels were then fixed for one hour in a solution of 10% acetic acid and 25% methanol (vol:vol). AMPD was stained for an hour with a solution of 0.25 % w/v Coomassie Brilliant Blue R-250, 50% v/v methanol and 7.5% v/v acetic acid and then destained in 25% v/v methanol / 10% v/v acetic acid. The subunit molecular weight was determined from a plot of R_f versus log molecular weight of the standards (kDa): rabbit muscle myosin (200), *E. coli* β -

galactosidase (116.0), glycogen phosphorylase *b* (97.0), bovine serum albumin (66.0), ovalbumin (45.0).

Data and statistics

Enzyme kinetic parameters were analyzed by standard procedures using a nonlinear least squares regression computer program developed for this purpose (Brooks, 1992). Data are expressed as the mean $\pm$ S.E.M. with statistical testing performed using the Student's t-test.

Results

AMP Deaminase binding

AMPD binds to myofibrils *in vivo* and changes in the distribution between free and myofibril-bound states have been shown to occur in response to physiological stimuli (such as exercise) in other systems. To determine whether the transition to the hibernating state altered the percentage of AMPD bound to myofibrils, the proportions of free versus bound AMPD were quantified in skeletal muscle sampled from hibernating versus euthermic *C. leucurus*. The percentage of bound AMPD in the skeletal muscle of hibernating prairie dogs, 39.3 ± 5.2 ($n = 6$), did not differ significantly from the percent bound, 37.5 ± 4.8 ($n = 12$), in skeletal muscle of euthermic animals. Recovery tests indicated that the sum of bound and free AMPD, as determined by this protocol, was equal to the total AMPD activity measured when tissues were initially homogenized in high salt buffer to extract all the AMPD (as for purification procedures).

Purification and SDS-PAGE of *C. leucurus* AMP deaminase

A typical purification of AMPD from *C. leucurus* skeletal muscle is summarized in Table 2.1. AMPD was purified using chromatography on phosphocellulose in a one-column procedure. Elution of AMPD was in a single peak as shown in Figure 3, with maximum activity eluting at 0.8 M KCl. This procedure typically gave a net purification factor of roughly 400 fold, an overall yield of 40% and resulted in a purified preparation with a final specific activity of approximately 60 U/mg protein (assayed at room temperature of approximately 22°C). This compared favorably with the 55 U/mg of protein for the rabbit skeletal muscle AMPD purchased from Sigma.

C. leucurus skeletal muscle AMPD purity and subunit molecular weight were assessed using SDS-PAGE. The enzyme migrated as one band indicating that the AMPD

preparation was homogeneous. Figure 2.2 shows the SDS-PAGE gel of *C. leucurus* AMPD with the molecular weight standards labeled with arrows. The standard curve for the molecular weight markers is shown in Figure 2.3. The apparent subunit molecular weight of AMPD was 68.8 KDa ($n=1$).

pH Curves

The pH optima of *C. leucurus* and rabbit muscle AMPD were assessed at both 5°C and 37°C and data are shown in Figure 2.4 and Figure 2.5, respectively. The assays were conducted in imidazole buffer which shows a 0.018 pH unit increase for every 1°C decrease in temperature. To account for this effect of temperature on pH, the pH values of all buffers were measured at room temperature (determined using a thermometer at the time of pH determination) and then the corresponding values at the 5°C or 37°C assay temperatures were calculated. Figure 2.4 shows that the pH optimum of *C. leucurus* AMPD was 6.65 at 5°C and decreased to 6.32 at 37°C. The pH optimum of rabbit AMPD also decreased with increasing temperature from 6.50 at 5°C to 6.04 at 37°C.

Arrhenius Plots

Arrhenius plots for *C. leucurus* and rabbit AMPD are shown in Figure 2.6. Both plots were linear ($r^2 = 0.99$) over the temperature range assayed from 3 to 35°C. Activation energies were calculated from the slopes of the plots. For *C. leucurus* AMPD the calculated activation energy was $90.90 \pm 3.00 \text{ kJ}\cdot\text{mol}^{-1}$ ($n=4$ determinations using the same enzyme preparation) whereas the E_a for rabbit muscle AMPD was 15% lower at $77.68 \pm 1.88 \text{ kJ}\cdot\text{mol}^{-1}$ ($n=4$ determinations on the same enzyme preparation).

Substrate affinities

Affinity constants for AMP at two assay temperatures are shown in Table 2.2. The K_m of the *C. leucurus* enzyme did not change significantly from 37°C to 5°C (0.79

to 0.81 mM AMP, respectively). The K_m for rabbit AMPD, while lower than the *C. leucurus* values, also did not change significantly with temperature.

Inhibitors of AMP deaminase.

The following compounds were tested for inhibitory effects on AMPD: $MgCl_2$, $MgSO_4$, NH_4Cl , $(NH_4)_2SO_4$, $CaCl_2$, inorganic phosphate (Pi), GTP and IMP. IMP (tested at concentrations up to 4 mM) was the only compound in this series that did not affect enzyme activity at either temperature. With the exception of magnesium inhibition of rabbit AMPD, sulphate salts were all stronger inhibitors than their comparable chloride salts. In addition, inhibition was generally stronger at 5°C compared with 37°C. I_{50} values for inhibitors of *C. leucurus* AMPD are shown in Table 2.3; data for rabbit AMPD are shown in Table 2.4.

The effect of salts on *C. leucurus* AMPD was influenced by the assay temperature. Decreasing the assay temperature from 37°C to 5°C increased the inhibitory effect of each modifier, although the magnitude of these changes varied dramatically. There were only slight (though statistically significant) increases in sensitivity to magnesium and calcium salts at 5°C; the I_{50} values for $MgCl_2$, $MgSO_4$ and $CaCl_2$ all decreased by about half (Table 2.3). The influence of assay temperature on the effect of ammonium salts was far more dramatic. At 5°C, I_{50} values for NH_4Cl and $(NH_4)_2SO_4$ decreased by 28-fold and 83-fold respectively.

In general, the rabbit AMPD pattern of response to inhibitors and temperature was similar to that of the *C. leucurus* enzyme, but the fold changes were of a lesser magnitude. Only the I_{50} for $MgSO_4$ did not change with assay temperature. The I_{50} for $CaCl_2$ dropped by 35 % whereas the I_{50} for $MgCl_2$ decreased 4-fold (Table 2.4). The I_{50} for NH_4Cl dropped 4-fold at 5°C, a lesser decrease than the 28-fold change for hibernator AMPD. In addition, the I_{50} for NH_4Cl for the rabbit enzyme at 5°C was 7-

fold greater than the I_{50} for *C. leucurus* AMPD. $(\text{NH}_4)_2\text{SO}_4$ was also a much stronger inhibitor at 5°C ; the I_{50} dropped by 26-fold (*C. leucurus* dropped 83-fold).

Inorganic phosphate is considered an important inhibitor of AMPD *in vivo*. At 37°C *C. leucurus* and rabbit AMPD were inhibited by P_i in a similar manner and the I_{50} values of the two enzymes were not significantly different (5.46 ± 0.46 mM and 6.13 ± 0.27 mM, respectively). Although P_i inhibition was stronger at 5°C for both enzymes, the extent of inhibition was much greater for the hibernator enzyme. *C. leucurus* AMPD showed an 8-fold increase in sensitivity to P_i (I_{50} dropped to less than 1 mM) whereas the comparable change for the rabbit enzyme was only 2-fold (I_{50} declined by half).

GTP is also considered to be an important regulator of AMPD activity *in vivo*. Unlike the previously mentioned salts, GTP is an allosteric inhibitor of AMPD activity. Both *C. leucurus* and rabbit AMPD responded to GTP and temperature change in the same manner. At 37°C the I_{50} for GTP of *C. leucurus* AMPD was 90.0 ± 5.2 μM , whereas rabbit AMPD was similarly inhibited with an I_{50} of 91.7 ± 2.4 μM . At 5°C the hibernator I_{50} dropped dramatically to 1.06 ± 0.07 μM (an 85-fold decrease) whereas the rabbit enzyme I_{50} fell to 0.73 ± 0.02 μM (a 125-fold decline). Despite the greater fold decrease by the rabbit enzyme, the I_{50} values for the two species assayed at 5°C were not statistically different.

Activator Constants

C. leucurus and rabbit AMPD were both sensitive to ATP and ADP as allosteric effectors (Table 2.5). At 37°C , *C. leucurus* AMPD was activated 3-fold by ATP with a K_a of 0.39 ± 0.09 mM; rabbit AMPD showed similar maximal activation and a K_a of 0.44 ± 0.06 mM, not significantly different from the hibernator enzyme. However, at 5°C , both enzymes showed a remarkable change of events and were strongly inhibited by micromolar amounts of ATP. *C. leucurus* AMPD showed an I_{50} for ATP of 1.13 ± 0.05

μM whereas the rabbit enzyme was even more strongly inhibited with an I_{50} of $0.103 \pm 0.015 \mu\text{M}$).

C. leucurus AMPD was also strongly activated by ADP at 37°C with a K_a of $0.65 \text{ mM} \pm 0.05$ and a 5-fold maximal activation observed. At 37°C rabbit AMPD was more sensitive to activation by ADP with a 4-fold lower K_a of $0.16 \text{ mM} \pm 0.005$, but with a slightly lower fold activation of 4.3 as well. However, at 5°C these characteristics changed dramatically. *C. leucurus* AMPD was strongly inhibited by ADP with an I_{50} of $14.4 \pm 1.5 \mu\text{M}$ at 5°C but the rabbit enzyme was not affected by ADP.

Table 2.1: Typical purification of AMPD from *C. leucurus* skeletal muscle.

Purification Step	Total Protein mg	Total Units	Specific Activity U·mg ⁻¹	Fold purification	Yield (%)
Crude homogenate	1549	226.2	0.146	-	100
Phosphocellulose Pooled sample	1.157	73.7	64.20	439.7	34.2
Final preparation (concentrated)	1.158	77.4	66.89	452.1	34.2

Table 2.2: K_m AMP for purified AMP deaminase from *C. leucurus* and rabbit skeletal muscle. Data are means $\pm$ S.E.M. (n = 4 determinations, using at least two different enzyme preparations).

	Assay Temperature	
	37°C	5°C
<i>C. leucurus</i>	0.79 ± 0.03	0.81 ± 0.04
Rabbit	0.65 ± 0.042	0.76 ± 0.021

Table 2.3: Allosteric effectors of *C. leucurus* muscle AMP deaminase.**All values are in mM except GTP which is in μ M.**

Inhibitor	37°C	5°C
P _i	5.46 $\pm$ 0.46	0.66 $\pm$ 0.025*
NH ₄ Cl	393 $\pm$ 40	14.2 $\pm$ 0.50*
(NH ₄) ₂ SO ₄	85.8 $\pm$ 2.45	1.03 $\pm$ 0.10*
MgCl ₂	3.30 $\pm$ 0.02	1.96 $\pm$ 0.050*
MgSO ₄	2.48 $\pm$ 0.22	1.28 $\pm$ 0.12*
CaCl ₂	4.76 $\pm$ 0.15	1.78 $\pm$ 0.15*
GTP	90.0 $\pm$ 5.2	1.06 $\pm$ 0.07*

AMPD was assayed at K_m level of substrate, 0.8 mM AMP.

Data are means $\pm$ S.E.M. (n=3), 2 different enzyme preparations.

* - Significantly different from the corresponding value at 37°C as determined by Student's t-test (2-tailed), * P < 0.001.

Table 2.4: Allosteric inhibitors of rabbit skeletal muscle AMP deaminase. All values are in mM except GTP which is in μ M.

Inhibitor	37°C	5°C
P _i	6.13 $\pm$ 0.27	2.99 $\pm$ 0.10*
NH ₄ Cl	430 $\pm$ 12	101 $\pm$ 6.0*
(NH ₄) ₂ SO ₄	98.7 $\pm$ 4.2	3.81 $\pm$ 0.39*
MgCl ₂	6.77 $\pm$ 0.12	1.64 $\pm$ 0.22*
MgSO ₄	5.39 $\pm$ 0.36	4.45 $\pm$ 0.27
CaCl ₂	9.41 $\pm$ 0.75	6.15 $\pm$ 0.65**
GTP	91.7 $\pm$ 2.4	0.73 $\pm$ 0.02 *

AMPD was assayed at K_m levels of substrate, 0.8 mM AMP.

Data are means $\pm$ S.E.M. (n=3).

* - Significantly different from the corresponding value at 37°C, P < 0.001; ** - P < 0.05.

Table 2.5: Effect of ATP and ADP on *C. leucurus* and rabbit skeletal muscle AMPD at 37°C and 5°C.

	37°C		5°C	
	<i>C. leucurus</i>	Rabbit	<i>C. leucurus</i>	Rabbit
	K _a (mM)	K _a (mM)	I ₅₀ (μM)	I ₅₀ (μM)
ATP	0.39 ± 0.08	0.44 ± 0.06	1.13 ± 0.05	103 ± 0.015
ADP	0.65 ± 0.05	0.16 ± 0.005	14.0 ± 1.50	NE

NE = no effect.

Data are means ± S.E.M. (n=3 determinations). Data was collected for 2 different *C. leucurus* AMPD preparations; rabbit data was collected using one AMPD preparation. Because ATP and ADP also absorb at 285 nm, AMPD enzymes were assayed at sub-K_m levels of substrate (0.3 mM AMP) to limit the total absorbance of the assay.

Figure 2.1. Typical elution profile of *C. leucurus* AMP deaminase from phosphocellulose. The enzyme was eluted in a single peak with a linear gradient of KCl (0.45 - 1.5 M). Activities are relative to the peak fraction.

< 20

< 116

< 97

< 66

< 45

< 31

< 270

< 116

< 97

< 66

< 45

< 31

Figure 2.3. Standard curve used to determine the sub-unit molecular weight of *C. leucurus* AMP deaminase. The standards and their molecular weights (kDa) are: (1) myosin, 200; (2) β -galactosidase, 116.25; (3) phosphorylase B, 97.4; (4) serum albumin, 66.2; (5) ovalbumin, 45.0; (6) carbonic anhydrase, 31.0 ($r = 0.97$).

:

Figure 2.4. pH profiles of *C. leucurus* AMP deaminase at 5°C and 37°C. The pH optimum shifted from 6.32 at 37°C (open circles) to 6.65 at 5°C (solid circles). Values are the average of 4 trials performed on a single enzyme preparation. Activities are relative to the maximum activity at the optimal pH which was set at 100.

Figure 2.5. pH profiles of rabbit AMP deaminase at 5°C and 37°C. The pH optimum shifts from 6.04 at 37°C (open circles) to 6.50 at 5°C (solid circles). Values are the average of 4 trials performed on a single enzyme preparation. Activities are relative to the maximum activity at the optimal pH which was set at 100.

Figure 2.6. Arrhenius plots for *C. leucurus* and rabbit AMP deaminase. Calculated activation energies (E_a values) were $90.9 \pm 3.0 \text{ kJ}\cdot\text{mol}^{-1}$ for *C. leucurus* (open circles) and $77.7 \pm 1.9 \text{ kJ}\cdot\text{mol}^{-1}$ for rabbit (solid circles). The data points represent the average of 4 trials performed on a single preparation of purified enzymes.

Discussion

The primary cause of death in hypothermic non-hibernators (rats and hamsters) has been reported to be respiratory failure due to glucose depletion (Musacchia, 1989). The role of carbohydrate metabolism in prolonged hypothermia has been studied in several labs; Musacchia concluded that AMP is a potent activator of both glycogen phosphorylase, which breaks glycogen stores into glucose, and PFK, a key regulatory point of glycolysis. A rise in AMP concentration indicates an energy crisis situation, and activates PFK to produce more ATP. This approach is an effective short-term mechanism which is useful under conditions such as strenuous exercise, but in a hibernating mammal, a drop in energy charge of the cell, followed with the activation of PFK would result in an increased metabolic rate and disastrous use of fuel (depletion of glucose and glycogen stores) at a time when it could not be replenished. A system to deal with a potential AMP build-up would therefore be advantageous for hibernating mammals. AMPD, an enzyme that consumes AMP to produce IMP and ammonia, has been implicated in the stabilization of cellular energy status under stressed conditions by effectively preventing an increase in AMP concentration. The impact of low temperatures upon purified AMPD was examined to determine if it could have a role in the regulation of cellular energy charge during hibernation in the prairie dog *C. leucurus*.

Bound and free fractions of AMPD

The activity of AMPD can be altered by changing the subcellular distribution between cytosol and myosin-bound fractions. Electrical stimulation and exhaustive exercise have been shown to increase the percentage of AMPD that binds to myosin in rat skeletal muscle (Rundell et. al., 1993). Acidification is characteristic of exercised muscle and may be the stimulus for binding (Barshop and Friedan, 1984). These authors

concluded that AMPD:myosin binding serves an important physiological role in the control of AMP deamination in contracting muscle. It has been demonstrated that myosin binding (in response to physiological stimuli) enhances the activity of AMPD by lowering the K_m , increasing the V_{max} , increasing sensitivity to ADP activation, and lowering susceptibility to P_i inhibition in the presence of ADP (Rundell et al., 1993). Hibernation did not appear to induce the binding of AMPD to myosin in *C. leucurus* skeletal muscle. The amount of bound AMPD did not change between the euthermic and hibernating states and the similar values for the euthermic and hibernating conditions show that hibernator AMPD activity is not altered by changing the subcellular localization during hibernation. This data, in combination with the observation that AMPD activity in crude homogenates was the same for euthermic and hibernating tissues (Appendix 1), demonstrated that any changes to AMPD activity during hibernation are most likely kinetic alterations.

Purification and subunit molecular weight of AMP deaminase

AMPD from the skeletal muscle of *C. leucurus* was purified to electrophoretic homogeneity (400 fold) with a specific activity of 60 U·mg⁻¹. Although this specific activity is lower than the value of 177.9 U·mg⁻¹ protein that Spychala and Marszalek (1991) reported for rat skeletal muscle (though this determination was made using a different assay protocol) it is similar to rabbit skeletal muscle AMPD purchased from Sigma (55 U·mg⁻¹, determined using the same assay protocol as for the *C. leucurus* AMPD).

The purification procedure modified from Smiley et al. (1967) was rapid and reproducible. Extensive washing of the phosphocellulose column was necessary to remove contaminating proteins and as shown in other examples (Smiley et al., 1967; Lushchak and Storey, 1994) was the key to a pure enzyme. As illustrated in Figure 2.3, *C. leucurus* eluted in one peak from the phosphocellulose column. Subsequent dialysis

against solid polyethylene glycol was necessary to increase the protein concentration, which has been shown to increase the stability of AMPD in solution (Smiley et al., 1967; Lushchak and Storey, 1994). A high concentration of KCl has also been shown to increase AMPD stability (Raffin et al., 1987) and therefore was not removed from the final preparation.

C. leucurus AMPD subunit molecular weight, as determined by SDS-PAGE, was 68.8 kDa. Literature values of subunit molecular weights for the enzyme from other sources are in the same range: 77 kDa for prawn (*Palaemon serratus*); 77 kDa for frog (*Rana temporaria*), 71.6 kDa for trout muscle, and 68 kDa for rabbit skeletal muscle (Raffin et al., 1987; Spychala et al., 1983; Lushchak et al., 1994; Barshop et al., 1984).

pH profiles

The cytoplasmic pH of most organs of cold-blooded animals increases 0.018 pH units for every 1°C drop in temperature (Somero, 1981). A constant ionization state, maintained on proteins over a wide temperature range, allows enzymes in the buffer system to retain their required charge groups to attain maximal activity. Malan (1978) proposed that pH is a key factor regulating the decrease in metabolic rate of a hibernating mammal. The relative acidosis found in the tissues of hibernators may decrease the activity of PFK (shutting down glycolysis and thereby saving glycogen stores) and reduce the metabolism of brown adipose tissue (Malan, 1978). The relatively acidic pH of hibernating skeletal muscle tissue may also serve to enhance optimal AMPD activity during hibernation.

AMPD activity is most necessary under stressed conditions such as exhaustive exercise where muscle pH can drop to approximately 6.5 (Dudley et al., 1985). Under stressed conditions ATP is consumed faster than it can be replenished which could potentially lead to a drop in energy charge. With the exercise example, the resulting acidic pH serves to enhance AMPD activity in skeletal muscle (Lushchak and Storey,

1993) and allow contraction to continue. While a hibernating mammal does not require sustained muscular contractions, skeletal muscle integrity is vital for shivering thermogenesis which is used throughout hibernation to maintain the hibernating Tb and to rewarm during arousal. *C. leucurus* and rabbit AMPD, in keeping with the function of the enzyme, behaved optimally under acidic conditions. The pH optima for *C. leucurus* and rabbit AMPD both increased when the assay temperature was dropped from 37°C to 5°C. The pH optimum of hibernator AMPD increased by 0.33 pH units, somewhat less than the 0.46 pH unit increase of rabbit AMPD; these shifts are quite slight and may not be reflective of physiological changes. The predicted pH increase of imidazole buffer from 37°C to 5°C is 0.58 pH units, however as demonstrated by McArthur et al. (1990) the actual pH increase in cells is not as high as predicted over any given temperature decrease. This relative cellular acidity which occurs during hibernation may therefore be one mechanism of slightly enhancing AMPD activity at no metabolic cost to the animal.

Arrhenius plots

The Arrhenius plots for *C. leucurus* and rabbit AMPD were both linear over the range of 2 to 35°C. This indicates that the enzymes do not undergo a temperature-induced conformational change as the temperature decreased, although the $V_{\max}$ of each enzyme does decline. The activation energy of *C. leucurus* AMPD was significantly higher than that of the rabbit enzyme, indicating that the maximal velocity for *C. leucurus* AMPD is not enhanced at colder temperatures with respect to a non-hibernator enzyme. In fact, hibernator AMPD activity declined more steeply than rabbit AMPD as the temperature decreased. These data seem to indicate that *C. leucurus* AMPD was more strongly depressed at cold temperatures than the enzyme from a comparative nonhibernator.

Kinetic Parameters

Substrate affinity

Both *C. leucurus* and rabbit AMPD showed unchanged substrate affinity at cold temperatures. Although the K_m values were consistent from 37°C to 5°C, they were much higher than values reported in the literature for physiological concentrations of AMP (less than 0.10 $\mu\text{mol/g}$ wet weight (Rundell et al., 1993; Storey and Kelly, 1995)). Comparison of K_m values that are so much higher than the physiological values of the substrate may be somewhat erroneous. However, in this situation, when physiological concentration is lower than the K_m , any change in the AMP concentration within the cell will result in a proportionally higher AMPD activity. In a sense then, the most potent "activator" of AMPD is its substrate (Rundell et al., 1992a and 1992b).

Allosteric effectors

At 37°C *C. leucurus* and rabbit AMPD were affected by various nucleotides (Table 2.3) in a manner that has also been reported for AMPD from other sources: inhibition by GTP and activation by ADP and ATP. However, low temperature altered the effect of these nucleotides on rabbit and *C. leucurus* AMPD. At 5°C rabbit muscle AMPD was no longer affected by ADP, and was inhibited by ATP, whereas *C. leucurus* AMPD was inhibited by both ADP and ATP (Table 2.5). In terms of AMPD function, inhibition by ATP is logical. Since AMPD functions to artificially elevate the energy charge by removing AMP, its activity would not be required under conditions where ATP levels are not depleted. Reasons given for ATP activation of the enzyme from other sources are obscure, but it has been proposed that as ATP levels decline physiologically, the activation would be lifted, causing AMPD activity to slow, perhaps as a mechanism to prevent undue loss of adenylates from the total pool (Chapman et al., 1973).

Both enzymes were inhibited by various effectors (Tables 2.3 and 2.4) at both 37°C and 5°C. *C. leucurus* AMPD was inhibited in the order $\text{GTP} > \text{MgSO}_4 > \text{MgCl}_2 > \text{CaCl}_2 > \text{Pi} > (\text{NH}_4)_2\text{SO}_4 > \text{NH}_4\text{Cl}$ at 37°C and $\text{GTP} > \text{Pi} > \text{MgSO}_4 > \text{CaCl}_2 >$

$(\text{NH}_4)_2\text{SO}_4 > \text{MgCl}_2 > \text{NH}_4\text{Cl}$ at 5°C . For each effector, *C. leucurus* AMPD was significantly more sensitive to inhibition at 5°C than at 37°C . Rabbit AMPD was inhibited by the same salts in the order $\text{GTP} > \text{MgSO}_4 > \text{Pi} > \text{MgCl}_2 > \text{CaCl}_2 > (\text{NH}_4)_2\text{SO}_4 > \text{NH}_4\text{Cl}$ at 37°C and $\text{GTP} > \text{MgCl}_2 > \text{Pi} > (\text{NH}_4)_2\text{SO}_4 > \text{MgSO}_4 > \text{CaCl}_2 > \text{NH}_4\text{Cl}$ at 5°C . Rabbit AMPD was also more sensitive to inhibition at 5°C as indicated by lower I_{50} values. In all cases, *C. leucurus* AMPD was inhibited more strongly than the rabbit enzyme at colder temperatures.

In physiological terms, inorganic phosphate (physiological levels of 2 - 10 mM) is considered to be the primary inhibitor of AMPD activity from many sources because the I_{50} values for GTP and other salt inhibitors are well above their physiological concentrations (Rundell et al. 1992a). Both enzymes were indeed affected by physiological levels of Pi at both high and low temperatures. However, whereas the impact of GTP upon AMPD activity (I_{50} value of approximately 90 μM) was far from physiological at 37°C , when assayed at 5°C the inhibitory effect was far more pronounced; the change in assay temperature resulted in a drop in the I_{50} for GTP to 1 μM or less. However, since these effects are seen in both a hibernator and a non-hibernator, any direct adaptive impact to the condition of hibernation is obscure.

Hibernation is not a static state, so changes to enzymes and/or biochemical flux must be transient in nature. Recently, covalent modification of AMPD has been shown to increase enzyme activity. Protein kinase C has been implicated in the phosphorylation of AMPD in both rabbit cardiac muscle and rat skeletal muscle, serving to increase AMPD activity by increasing V_{max} in concert with a lowering of the K_{m} to near physiological values (Thakkar et al., 1993 and Tovmasian et al., 1990). Covalent modification studies were not undertaken in this study, but they may have relevance to hibernation because phosphorylation is a rapid and readily reversible mechanism by which enzyme activity can be altered. If it is determined that *C. leucurus* AMPD is indeed subject to reversible phosphorylation, a further study would be justified to investigate both phosphorylation

effects on AMPD and the appropriate kinase that is responsible for AMPD control. The effect of phosphorylation on hibernator AMPD, including changes to K_m , V_{max} , inhibitor and activator constants, could be examined at warm and cold temperatures to determine if the *C. leucurus* enzyme would respond to phosphorylation differently than rabbit AMPD.

The data presented in this chapter do not provide evidence of enhanced AMPD activity at cold temperatures. The effects of ADP, ATP, GTP, and salts would effectively shut down *C. leucurus* AMPD activity in vivo at cold temperatures. In fact, *C. leucurus* AMPD activity is depressed more intensely than rabbit AMPD at cold temperatures; the hibernator enzyme is more sensitive to inhibition (as indicated by lower I_{50} values), and has a higher activation energy than rabbit AMPD. Therefore, the results of this kinetic study do not indicate an enhanced activity of hibernator AMPD at cold temperatures, which would in turn support a role in the maintenance of energy charge balance during hibernation. However, this does not negate the need to maintain the energy charge during hibernation, so how, then, is this accomplished by *C. leucurus*? Adenylate kinase (AK) an enzyme present in much higher concentrations with a higher activity than AMPD, is known to regulate the adenylate pool by interconverting ATP + AMP with two ADP. The next section of this study presents the purification and kinetic analysis of *C. leucurus* and rabbit AK.

Chapter 3

Effects of temperature on the kinetic and thermodynamic properties of skeletal muscle adenylate kinase from the hibernating prairie dog, *Cynomys leucurus*

Introduction

The primary function of muscle, contraction, requires large amounts of energy. Therefore, optimal muscle function requires a precise coupling of energy supplies and utilization. ATP provides the free energy for most cell functions, including muscle contraction, and although the rate of ATP usage in the process of contraction and relaxation is large, the coordination of ATP synthesis with its utilization is so efficient that no net change in ATP concentration is often detected under normal circumstances (Mommarts, 1969). During hibernation, however, the total cellular concentrations of adenylates decrease, whereas the relative amounts of ATP, ADP and AMP each remain the same (i.e. energy charge is stable) (Storey and Kelly, 1995; Appendix I). Adenylate kinase (AK, E.C. 2.7.4.3, also called myokinase in muscle) influences the relative size of the three adenine nucleotide pools as it catalyzes the interconversion of ATP and AMP with two ADP. The present study was undertaken to determine whether AK from the muscle of hibernating versus non-hibernating mammalian species are differentially affected by low temperatures and, hence, whether this enzyme could influence the pool sizes of adenylates under the low body temperature conditions of the hibernating state.

The constancy of ATP levels in the cell promoted the current role of AK as one of a steady-state enzyme, serving to equilibrate the pools of adenine nucleotides. The direction of catalysis is regulated by the availability of AMP or ADP, the concentrations of which increase when primary energy-providing metabolic pathways have been compromised (Zelevnikar et al., 1990). In addition, it has been speculated that AK (in concert with creatine kinase) may play an integral role in cellular high energy phosphate metabolism because it interconverts energy equivalents in the cell and is bound to subcellular structures that are involved in energy production and utilization (Savabi, 1994).

AK is a small, monomeric enzyme, having a molecular weight of approximately 21 kDa in mammalian muscle. Isozymes have been identified in several animal tissues including human (Luz, et al., 1990). The two main isozymes are AK1 (cytosolic, basic) and AK2 (mitochondrial, acidic). The mitochondrial AK has a lower K_m and a different pH profile for AMP + ATP than cytosolic AK (Watanabe et. al., 1979). AK has been purified and characterized from a variety of sources, but no studies have, to date, examined the impact of temperature upon its kinetic properties.

Given the importance of adenylate kinase to the regulation of the cellular adenine nucleotide pools, a comparative study was undertaken to elucidate the impact of temperature fluctuations upon the kinetic properties of this important equilibrium enzyme from hibernating (*Cynomys leucurus*) versus non-hibernating (rabbit) mammals.

Material and Methods

Animals and biochemicals

Animals were obtained as per Chapter 2. All biochemicals and coupling enzymes were obtained from either Sigma Chemical Co. or Boehringer Mannheim Corp. Rabbit muscle AK was purchased from Boehringer Mannheim in an ammonium sulfate suspension.

C. leucurus Adenylate Kinase Purification

The AK purification protocol was initially elucidated using tissues from euthermic animals. Later purification of AK from samples of hibernating tissues resulted in a protein with the same molecular weight and K_m characteristics.

Tissue samples were homogenized 1:5 (wt:vol) in 10 mM KPi, 10 mM β -mercaptoethanol, pH 5.5. The homogenate was centrifuged for 20 minutes at 23,000 x g at 4°C in a Sorvall refrigerated centrifuge. The supernatant was removed and the pellet discarded. The supernatant was brought to 20% w/v PEG (polyethylene glycol 8000) by the addition of an appropriate volume of a 50% stock solution and then incubated with mixing for 20 minutes at 7°C. The mixture was then centrifuged at 23,000 x g for 5 minutes to spin down precipitated proteins. The supernatant was removed and diluted 1:1 with buffer (10 mM KPi, pH 5.5) and then applied to a 2 x 1 cm hydroxylapatite column that had been previously equilibrated in 10 mM KPi, pH 5.5. The column was washed with this buffer until no further protein was eluted ($A_{280\text{ nm}}$ was low and constant). AK was then eluted with a KPi gradient (10 - 250 mM KPi, pH 5.5) and collected in 1 mL fractions. Fractions containing the majority of AK activity were pooled, dialyzed for 1-2 hours against 2 L of 10 mM KPi, pH 5.5, and then applied to a blue dextran affinity column. The blue dextran column was washed with 40 - 50 mL of dialysis buffer to elute unbound proteins and then AK was eluted with a KCl gradient (0-1.25 M in the same

buffer) and collected in 1.0 mL fractions. Peak activity fractions were pooled and concentrated against solid PEG to a volume of less than 1 mL. This sample was then injected onto an Varian TSK 4000 HPLC size exclusion column (7.5 mm x 60 cm, equipped with guard column). The elution buffer was 10 mM KPi, 100 mM KCl, pH 7.2. The column was run over 40 minutes with a flow rate of 0.5 mL per minute. One minute (0.5 mL) fractions were collected, assayed, and peak activity fractions were pooled and brought to 300 mM KCl. The final preparation was stored at 4°C.

Molecular weights

Purity and molecular weight of AK were assessed with SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The running gel was 12% acrylamide and the stacking gel was 5% acrylamide. The running buffer was 24.8 mM Tris, 192 mM glycine, pH 8.8 containing 0.1% w/v SDS. Before application to the gel, protein samples were mixed 1:1 v/v with a solution of 46.8 mM Tris buffer, pH 6.8, containing 7.5% v/v glycerol, 2.25% w/v SDS and 10 % v/v β -mercaptoethanol. Samples were incubated in a boiling water bath for 2 minutes and then loaded onto the gel. The gel was run at 150 volts until the bromophenol blue dye reached the bottom of the gel (approximately an hour) and then fixed for one hour in a solution of 10% acetic acid and 25% methanol (vol:vol). The gel was then stained with silver in the following method: washed for 3 X 20 minutes in distilled water followed with a wash for 30 minutes in a solution of silver nitrate (2 g per litre). The protein bands were developed with the addition of reducing agent, a solution of sodium carbonate and formaldehyde (14.5 grams NaCarbonate and 0.25 mL of a 0.37% solution of formaldehyde per 500 mL double distilled water); development was stopped with the addition of 5% acetic acid.

Adenylate kinase is a monomeric protein so the native molecular weight can be determined from a plot of log molecular weight versus R_f of the SDS-PAGE standards. The standards (kDa) used for MW determination were: bovine serum albumin (66.0),

ovalbumin (45.0), carbonic anhydrase (31), trypsin inhibitor (21.5) and lysozyme (14.5).

As a confirmation of this molecular weight, *C. leucurus* AK was applied to a gel filtration column (Sephacryl-200 equilibrated in 100 mM KCl and 10 mM phosphate buffer pH 7.2) and the elution profile compared to that of rabbit AK (known molecular weight).

Adenylate kinase assay (E.C. 2.7.4.3)

Adenylate kinase (AK) interconverts one AMP and one ATP with 2 ADP in a reversible reaction. For assays in the direction of ADP production, AK activity was linked with the coupling enzymes pyruvate kinase (PK) and lactate dehydrogenase (LDH). Enzyme activity was followed by measuring the decrease in NADH absorbance at 340 nm in the following assay system:

Maximal adenylate kinase activity was determined in the presence of 1 unit each of LDH and PK, 1.0 mM ATP, 2.0 mM AMP, 2.0 mM PEP, 0.15 mM NADH, 2.5 mM Mg^{2+} , 20 mM KCl and 20 mM phosphate buffer (pH 7.2) in a cuvette volume of 1.0 mL.

In the direction of ADP utilization, AK activity was coupled to that of hexokinase (HK) and glucose-6-phosphate dehydrogenase (G-6-PDH) in the following assay system:

Maximal AK activity was determined in the presence of 15 mM glucose, 0.2 mM NAD⁺, one unit each of HK and G-6-PDH (NAD-dependent from *Leuconostoc mesentoides*), 2.0 mM ADP, 20 mM KCl, 2.5 mM MgCl₂, and 20 mM KPi, pH 7.2.

Isoelectric Focusing

Isoelectric focusing was performed by the method of Vesterberg (1971). Isoelectric points (pI's) were determined for both hibernating and euthermic *C. leucurus* muscle samples. Tissue samples from hibernating and euthermic muscle were homogenized as per the purification procedure above. After centrifugation, equal volumes of euthermic and hibernating muscle extracts were combined and used for the focusing experiments. The samples were focused at 450 V for approximately 14 hours in an LKB Products 8101 (110 mL) column with a pH 3.5 to 10 gradient of LKB ampholines in a sucrose gradient. After focusing, the column was drained and collected in 2 mL fractions using a Gilson fraction collector. The fractions were assayed for AK activity and the pH of every second fraction was recorded.

Arrhenius Plots

Arrhenius plots were obtained for *C. leucurus* and rabbit AK in both the forward and reverse directions. Maximal velocity assay conditions were employed over the range of 2 to 43°C. Cuvette temperature was regulated with a water-jacketed cell holder attached to an adjustable temperature circulating water bath.

pH curves

To determine pH optimum, the enzymes were assayed at several pH values under optimal substrate conditions as described above (ADP was the substrate). The enzymes were assayed at 37°C and 5°C.

Kinetics: Substrate Affinity, Inhibitor Constants, Activator Constants

To investigate the effect of temperature on substrate affinity, K_m values were determined for *C. leucurus* and rabbit AK for ATP, ADP, and AMP over a range of temperatures from 5°C to 44°C. Inhibitor constants were determined for KCl, NaCl, NH_4Cl and $(\text{NH}_4)_2\text{SO}_4$ for both species at both 5°C and 37°C. Activator constants were determined for MgCl_2 and MgSO_4 . Assays were performed using a Dynatech MR500 Microplate reader or a Gilford spectrophotometer.

Degradation Curves: Urea and Temperature

The effect of urea concentration and temperature change on AK stability were assessed. For both the urea and temperature studies, the rabbit enzyme was diluted from the Boehringer stock such that the activity (under the same assay conditions) was equivalent to that of the *C. leucurus* AK. Because the specific activities were the same for both enzyme preparations, the dilution resulted in a similar amount of protein in each incubation. Hence protein concentration was not a limiting factor for the death rate of the enzymes. In addition, neither protein was dialysed to remove salts; the final concentration of which was somewhat higher in the hibernator preparation used for these studies.

To examine the effects of a chemical denaturant, urea, on AK activity, aliquots of enzyme were added to a series of Eppendorf tubes containing the following concentrations of urea: 0, 0.1, 0.25, 1, 1.25, 1.75, 2, 4, 8 M (stock solution of urea was in 10 mM KPi buffer, and was diluted with the same buffer to reach the final concentration; the volume in each tube was the same). Incubations were carried out at 5°C (using an Omega CN390 recirculating water bath) and 37°C (using a Baxter heating block). After 60 minutes, an aliquot was removed from each tube and assayed for AK activity ($n = 4$ assays of each sample were conducted using the Dynatech MR500 Microplate reader, under optimal assay conditions, at room temperature, with ADP as the substrate). For

temperature studies, samples of enzyme were incubated at various temperatures in Baxter heating blocks. Aliquots were removed at time intervals and assayed for residual AK activity (ADP as substrate) at 22°C. All activities were compared with the initial activity of the preparation at time zero.

Data and statistics

Enzyme kinetic parameters were analyzed using a computer program developed for this purpose (Brooks, 1992). Data are expressed as the mean $\pm$ SEM with statistical analysis performed using the Student's t-test.

Results

Purification

C. leucurus AK was purified to a final specific activity of 373 U/mg protein. Purified rabbit muscle AK has a specific activity of 285 U/mg protein (determined using the same assay conditions). Table 3.1 illustrates the progressive increase in enzyme specific activity through each of the purification steps. The purification procedure used a combination of ion-exchange, affinity and size exclusion chromatography. AK eluted in a single sharp peak off each column. Typical elution profiles for hydroxyapatite (peak centered at 110 mM KPi), blue dextran (peak at approximately 400 mM KCl) and HPLC size exclusion (peak at 27 minutes) are shown in Figures 3.1, 3.2 and 3.3, respectively. Though the 20% PEG cut resulted in some loss of activity from the crude homogenate, it was the only step that eliminated a problematic protein. Similarly, the hydroxyapatite step resulted in a substantial loss of activity, but was the only ion-exchange resin that bound at least some of the AK. The final purified enzyme was stored in 300 mM KCl at 4°C and showed no loss of activity over at least 4 weeks.

SDS-PAGE and Molecular weight

Figure 3.4 shows a photographic representation of the silver-stained SDS-PAGE gel used to assess purity and molecular weight of AK. A single band in the lane with the purified enzyme indicated an essentially homogeneous preparation. A molecular weight standard curve (log MW vs. R_f, shown in Figure 3.5) was constructed from the SDS-PAGE data and used to calculate a molecular weight of 22.8 kDa for *C. leucurus* AK. Figure 3.6 shows the overlapping elution profiles for *C. leucurus* and rabbit AK from the S-200 size exclusion column and indicates that the prairie dog enzyme was the same size as the typical mammalian skeletal muscle enzyme.

Isoelectric Focusing

Figure 3.7 illustrates a representative IEF profile of a 1:1 v/v mixture of crude extracts from muscle of euthermic and hibernating animals (prepared using the same homogenization techniques). This procedure was performed in duplicate and each trial indicated only one peak of AK activity with a mean pI of 7.25 ± 0.04 .

Arrhenius Plots

Arrhenius plots were constructed for both the forward and reverse directions for *C. leucurus* and rabbit muscle AK (Figures 3.8 and 3.9, respectively) from assays of $V_{\max}$ enzyme activity over the temperature range 2°C to 42°C. In all cases, the plots were linear over the entire range of assay temperatures. Activation energies were determined from these plots and are shown in Table 3.2.

For both enzymes, the reaction direction with ATP and AMP as substrates had a slightly higher E_a than that with ADP. The hibernator values were not significantly different, but the rabbit values were ($P < 0.001$). Though the difference between the rabbit values was 20%, the significant difference is most likely due to very tight SEMs.

pH Optima

Figure 3.10 and 3.11 show the pH optima for the ADP-utilizing direction at both 5°C and 37°C for *C. leucurus* and rabbit AK, respectively. Assays were performed over a pH range of 5.5 to 8.5 in phosphate buffer under optimal substrate conditions. Both *C. leucurus* and rabbit AK showed broad pH optima (generally pH 6-7 at 37°C and pH 7-8 at 5°C). Both enzymes were relatively insensitive to pH changes over the physiological range, though the hibernator curves do have steep slopes at the extremes. Both enzymes had pH optima of approximately 6.5 at 37°C. In both cases, activity remained above 80% of maximum between pH 5.8 and 7.5, dropping to 50% at pH 8.5. At 5°C, both *C. leucurus* and rabbit AK pH optima increased to approximately 7.40, however, the

C. leucurus enzyme was more sensitive to changes in pH. Hibernator AK activity dropped to 40% of optimal over the pH range 7.5 to 8.2, whereas the rabbit enzyme remained over 60 % active up to pH 8.70.

Substrate Affinities and Relationships to Temperature

Substrate affinity constants were determined at 5°C and 37°C for ATP, ADP and AMP for both *C. leucurus* and rabbit muscle AK; data are shown in Table 3.3. In all cases, K_m values dropped by approximately 40-60 % with the change in assay temperature from 37°C to 5°C, indicating that both enzymes had a greater affinity for substrates at cold temperatures. The changes at low temperature were statistically significant in all cases (statistical differences are indicated in Table 3.3).

Both Table 3.3 and Figure 3.12 show that, in most cases, the absolute K_m values for the three substrates were different between the species at any given temperature. However, Figure 3.12 shows that the effect of temperature on these K_m 's followed the same trends for both enzymes. The K_m for each substrate decreased with temperature, gradually dropping over the range of 42°C to 5°C. In both cases, the K_m for ADP increased sharply at 42°C, rising by 2-fold above the value at 37°C.

Salt Effects

Various salts inhibited AK and the inhibitory effects of KCl, NaCl, NH_4Cl , and $(\text{NH}_4)_2\text{SO}_4$ were analyzed for both hibernator and rabbit AK at 37°C and 5°C. I_{50} values for these salts are shown in Table 3.4; statistical differences between values at 37 and 5°C within a species were assessed using the Student's T-test. I_{50} values for these salts changed significantly from 37°C to 5°C for both *C. leucurus* and rabbit AK. The I_{50} values increased at low temperature for KCl, NaCl, and $(\text{NH}_4)_2\text{SO}_4$ but decreased for NH_4Cl in the same manner for both enzymes. The change in I_{50} values between the two

temperatures was in most cases about 20 %, but the absolute values at each temperature were much the same for both species; most statistical differences are likely the result of very tight SEMs.

Magnesium is an essential cofactor for the AK reaction. Activator constants for Mg^{2+} salts, determined at 37°C and 5°C for *C. leucurus* and rabbit muscle AK, are found in Table 3.4. As indicated by the higher K_a values, *C. leucurus* AK was significantly less sensitive to Mg^{2+} activation than rabbit AK, regardless of the magnesium salt used or the assay temperature. For any given temperature and Mg^{2+} salt, the K_a was significantly greater ($P < 0.001$) for the hibernator enzyme, compared with rabbit AK. Assay temperature also significantly affected the K_a for Mg^{2+} for the enzyme from each species. Hibernator AK K_a for MgCl_2 at 5°C dropped 14% from the 37°C value, whereas the K_a for MgSO_4 dropped 20% under the same temperature change ($P < 0.01$). Rabbit AK showed a similar decreasing trend, as the K_a MgCl_2 at 5°C dropped 44% from the 37°C value ($P < 0.001$); the K_a MgSO_4 also decreased, but less dramatically, by 23% ($P < 0.01$). The Mg^{2+} salt used had little influence on the activator constant; only rabbit AK assayed at 37°C showed a significant difference between the K_a for MgCl_2 and MgSO_4 , the K_a being 17 % lower for the sulfate salt ($P < 0.005$).

AK Susceptibility to Denaturation by Urea and Temperature

Exposure to different concentrations of urea or to high temperatures are commonly used as probes to examine the stability of an enzyme. The effects of both were tested on hibernator and rabbit AK. Figure 3.13 illustrates the decrease in AK activity with increasing concentrations of urea. Concentrations higher than 2 M reduced AK activity to near zero in all cases. Urea had the same effect on both enzymes at 5°C with the greatest decline in activity occurring between 1 M and 2 M. Both enzymes showed an increased susceptibility to urea when incubated at 37°C. At 1.0 M urea, for

example, residual *C. leucurus* and rabbit AK activity had dropped to approximately 60% and 50% of initial activity, respectively.

Thermostability of the enzymes was monitored over time during enzyme incubation at various high temperatures. The two enzymes differed markedly in their thermostability (Figure 3.14). The maximal activity of *C. leucurus* AK decreased over time at all incubation temperatures (40°C, 50°C, 55°C). The rate of enzyme activity decrease was greater as temperature rose, as demonstrated by the half-times of activity loss (63 minutes, 16 minutes and 9.4 minutes for 40, 50, and 55°C, respectively). At 55°C, *C. leucurus* AK activity dropped by 65 % within 20 minutes of incubation and was reduced to zero by 30 minutes. By 130 minutes, AK activity had declined to zero at all temperatures. By contrast, rabbit AK largely maintained its activity over time at all three elevated temperatures; even with the longest incubation time, activity was still 70-80% of the initial value.

Table 3.1: Purification of adenylate kinase from *C. leucurus* skeletal muscle.

Purification Step	Total Protein (mg)	Total Units	Specific Activity (U·mg ⁻¹)	Fold purification	Yield (%)
Crude Homogenate	60.68	273	4.50	1.0	100
PEG cut	22.08	218	9.6	2.1	79.8
Hydroxyapatite	0.714	45.1	67.4	15	16.5
Blue Dextran	0.21	30.3	189	42	11
Gel Filtration	0.021	7.80	373	82	3.5

Table 3.2: Activation energies for adenylate kinase from *C. leucurus* and rabbit muscle. Values are presented in kJ/mol and are the means $\pm$ SEM, n = 4 trials. * -

Significantly different from the corresponding value for the opposite direction ($P < 0.01$).

	Substrate: ATP + AMP	Substrate: ADP
<i>C. leucurus</i>	49.83 $\pm$ 1.12	46.30 $\pm$ 1.05
Rabbit	52.90 $\pm$ 1.45	41.50 $\pm$ 0.41*

Table 3.3: *C. leucurus* and rabbit muscle AK substrate affinity constants for ATP, ADP, and AMP at 37°C and 5°C. Values are in μM and represent the means $\pm$ SEM. $n=3$ or 4 trials (different *C. leucurus* AK preparations, the same rabbit AK preparation). Significant differences between values at 37°C and 5°C are represented as follows: $P < 0.05$ (a), $P < 0.005$ (b).

Substrate	<i>C. leucurus</i> AK		Rabbit AK	
	37°C	5°C	37°C	5°C
ATP	113 ± 17.0	63 ± 1.00^a	73.3 ± 0.70	29.0 ± 4.70^b
ADP	256 ± 6.00	105 ± 10.0^b	256 ± 18.0	147 ± 12.40^b
AMP	218 ± 2.00	104 ± 2.00^b	120 ± 14.0	79.0 ± 2.30^a

Table 3.4: Effect of salts on adenylate kinase activity at 37°C and 5°C. Data are means $\pm$ SEM, n = 4 trials using the same enzyme preparation; all values are in mM.

Effector	<i>C. leucurus</i> AK		Rabbit AK	
	37°C	5°C	37°C	5°C
Inhibitor. Constant (I_{50})				
KCl	186 $\pm$ 4.3	239 $\pm$ 11.3 ^a	146 $\pm$ 4.0	190 $\pm$ 2.2 ^a
NaCl	161 $\pm$ 11.7	198 $\pm$ 9.46 ^b	137 $\pm$ 5.8	173 $\pm$ 5.2 ^a
NH ₄ Cl	147 $\pm$ 4.13	127 $\pm$ 4.12 ^b	187 $\pm$ 2.6	153 $\pm$ 1.2 ^a
(NH ₄) ₂ SO ₄	13.0 $\pm$ 0.74	21.4 $\pm$ 0.54 ^c	30.0 $\pm$ 0.33	40.3 $\pm$ 2.28 ^a
Activator Constant (K_a)				
MgCl ₂	0.197 $\pm$ 0.005	0.170 $\pm$ 0.005 ^c	0.124 $\pm$ 0.002	0.070 $\pm$ 0.002 ^a
MgSO ₄	0.181 $\pm$ 0.006	0.145 $\pm$ 0.006 ^c	0.103 $\pm$ 0.003	0.079 $\pm$ 0.005 ^c

a - Significantly different from the corresponding value at 37°C, $P < 0.005$; b - $P < 0.05$;

c - $P < 0.01$.

Figure 3.1: Hydroxylapatite chromatography. Typical elution profile of *C. leucurus* adenylate kinase from hydroxylapatite. The column was equilibrated in 10 mM KPi, pH 5.5 and AK was eluted with a linear (10 mM - 250 mM) gradient of KPi (dashed line) and collected in 40 x 1.0 mL fractions.

Figure 3.2: Blue dextran chromatography. Elution profile of *C. leucurus* AK from blue dextran agarose. The column was equilibrated in 10 mM KPi, pH 5.5. AK was eluted with a linear salt gradient of 0 - 1.25 M KCl (dashed line); the effluent was collected in 40 x 1.0 mL fractions.

Figure 3.3: HPLC size exclusion chromatography. Typical elution profile of *C. leucurus* AK from the TSK gel filtration HPLC column; elution buffer was 100 mM KCl, 10 mM KPi, pH 7.2. The flow rate was 0.5 mL per minute; 40 one minute fractions were collected.

Figure 3.4: Representation of the SDS-PAGE gel for the final preparation of *C. leucurus* adenylate kinase. Gel was stained with silver and molecular weight standards are indicated with arrows. The standards were: bovine serum albumin (66.0 kD), ovalbumin (45.0 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD) and lysozyme (14.5 kD).

---66

---45

---31

---21.5

---14.5

Figure 3.5 : SDS-PAGE molecular weight standard curve. The log MW of the following standards are plotted against the Rf values for protein migration: bovine serum albumin (66.0 kD), ovalbumin (45.0 kD), carbonic anhydrase (31 kD), trypsin inhibitor (21.5 kD) and lysozyme (14.5 kD) ($r = 0.98$)

Figure 3.6: Sephacryl-200 size exclusion chromatography. The top figure shows the elution profile of *C. leucurus* AK from a S-200 size exclusion column; data for rabbit AK are presented in the lower figure. Enzyme activities are presented relative to the activity in the peak fraction.

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Figure 3.7 : Isoelectric focusing profile. Typical IEF profile of *C. leucurus* AK. A mixture of crude and euthermic muscle extracts was focused in a pH 3.5-10 gradient at 400 V for 14 hours. Fraction pH values were assayed after enzyme activity was measured and are denoted by the open circles. AK enzyme activity is shown by the closed circles.

Figure 3.8: Arrhenius plots for *C. leucurus* Ak. Arrhenius plots were constructed for both reaction directions. Closed circles represent ADP as the substrate, open circles represent ATP and AMP as the substrate. Data are means $\pm$ SEM, n = 4 trials.

Figure 3.9: Arrhenius plots for rabbit AK. Arrhenius plots were constructed for both reaction directions. Closed circles represent ADP as the substrate, open circles represent ATP and AMP as the substrate. Data are means $\pm$ SEM, $n = 4$ trials.

Figure 3.10: *C. leucurus* AK pH profiles. Assays were performed under optimal conditions at 37°C (open circles) and 5°C (closed circles) with ADP as the substrate. Data are presented as the mean $\pm$ SEM, n = 4 trials.

Figure 3.11: Rabbit AK pH profiles. Assays were performed under optimal conditions with ADP as the substrate at 37°C (open circles) and 5°C (closed circles). Data are presented as the mean $\pm$ SEM, n = 4 trials.

Figure 3.12: Effect of temperature on substrate affinity constants for AK. C.

leucurus AK is denoted with open symbols, rabbit AK with closed symbols. Substrates are denoted as follows: ATP = triangles, ADP = squares, AMP = circles. Values are means $\pm$ SEM (n = 4 trials). K_m values are in mM.

Figure 3.13: Effect of urea on the activity of *C. leucurus* and rabbit AK. Aliquots of AK were incubated at 37°C or 5°C with varying concentrations of urea (a protein denaturant) for one hour and then assayed at 22°C for residual activity. The upper graph shows *C. leucurus* AK at 37°C (closed circles) and 5°C (open circles), the lower graph shows comparable results for rabbit AK at 37°C (closed circles) and 5°C (open circles). Values are presented as the mean $\pm$ SEM (n=4 trials using the same enzyme preparation, for each animal).

Figure 3.14: Effect of high temperature incubations on the activity of *C. leucurus* and rabbit AK. Aliquots of AK were incubated at 40°C, 50°C and 55°C, then assayed at 22°C for residual activity. *C. leucurus* activity is indicated with open symbols, rabbit activity with closed symbols. Incubation temperatures are: 40°C, inverted triangles; 50°C, triangles; 55°C, circles. Values are presented as the mean $\pm$ SEM (n=4 trials using the same enzyme preparation, for each animal).

Discussion

The constancy of cellular energy charge during ATP utilization prompted the suggestion that adenylate kinase functions only to equilibrate the stores of adenine nucleotides. Though it is generally thought that the equilibrium is only perturbed when the concentrations of AMP or ADP increases, as occurs when the primary energy-producing metabolic pathways become compromised, recent studies purpose that AK acts in concert with creatine kinase to transfer energy-rich phosphoryls at mitochondrial and myofibular sites and, hence, may have even great importance for regulation of cellular energy status (Zelevnikar et al., 1990; Savabi, 1994).

Adenylate kinase is a unique enzyme of adenylate regulation pathway, catalyzing the only reversible reaction in the pathway. Because it interconverts all three components of the energy charge equation (ATP, ADP, AMP) it is considered an important component of adenylate regulation. Under conditions of stress (such as intense exercise) AK operates in the direction of ATP production (Savabi, 1994). In addition, because no metabolites act as allosteric effectors of mammalian skeletal muscle AK, this enzyme is sensitive to changes in substrate.

The purification protocol resulted in an essentially pure *C. leucurus* AK preparation, as evidenced by the SDS-PAGE gel showing one definitive band. The PEG cut resulted in the loss of some AK activity but it effectively removed a problematic contaminating protein. Similarly, though the hydroxyapatite step resulted in a low yield, it was the only ion-exchange resin that bound AK.

Nearly all mammalian adenylate kinases studied to this point (including bovine heart (Batra and Skinner, 1990), porcine heart (Watanabe et al., 1979) and rabbit muscle (Noda, 1973)) have a molecular weight in the range of approximately 22.5 kDa. The molecular weight of 22.8 kDa, as calculated from the SDS-PAGE standards, is therefore

in agreement with literature values. In addition, *C. leucurus* and rabbit AK showed identical activity profiles from the S-200 size exclusion column, illustrating that both the hibernator and rabbit enzymes have very similar molecular weights.

Isoelectric focusing, a useful technique for separating enzymes the basis of their isoelectric points, was used to eliminate the possibility that different enzyme forms of AK occurred in euthermic and hibernating animals. After focusing a mixture of euthermic and hibernating supernatants of crude muscle homogenates, AK activity was detected at only one point along the gradient, demonstrating the presence of only form of *C. leucurus* AK. In addition, maximal activities of *C. leucurus* AK in skeletal muscle extracts (shown in Appendix 1) were not significantly different between the hibernating and euthermic conditions. These activities in crude supernatants, in concert with the IEF data, demonstrate that entrance into torpor does not alter total enzyme activity nor the complement of AK. However, because all experimental animals were collected at the same time of the year, this experiment does not eliminate the possibility of winter and summer forms of AK, a phenomenon that has been seen with respect to other enzymes in other hibernating species, such as LDH in the little brown bat (Moon, 1978). In addition, Borgmann and Moon (1975) demonstrated changes in PK isozyme patterns in liver and muscle of little brown bats which could also be seasonal since hibernating bats were collected in early April while the normothermic animals were collected in August.

A clear break in the linearity of an Arrhenius plot indicates a conformational change in the protein that affects the rate of catalysis. Over the wide temperature range tested, no such temperature-dependent change in protein conformation was identified for either *C. leucurus* or rabbit AK regardless of reaction direction, as demonstrated by the linear Arrhenius plots. With ADP as the substrate, E_a values were slightly lower than when ATP + AMP were substrates, perhaps reflecting a greater tendency for the enzyme to function in the direction of ATP + AMP production. However, only the values for rabbit AK were significantly different between the directions. Physiologically, the

similarity of *C. leucurus* and rabbit E_a values do not indicate a major difference between the enzymes that would imply a specific adaptation of the *C. leucurus* protein to facilitate enzyme function during hibernation. In addition, the E_a values are also similar to those determined for purified bovine cardiac AK isozymes (Walker and Dow, 1982).

Hypercapnia-induced acidosis has received a great deal of attention in the literature as a potential modulator of enzyme activity in hibernating species (Malan *et al.*, 1985; Malan and Mioskowski, 1988). A relative acid shift in cellular pH is said to occur during hibernation because as temperature drops, the pH of the cytosol does not increase as far as theory would predict. This pH shift could influence the activities of enzymes and pathways that are pH sensitive. pH optima, determined at 37°C and 5°C for purified AK from both species with ADP as the substrate, did increase at the lower temperature, but the changes were similar for both species (6.70 to 7.40). The hibernator AK was more sensitive to higher pH's at 5°C, as indicated by the sharp decline in activity between pH 7.40 and 8.50, but the two enzymes behaved in essentially the same manner within the physiological pH range. Although a temperature drop does change the optimal pH range for hibernator AK, the enzyme is not inherently different from that of a non-hibernator (in this case, rabbit) with respect to temperature induced pH changes and the data, therefore, do not support a hibernation-specific regulation mechanism.

Ligand-binding is considered to be a particularly temperature-sensitive event because it depends on three factors that are easily disrupted by temperature: (a) maintenance of binding site geometry, (b) establishment of noncovalent bonds between the binding site and ligand, and (c) a change in conformation as a part of the binding event (Somero, 1995). An examination of substrate affinity changes with temperature can lead to inferences about catalytic efficiency (a function of active site integrity) and provide a means of comparing the impact of temperature upon enzyme function between species. K_m values for both species were similarly affected by temperature, regardless of substrate. K_m for ATP, ADP and AMP declined approximately 40-60% from 37°C to 5°

C for *C. leucurus* and rabbit. The decrease in K_m indicates a continued functional activity for both enzymes at cold temperatures. However, the similarity between the species precludes a conclusion of an adaptation to hibernation.

A variety of salts affected *C. leucurus* and rabbit AK. The chloride salts showed consistent inhibitory effects with ammonium being the most inhibitory, then sodium, and potassium. Comparison of the two ammonium salts indicated strong inhibition by the sulfate anion compared with chloride, a trend that was seen with AMPD as well (see Chapter 2). Again, definitive studies of ammonium levels in hibernating mammals do not exist, but considering its effect on the enzymes in this study, such levels are of interest.

Although AK from both animals was inhibited by all salts tested, the fold changes from 37°C to 5°C were not of a level to affect enzyme activity with a physiological change in temperature. Most of the between-species comparisons were significantly different due to very tight SEM values, but do not reflect a consistent behaviour or meaningful difference that can be promoted as adaptive for *C. leucurus*.

The effect of chemical denaturants and temperature on enzyme activity are common probes of enzyme stability, providing an indication of its structural integrity. Samples of AK from both species were incubated with increasing concentrations of urea at 37°C and 5°C and monitored for the effect on activity. Both enzymes responded to this treatment in a similar manner, with residual activity reduced to almost zero by 1.0 M urea, demonstrating that the denaturing effects of urea (unfolding) have the same impact upon activity for both enzymes.

Thermostability was a distinguishing factor between the AK enzymes. Data in Figure 3.14 show a greater thermolability of the hibernator enzyme. At each incubation temperature (40°C, 50°C and 55°C), *C. leucurus* AK activity gradually declined to near zero by 120 minutes of incubation. Rabbit AK however remained at 80% or greater of its initial activity over the same incubation period, regardless of the incubation temperature.

This demonstrates a greater thermostability for the rabbit enzyme, perhaps indicating a structurally tighter enzyme, indicative of an enzyme designed to function physiologically at only one temperature. By contrast, the hibernator enzyme is designed to function physiologically over a range of temperatures and is consequently more flexible.

This study compared skeletal muscle AK from hibernating and nonhibernating mammalian species and showed them to share many kinetic and functional parameters. Many properties including the inhibitory effects of salts, activation by magnesium, pH optima, and E_a values were all similar between the species. Only the high temperature incubations showed the two enzymes to be distinct, with the hibernator enzyme being more sensitive to high temperature. It was proposed that this may be the result of a more flexible structure for the *C. leucurus* enzyme.

Chapter 4

General Discussion

The studies presented in this thesis were initiated with two aims: (1) to compare the impact of cold temperature on the kinetic behavior of enzymes of adenylate metabolism from a mammalian hibernator and a non-hibernator, and (2), from the accumulated data, attempt to elucidate an enzymatic mechanism of adenylate regulation that is unique to a hibernating mammal. The enzymes studied, AMP deaminase and adenylate kinase, are integral to the maintenance of cellular energy charge. In studies using isolated cell systems, Chapman (1973) found that under conditions of increased ATP utilization AMPD provided a mechanism that, in concert with AK, stabilized the relative concentrations of the three adenylates at the cost of a net loss to the adenine nucleotide pool. Chapman (1973) proposed that AMPD removed AMP from the adenylate pool and as a result pulled the AK reaction towards ATP production, thereby tempering a build-up in ADP and equilibrating relative amounts of ATP, ADP and AMP. So, while AMPD removes adenylates from the total pool, AK acts to equilibrate the remaining pool.

In many situations where ATP is depleted (for example: muscle exercise, hypoxia/ischemia) the concomitant increase in AMP concentration is a key signal that activates catabolic pathways (glycolysis in particular, via AMP activation of PFK) to increase ATP synthesis. This situation is clearly disadvantageous for a hibernator during torpor but may be useful during arousal. Although El Hachimi et al. (1990) did not detect a drop in ATP content during hibernation in the jerboa, many other studies have demonstrated ATP depletion during torpor. For example, the total adenylate pool (ATP + ADP + AMP) dropped to 47% of control values in *Z. hudsonius* skeletal muscle after 24 days of hibernation (Storey and Kelly, 1995). Bronnikov et al. (1990) found that levels of ATP + AMP dropped 5-fold in liver mitochondria from hibernating ground squirrels, while Gehnrich and Aprille (1988) found intramitochondrial adenine nucleotides declined

by 30% during hibernation. A similar drop was seen in *C. leucurus* muscle, as the total level of adenylates in hibernating muscle samples dropped 33% from euthermic values (Appendix 2). In all these studies the animals maintained the energy charge of the cell despite the drop in the total concentration of adenylates.

In terms of the kinetic and thermodynamic data obtained, AMPD activity was more sensitive to decreased temperatures than AK. *C. leucurus* AMPD had a higher activation energy than both AK and the comparison rabbit AMPD. Substrate sensitivity was very different between the enzymes as AK was generally more sensitive to substrate than AMPD. AK affinity for AMP (a common substrate for the two enzymes) was much higher than that of AMPD (3.6-fold higher at 37°C and 8-fold higher at 5°C). In addition, where the AK K_m for AMP dropped with temperature, AMPD K_m remained steady. Therefore, although AMPD maintained its substrate affinity and hence functional ability at cold temperatures, AK is most likely far more sensitive to changes in AMP levels than AMPD. Finally, at cold assay temperatures, *C. leucurus* AMPD was strongly inhibited by salts, inorganic phosphate and GTP at physiological levels. In contrast, AK, while inhibited by salts at a physiological level, showed I_{50} values that were not drastically different between cold and warm temperatures. So while both enzymes studied maintained catalytic ability at cold temperatures, neither was enhanced with respect to a non-hibernator, the rabbit.

As demonstrated by the enzyme kinetic and metabolite data, the well-theorized role of AMPD and AK acting in concert to maintain the energy charge at a loss to the total adenylate pool does not appear to be at work in this hibernator. However, as depicted in Appendix 2, there is a statistically significant drop in the total adenylate level which is predominantly due to the decrease in ATP levels, indicating that ATP utilization is not matched by synthesis during hibernation. But, as described previously, AMPD and AK

do not appear to be catabolizing AMP and equilibrating the residual adenylates to stabilize cellular energy charge during hibernation. The concentrations of AMP and IMP did not increase, and the key enzymes of this system were inhibited at low temperatures forgoing any indication of enhanced enzyme function.

What then is the mechanism by which adenylate levels decline, if not via AMPD? AMP degradation however, can also occur via the enzyme 5'nucleotidase, but AMP catabolizing activity by 5'NT was not detected with the assay system used. Further study with a more sensitive assay system may allow for detection of this activity. Alternatively, it is possible that 5'NT is responsible for IMP degradation to inosine. A first step to such a study would be the accurate determination of inosine, followed by a kinetic study of 5'NT IMP catabolic activity. Further catabolism of adenylates beyond inosine is unlikely because: (1) activity of the final enzymes in the degradation pathway, XO and XDH, were not detected in skeletal muscle (unpublished data), and (2) these reactions are irreversible and represent a permanent loss to the adenylate system.

While a mechanism of adenylate regulation was not elucidated, there is evidence that the decline of adenylate levels may be an important mechanism of metabolic depression. Doherty et al. (1993) showed that erythrocytes from hibernating ground squirrels contained about 50 % of the ATP content of cells from euthermic animals and that cold storage of blood from euthermic animals resulted in a decrease in ATP levels to values comparable to those of hibernators whereas cold storage of hibernator blood did not alter ATP levels. Hypothermia disrupts energy metabolism in non-hibernators (Hochachka 1986) but hibernators may exploit a low temperature-induced suppression of adenylate and levels as part of the transition to hibernation. Thus, a net reduction in the overall cellular pools of high energy compounds (such as ATP) may be part of the mechanism of metabolic suppression in hibernation. Aprile (1988) reviewed the strong

evidence that shows that changes in the mitochondrial adenylate pool size can lead to selective regulation of metabolic activities that have adenine nucleotide dependent steps localized in the mitochondria, such as gluconeogenesis and urea biosynthesis. Thus, one way to facilitate metabolic rate depression by hibernating mammals may be to alter the activities of the mitochondrial membrane ATP-Mg/P_i transporters to modify ATP-Mg uptake/release and via the consequent change in the net mitochondrial adenylate pool size, suppress the rates of ATP generation and ATP-dependent reactions in the organelle (Aprille 1988). However, in the non-hibernating mammalian species studied to date, the changes in mitochondrial adenylate pool size are usually caused by a redistribution of adenylates between mitochondria and cytoplasm with no net change in overall tissue adenylate levels (Aprille 1988). Hibernators may take the process one step further with a net decrease in total cellular adenylate levels that could help to sustain long term metabolic suppression in all subcellular compartments at low body temperatures (Storey and Kelley, 1995). However, the mechanism by which this decrease would occur is still unclear.

Appendix 1

Activity of adenylate metabolism enzymes in supernatants of crude homogenates from euthermic and hibernating *C. leucurus* tissues. Data are presented as the mean $\pm$ SEM (n of 3 to 5 trials) in units (μ mol product produced per minute) per gram wet weight (nd = not detected). No statistical differences were determined between euthermic and hibernating states using Student's t-test (two-tailed).

	Euthermic U/g	Hibernator U/g
<i>C. leucurus</i> liver		
ADA	0.41 $\pm$ 0.03	0.44 $\pm$ 0.01
NP	5.88 $\pm$ 0.36	5.59 $\pm$ 0.36
AK	7.95 $\pm$ 0.80	8.56 $\pm$ 0.53
5'NT	0.491 $\pm$ 0.11	0.65 $\pm$ 0.06
AMPD	nd	nd
<i>C. leucurus</i> heart		
ADA	0.171 $\pm$ 0.01	0.198 $\pm$ 0.007
NP	1.63 $\pm$ 0.13	1.61 $\pm$ 0.09
AK	86.18 $\pm$ 8.12	107 $\pm$ 3.46
5'NT	nd	nd
AMPD	nd	nd
<i>C. leucurus</i> skeletal muscle		
ADA	0.061 $\pm$ 0.004	0.071 $\pm$ 0.010
NP	0.643 $\pm$ 0.05	0.714 $\pm$ 0.035
AK	391.6 $\pm$ 55.2	353.3 $\pm$ 30.6
5'NT	nd	nd
AMPD	31.22 $\pm$ 4.76	35.25 $\pm$ 3.30
<i>C. leucurus</i> white adipose tissue		
ADA	0.0126 $\pm$ 0.002	0.011 $\pm$ 0.061
NP	0.455 $\pm$ 0.058	0.438 $\pm$ 0.032
AK	0.357 $\pm$ 0.041	0.497 $\pm$ 0.053
5'NT	0.016 $\pm$.002	0.022 $\pm$.0031
AMPD	nd	nd

Optimal assay conditions for the determination of enzyme activity:

Tissues were homogenized in 20 mM imidazole, 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 10 mM β -mercaptoethanol (pH 7.50) for all enzymes but AMPD, in which case the homogenization buffer was 20 mM imidazole (pH 7.0) and 500 mM KCl.

•AMPD and AK activities were determined as per chapters 2 and 3 respectively.

•5'NT: 20 mM imidazole buffer (pH7.0), 5 mM $MgCl_2$, 5 mM AMP, 0.15 mM NADH, 2 mM α -ketoglutarate and 2 U each of GDH and ADA; monitor change in absorbance at 340 nm (extinction coefficient = 6.22 μ M). Note: 5'NT can also be found in a membrane-bound fraction, but detection of 5'NT activity in the cytosolic versus membrane fractions was not investigated.

•NP: 50 mM KPi buffer (pH 7.4), 4 mM inosine and 0.02 U XO in a final volume of 1.0 mL. Change in absorbance was monitored at 293 nm (extinction coefficient = 12.6).

•ADA: 50 mM imidazole (pH 7.0) and 0.15 mM adenosine in a final volume of 1 mL. Change in absorbance was monitored at 265 nm (extinction coefficient = 14.6).

Appendix 2:

Metabolite levels (adenylates and IMP) in *C. leucurus* skeletal muscle.

Data are presented as the mean $\pm$ SEM, n=4 trials of different tissue extracts.

	Euthermic Muscle	Hibernating Muscle
	$\mu\text{mol/gww}$	$\mu\text{mol/gww}$
Total adenylates	6.97 ± 0.31	4.66 ± 0.58^a
ATP	5.98 ± 0.27	3.51 ± 0.40^b
ADP	0.89 ± 0.02	1.01 ± 0.09
AMP	0.10 ± 0.02	0.14 ± 0.03
E.C.*	0.91 ± 0.005	0.87 ± 0.11
IMP	0.083 ± 0.01	0.10 ± 0.03

* E.C. refers to energy charge and is defined as $[\text{ATP} + \text{ADP}/2] / [\text{ATP} + \text{ADP} + \text{AMP}]$

a- values are significantly different, $P < 0.01$; b- significantly different, $P < 0.005$.

Metabolite measurement protocol:

Metabolites were measured using an HPLC technique as per Sayos et. al. (1994) with modifications. HPLC analysis allows quantification of several adenylate species in one trial run; in this case ATP, ADP, AMP and IMP were determined.

Preparation of tissue extracts for metabolite measurements

Tissues from hibernating and euthermic animals were obtained and stored as per chapter 2). Samples of muscle were ground to a powder under liquid nitrogen and deproteinized by acid precipitation; 1:5 w/v with perchloric acid (PCA; 6% solution containing 1 mM EDTA). Samples were then centrifuged and the supernatants removed and neutralized with 3 M KOH, 0.4 M KCl in 0.3 M Tris base. The sample is centrifuged and the supernatant collected for HPLC analysis. All volumes of acid and base added during homogenization and neutralization were recorded so the dilution factor could be calculated and allow conversion of metabolite concentrations in extracts back to tissue concentrations.

Metabolite measurement

The perchloric acid extracts were analyzed for ATP, ADP, AMP, and IMP on a Bio-sil C18 HL 90-5 S, 250 x 4.6 mm column from Bio-rad. Elution of the adenylates and IMP

was obtained with a binary gradient, composed of two buffers, A and B. Each buffer was made in deionized, degassed water as follows:

Buffer A:

30 mM KH_2PO_4

7.5 mM tetrabutylammonium phosphate (TBAP)

pH 5.45

Buffer B:

30 mM KH_2PO_4

7.5 mM TBAP

50% v/v acetonitrile

pH 7.0

The elution consists of three steps:

Step 1: A 7 minute linear gradient of 5% to 23% buffer B.

Step 2: A isocratic 7 minute period at 23% buffer B.

Step 3: A 16 minute linear gradient from 23% to 80% buffer B.

The flow rate was held at 0.7 mL/minute. Metabolite concentrations were determined from a standard curve which was determined from the results of a series of stock solution injections (0.0, 0.01, 0.05, 0.1, 0.5, 5 mM), each containing the metabolites under study.

References

- Aprillie, J.R. 1988. Regulation of the mitochondrial adenine nucleotide pool size in liver: mechanism and metabolic role. *FASEB J.* **2**: 2547-2556.
- Ashby, B., and Frieden, C. (1972) Interaction of AMP-aminohydrolase with myosin and its subfragments. *J. Biol. Chem.* **252**: 1869-1872.
- Ashby, B. and Frieden, C. (1978) Adenylate deaminase. Kinetic and binding studies on the rabbit muscle enzyme. *J. Biol. Chem.* **253**: 8728-8735.
- Atkinson, D. (1977) *Cellular energy metabolism and its regulation*. New York: Academic Press.
- Bakko, E.B. and Nahorniak, J. (1986) Torpor patterns in captive white-tailed prairie dogs (*Cynomys leucurus*) *J. Mammal.* **67**:576-578.
- Barshop, B.A., and Frieden, C. (1984) Analysis of the interaction of rabbit skeletal muscle adenylate deaminase with myosin subfragments. *J. Biol. Chem.* **259**: 60-66.
- Batra, P.P. and Skinner, G. (1990) A kinetic study of the photochemical inactivation of adenylate kinases of *Mycobacterium marinum* and bovine heart mitochondria. *Biochem. Biophys. Acta.* **1038**: 52-59.
- Borgmann, A.I., and Moon, T.W. (1976) Enzymes of the normothermic and hibernating bat, *Myotis lucifugus*: temperature as a modulator of pyruvate kinase. *J. Comp. Physiol.* **107**: 185-199.
- Bradford, M.M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein using the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-255.
- Brooks, S.P.J. (1992) A simple computer program with statistical tests for the analysis of enzyme kinetics. *BioTechniques* **13**: 906-911.
- Clark, T.W., Hoffmann, R.S., and Nadler, C.F. (1971) *Cynomys leucurus*. *Mammalian Species.* **7**: 1-4.
- Dark, J. and Ruby, N. F. (1993). Metabolic fuel utilization during hibernation. In: *Life the Cold*. (eds.) Carey, C., Florant, G. L., Wunder, B. A., and Horwitz, B. Westview Press Inc., pp. 167-174.

- Davenport, J. (1992). *Animal Life at Low Temperature*. Chapman and Hall. London, pp. 111-135.
- Doherty, J.C., Kronon, M.T., and Rotermund, A.J. (1993) The effects of short term cold storage upon ATP and 2,3-BPG levels in the blood of euthermic and hibernating thirteen-lined ground squirrels *Spermophilus tridecemlineatus*. *Comp. Biochem. Physiol. A* **104**: 87-91.
- Dudley, G.A., and Terjung, R.L. (1985) Influence of acidosis on AMP deaminase activity in contracting fast-twitch muscle. *Am. J. Physiol.* **248**: C43-C50.
- El Hachimi, Z., Tijane, M., Boissonnet, G., Benjouad, A., Desmadril, M., and Yon J.M. 1990. Regulation of the skeletal muscle metabolism during hibernation of *Jaculus orientalis*. *Comp. Biochem. Physiol. B* **96**: 457-459.
- Geiser, F. (1988). Reduction of metabolism during hibernation and daily torpor in mammals and birds: temperature effect or physiological inhibition? *J. Comp. Physiol. B*. **158**: 25-37.
- Hayward, J. S. (1968). The magnitude of noradrenaline-induced thermogenesis in the bat (*Myotis lucifugus*) and its relation to arousal from hibernation. *Can. J. Physiol. Pharmacol.* **46**: 713-718.
- Heldmaier, G., and Ruf, T. (1992a). Body temperature and metabolic rate during natural hypothermia. *Pflügers. Archiv.* **369**: 55-59.
- Heldmaier, G., and Ruf, T. (1992b). Body temperature and metabolic rate during natural hypothermia in endotherms. *J. Comp. Physiol. B*. **162**: 696-706.
- Heldmaier, G., Steiger, R. and Ruf, T. (1993) Suppression of metabolic rate in hibernation. In: *Life in the Cold*. Carey, C., Florant, G., Wunder, B.A. and Horowitz, B., (eds) Boulder, Colorado: Westview Press, pp. 544-548.
- Hochachka, P. and Guppy, (1987). *Metabolic arrest and the control of biological time*. Cambridge, Mass: Harvard University Press.
- Horwitz, B. A. (1989). Biochemical mechanisms and control of cold induced cellular thermogenesis in placental mammals. In: *Comparative and Environmental Physiology: Animal Adaptation to Cold*. Wang, L. C. H. (ed.). Berlin: Springer-Verlag, pp. 83-116.
- Hu B., Altschuld R.A. and Hohl C.M. (1991) Phorbol esters and cyclic AMP activate AMP deaminase in adult rat cardiac myocytes. *Arch. Biochem. Biophys.* **291**: 100-106.
- Hudson, J.W. (1973) Torpidity in mammals. In: *Comparative physiology of thermoregulation*. Whittow, G.C., (ed) New York: Academic Press, Vol. III, pp. 97-165.

Kaletha, K., and Nowak, G. (1988) Developmental forms of human skeletal muscle AMP deaminase. *Biochem. J.* **249**: 255-261.

Lee, Y.P., and Wang, M.H. (1968) Studies of the nature of the inhibitory action of inorganic phosphate, fluoride, and detergents on 5'-adenylic acid deaminase activity and on the activation by adenosine triphosphate. *J. Biol. Chem.* **243**: 2260-2265.

Lowenstein, J.M. (1972) Ammonia production in muscle and other tissues: the purine nucleotide cycle. *Physiol. Rev.* **52**: 382-414.

Lushchak, V.I. and Storey, K.B. (1994) Effect of exercise on the properties of AMP-deaminase from trout white muscle. *Int. J. Biochem.* **26**: 1305-1312.

Luz, C.M., Konig, I., Schirmer, R.H., and Frank, R. (1990) Human cytosolic adenylate kinase allozymes; purification and characterization. *Biochem. Biophys. Acta.* **1038**: 80-84.

Lyman, C.P., Willis, J.S., Malan, A., and Wang, L.C.H. (1982) *Hibernation and Torpor in Mammals and Birds*. New York: Academic Press.

Malan, A. (1982). Respiration and acid-base state in hibernation. In: *Hibernation and Torpor in Mammals and Birds*. Lyman, C. P., Willis, J. S., Malan, A. and Wang, L.C.H. New York: Academic Press, pp. 237-282

Makarwicz, W. (1969). AMP-aminohydrolase in muscle of elasmobranch fish. Purification and properties of the purified enzyme. *Comp. Biochem. Physiol.* **29**: 1-26.

Malan A. (1985). Intracellular pH in hibernation and respiratory acidosis in the European hamster. *J. Comp. Physiol. B.* **156**: 251-258.

Marchand P.J. (1991) *Life in the Cold*. Hanover, NH: University Press of New England.

McArthur, M.D., Hanstock, C.C., Malan, A., Wang, L.C.H., and Allen, P.S. (1990) Skeletal muscle pH dynamics during arousal from hibernation measured by ³¹P NMR spectroscopy. *J. Comp. Physiol. B.* **160**: 339-347.

Mommaerts, W.F. (1969) Energetics of muscle contraction. *Physiol. Rev.* **49**: 427-508.

Mommsen, T.P. and Hochachka, P.W. (1988) The purine nucleotide cycle as two temporarily separated units: a study on trout muscle. *Metabolism.* **37**: 552-558.

Moon, T.W. (1978) Enzymes of heterotherms: LDH of hibernating and normothermic little brown bats, *Myotis lucifugus*. *Comp. Biochem. Physiol.* **59B**: 183-190.

Mrosovsky, N. (1971). *Hibernation and the Hypothalamus*. New York: Appleton-Century-Crofts.

Musacchia, X.J., Steffen, J.M., Steffen, M.C., Geoghegan T.E., Dombrowski, J.M., Milsom, W.K., and Burlington, R.F. (1989) Morphometric and biochemical adaptations of skeletal muscle in hibernating and non-hibernating ground squirrels, *Spermophilus lateralis*. In *Living in the Cold II*. Malan, A., and Canguilhem, B. (eds) London: John Libbey, pp. 217-224.

Nedergaard, J. and Cannon, B. (1990) Mammalian hibernation. *Phil. Trans. R. Soc. Lond. B* **326**: 669-686.

Noda, L. (1973) In: *The Enzymes*. Lardy, P. (ed) Vol 9. New York: Academic Press, pp. 285-295.

Ogasawara, N., Goto, H. and Wantanabe, T. (1975) Isozymes of rat AMP deaminase. *Biochim. Biophys. Acta* **403**: 530-537.

Ogasawara, N., Goto, H., Wantanabe, T., Kawamura, Y., and Yoshino., M. (1974) Multiple forms of AMPdeaminase in various rat tissues. *FEBS Letters*. **44**: 63-66.

Raffin, J.P. (1983) AMP deaminase from the gill of the mullet *Chelon labrosus* R.: purification and effects of pH, phosphate and monovalent cations. *Comp. Biochem. Physiol. B*. **75**: 465-469.

Raffin, J.P. (1984) Purification and properties of trout gill AMP deaminase. *J. Comp. Physiol. B*. **154**: 55-63.

Raffin, J.P. and Thebault, M.T. (1987) Purification and partial characterization of an AMP deaminase from the marine invertebrate *Palaemon serratus*. *Comp. Biochem. Physiol.* **88**: 1071-1076.

Raggi, A. and Raniferi-Raggi, M. (1987) Regulatory properties of AMP deaminase isozymes from rabbit redl muscle. *Biochem. J.* **242**: 875-879.

Rundell, R.W., Tullson, P.C., and Terjung, R.T. (1992a) AMP deaminase binding in contracting rat skeletal muscle. *Am. J. Phys.* **263**: C287-C293.

Rundell, R.W., Tullson, P.C., and Terjung, R.L. (1992b) Altered kinetics of AMP deaminase by myosin binding. *Am. J. Phys.* **263**: C294-C299.

Rundell, R.W., Tullson, P.C., and Terjung, R.T. (1993) AMP deaminase binding in rat skeletal muscle after high-intensity running. *J. Applied Physiol.* **74**: 2004-2006.

- Savabi, F. (1994) II-6 Interaction of creatine kinase and adenylate kinase systems in muscle cells. *Mol. Cell. Biochem.* **133/134**: 145-152.
- Sayos, J., Solsona, C., Mallol, J., Lluís, C., Franco, R.. (1994) Phosphorylation of adenosine in renal brush-boarder membrane vesicles by an exchange reaction catalysed by adenosine kinase. *Biochem. J.* **297**: 491-496.
- Shiraki, H., Ogawa, H., Matsuda, Y., and Nakagawa, H. (1979) Interaction of rat muscle AMPD with myosin, II. Modification of the kinetic and regulatory properties of rat muscle AMP deaminase by myosin. *BBA* **566**: 345-352.
- Smiley, K.L., Berry, A.J., and Suelter, C.H. (1967) An improved purification, crystalization and some properties of rabbit muscle 5'-adenylic acid deaminase. *J. Biol. Chem.* **242**: 2502-2506.
- Smiley, K.L. and Suelter, C.H. (1967) Univalent cations of muscle adenosine 5-phosphate deaminase. *J. Biol. Chem.* **242**: 1980-1981.
- Snapp, B.D. and Heller, H.C. (1981) Suppression of metabolism during hibernation in ground squirrels (*Citellus lateralis*). *Physiol. Zool.* **54**: 297-307.
- Somero, G. N. (1995) Proteins and temperature. *Ann. Rev. Physiol.* **57**: 43-68.
- Spychala, J., and Marszalek, M. (1991) Regulatory properties of AMP deaminases from rat tissues. *Int. J. Biochem.* **23**: 1155-1159.
- Spychala, J., Stankiewicz, A., and Makarewicz, W. (1983) High substrate affinity and GTP insensitive AMP deaminase from frog liver. *Comp. Biochem. Physiol.* **74B**: 851-858.
- Stankiewicz, A., Spychala, J., Skladanowski, A., and Zydowo, M. (1979) Comparative studies on muscle AMP deaminase. I. Purification, molecular weight, subunit structure, and metal content of the enzymes from rat, rabbit, hen, frog, and pikeperch. *Comp. Biochem. Physiol.* **62**: 363 - 369.
- Stankiewicz, A. and Spychala, J. (1979) Comparative studies of muscle AMP-deaminase. II. Regulation by monovalent cations, ATP, and orthophosphate of the enzyme from hen, frog, and pikeperch muscle. *Comp. Biochem. Physiol. B.* **62**: 371-374.
- Storey, K.B. and Kelly, D.A. (1995) Glycolysis and energetics in organs of hibernating mice (*Zapus hudsonius*). *Can. J. Zool.* **73**: 202-207.
- Storey, K.B. and Storey, J.M. (1990) Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. *Quart. Rev. of Biol.* **65**: 145-174.

- Swan, H. (1982) Neuroendocrine aspects of hibernation. In: *Survival in the cold*. (eds.) Musacchia, X.J. and Jansky, L. Elsevier, Amsterdam, pp. 121-138.
- Steffen, J.M. and Riedsel, M.L. (1982) Pulmonary ventilation and cardiac activity in hibernating and arousing golden-mantle ground squirrels (*Spermophilus lateralis*). *Cryobiol.* **19**: 83-91.
- Thakkar J.K., Janero D.R., Yarwood, C., and Sharif, H.M. (1993) Modulation of mammalian cardiac AMP deaminase by protein kinase C-mediated phosphorylation. *Biochem. J.* **291**: 523-527.
- Tileston, J.V. and Lechleitner, R.R. (1966) Some comparisons of the black-tailed and white-tailed prairie dogs in north central Colorado. *Amer. Midland Nat.* **75**:292-316.
- Tovmasian, E.K., Hairapetain, R.L., Bykova, Severin, S.E. and Haroutunian, A.V. (1990) Phosphorylation of skeletal muscle AMP-deaminase by protein kinase C. *FEBS Lett.* **259**: 321-323.
- Twente, J.W., Twente, J., and Brack, V. Jr. (1985) The duration of the period of hibernation of three species of vespertilionid bats II. Laboratory studies. *Can. J. Zool.* **63**: 2955-2961.
- Vesterberg, O. (1971) Isoelectric focusing. In: *Methods in Enzymology*. (eds) Colowick, S.P. and Kaplan, N.O.. New York, Academic Press, pp. 389-412.
- Walker, E.J. and Dow, J.W. (1982) Location and properties of two isozymes of cardiac adenylate kinase. *Biochem. J.* **203**: 361-369.
- Wang, L.C.H and Hudson, J.W. (1970) Some physiological aspects of temperature regulation in the normothermic and torpid hispid pocket mouse, *Perognathus hispidus*. *Comp. Biochem. Physiol.* **32**: 275-293.
- Wang, L.C.H. (1979) Time patterns and metabolic rates of natural torpor in the Richardson's ground squirrel. *Can. J. Zool.* **57**: 149-155.
- Wang, L.C.H. (1989) Ecological, physiological and biochemical aspects of torpor in mammals and birds. In *Advances in Comparative and Environmental Physiology, Vol. 4.*, pp. 361-401.
- Watanabe, K., Itakura, T., and Kubo, S. (1979) Distribution of adenylate kinase isozymes in porcine tissues and their subcellular localization. *J. Biochem.* **85**: 799-805.
- Wheeler, T.J. and Lowenstein J.M. (1979) Adenylate deaminase from rat muscle. *J. Biol. Chem.* **254**: 8994-8999.

Willis, J. S. (1982). The mystery of periodic arousal. In: *Hibernation of Mammals and Birds*. Lyman, C. P., Willis, J. S., Malan, A., and Wang, L. C. H. (eds.) London: Academic Press, pp. 92-103.

Zelevnikar, R.J., Heyman, R.A., Graeff, R.M., Walseth, T.F., Dawis, S.M., Butz, E.A. and Goldberg, N.D. (1990) Evidence for compartmentalized adenylate kinase catalysis serving a high energy phosphoryl transfer function in rat skeletal muscle. *J.B.C.* **265**: 300-311.

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