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Metabolic adjustment and biochemical adaptation to torpor in mammalian hibernators

by

Marc Andrew de la Roche, B.Sc. (Hons)

**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 Chemistry Institute
Carleton University
Ottawa, Ontario
July, 1995**

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torpor in mammalian hibernators"**

submitted by
Marc Andrew de la Roche, B.Sc. (Hons)

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

Chair, Department of Chemistry

Thesis Supervisor

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1995

Abstract

Hibernating mammals have evolved the ability to lower their metabolic rate in response to temperate conditions and/or lack of food and water. At the level of enzyme control, this is accomplished by the coordinated suppression of all metabolic processes and the rearrangement of metabolic flux to maximize fuel stores. The quantification of 32 enzyme activities in 5 tissues of hibernating versus euthermic golden-mantled ground squirrels (*Spermophilus lateralis*) demonstrated a decreased potential in glycolysis, biosynthetic processes and carbon entry from glycolysis into the TCA cycle relative to an increased potential in fatty acid oxidation during hibernation. This was further supported by studies on ketone body metabolism, a product of fatty acid oxidation. Kinetic characterization of β -hydroxybutyrate dehydrogenase (β BDH) and glycerol-3-phosphate dehydrogenase (G3PDH) established unique functional adaptations of these enzymes in *S. lateralis* and *Cynomys ludicivicianus*. Using temperature and chemical denaturants as probes, purified G3PDH from a hibernator was found to have a greater chemical and thermal stability, and maintained both structural and functional integrity at lower temperatures relative to the enzyme from rabbits. These results purport a unique metabolic state during hibernation and demonstrate both structural and functional adaptations of hibernator enzymes to cold stress.

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List of Abbreviations

AA - acetoacetate

ALAAT - alanine aminotransferase

ALD - aldolase

ASPAT - aspartate aminotransferase

ATP, ADP, AMP - Adenosine tri-, di- and mono-phosphate

ATPCL - ATP-citrate lyase

KADH - branched chain ketoacid dehydrogenase

COT - carnitine-octanoyl transferase

CPT - carnitine-palmitoyl transferase

CS - citrate synthase

DE-52 - diethylamino cellulose

DHAP - dihydroxyacetone phosphate

EDTA - ethylenediamine tetraacetate

EGTA - ethyleneglycol bis(β -aminoethyl ether) tetraacetate

F2,6P₂ - Fructose-2,6-bisphosphate

FAS - fatty acid synthetase

FBPase - fructose-1,6-bisphosphatase

GK - glucokinase

G6PDH - glucose-6-phosphate dehydrogenase

GDH - glutamate dehydrogenase

G3PDH - glycerol-3-phosphate dehydrogenase

G3P - glycerol-3-phosphate

HK - hexokinase

HOAD - 3-hydroxyacyl-CoA dehydrogenase

β DH - β -hydroxybutyrate dehydrogenase

β OHB - β -hydroxybutyrate

NADIDH - (NAD⁺ dependent) isocitrate dehydrogenase

NADPIDH - (NADP⁺ dependent) isocitrate dehydrogenase

KGDH - α -ketoglutarate dehydrogenase

K_m - Concentration of substrate at which the enzyme reaction proceeds at 50% of its maximal velocity

LDH - lactate dehydrogenase

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

NADP⁺, NADPH - oxidized and reduced forms of nicotinamide dinucleotide phosphate

NADMDH - (NAD⁺ dependent) malate dehydrogenase

NADPMDH - (NADP⁺ dependent) malate dehydrogenase

NaF - Sodium fluoride

ME - malic enzyme

PDH - pyruvate dehydrogenase

PEPCK - phosphoenolpyruvate carboxykinase

PFK - 6-phospho-1-fructokinase

6PGDH - 6-phosphogluconate dehydrogenase

PK - pyruvate kinase

pKa - proton dissociation constant

PMSF - phenylmethylsulfonyl fluoride

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

SDH - serine dehydratase

TCA Cycle - Tricarboxylic acid cycle

U/gww - International units per gram wet weight

$V_{\max}$ - maximal enzyme velocity

WAT - white adipose tissue

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Chapter 1

General introduction

To avoid the metabolic cost of maintaining a constant high body temperature throughout the winter, some mammals have developed the ability to hibernate. This is an energy sparing strategy characterized by a reduction in body temperature concurrent with the reduction in ambient temperature. As well, metabolic processes within these animals are substantially reduced in order to minimize the consumption of metabolic fuel and production of metabolic waste. In nature, diverse species employ similar strategies in response to various environmental insults. A short list is presented in Table 1.1. Each of these strategies is a unique adaptation, however, they are all characterized by a profound reduction in metabolic rate.

Hibernation represents a major thermoregulatory adaptation in some mammalian species in response to cold conditions and lack of food. Whereas homeothermic species must utilize the majority of their metabolic fuel to maintain a constant body temperature (Wang 1989), hibernating species have abandoned this necessity. The effectiveness of such a strategy has been demonstrated in Richardson's ground squirrels (*Spermophilus richardsonii*) where Wang (1979) has calculated an 88% energy savings for the hibernator relative to the cost of remaining awake throughout the winter season.

The course of hibernation consists of a series of torpor bouts which can be divided into four metabolic states; preparation/storage, entry, hibernation and arousal. The signal for priming metabolism into a storage mode prior to hibernation is unknown, as is the signal for the entrance into hibernation. Many attempts have been made to prove the existence of a neuropeptide called the Hibernation Induction Trigger (HIT molecule; Galster 1978; Nedergaard and Canon 1990), however the present author believes a more plausible signal is a

combination of photoperiod, ambient temperature, diet composition (food composition changes over the course of summer) and food availability. These factors would be expected to effect hormone levels which in turn implicates genetic regulation of this processe; however there is a disparity of literature concerning genetic control over the entrance into hibernation.

The extreme metabolic changes that occur throughout a bout of torpor belie deliberate and controlled changes in metabolic flux to ensure optimal use of stored fuel resources. Four to six weeks prior to the entrance into hibernation, metabolic fuel, in the form of triglycerides, is stored in lipid depots throughout the body of the animal. This is readily seen during the fall when ground squirrels effectively double their body size and maximizing their triglyceride stores. This is the most efficiently stored source of metabolic fuel for mammals and can satisfy the energy requirements of the animal throughout a winter season (Nedergaard and Canon 1984).

The entrance into hibernation can occur over the course of a few hours to several weeks depending on the species and depth of hibernation (Wang 1978). Body temperature falls with ambient temperature until the hypothalamic set-point is reached. In *S. richardsoni*, this corresponds to a body temperature of 2-3°C, below which point the animal will actively thermoregulate regardless of ambient temperature (Wang 1978). During torpor, metabolic rate is substantially reduced, demonstrated by the reduction of oxygen consumption to less than 1% of euthermic, resting levels. The energy needs of the animal are almost entirely met by lipids, with some small contribution by carbohydrate stores.

Relative to the entrance into torpor, arousal is an explosive event that takes from 20 minutes to a few hours depending on the size of the animal. Non-shivering thermogenesis in brown adipose tissue provides initial rewarming, followed by eventual carbohydrate mobilization used to fuel shivering in peripheral muscle tissues (Wang 1987). Arousals occur periodically throughout the hibernating season (for ground squirrels every 2-3 weeks (Wang 1978)) and are very costly to the animal. Wang (1978) has estimated that these arousals represent as much as 90% of overall fuel consumption during the hibernation season. The reason for these periodic arousals is unknown, however many hypotheses have been put forward. These include the restoration of ionic balance, the replenishment of carbohydrate reserves, the elimination of metabolic waste, or simply 'catching up' on REM sleep.

The duration of torpor bouts varies widely among species and may last from a single night in daily heterotherms such as the deer mouse (*Peromyscus maniculatus*; Nestler 1992), to as long as 90 days in the big brown bat (*Eptesicus fuscus*; Yacoe 1983). The depth of hibernation is the degree to which metabolic rate is depressed and can be represented by the body temperature during torpor relative to euthermia. Both duration and depth of torpor bouts are indices of relative hibernation 'ability' among species which can be applied to the two hibernators in this study.

Golden-mantled ground squirrels (*Spermophilus lateralis*) are profound hibernators with an obligatory hibernation season. They are approximately 30 cm in length (Michener and Koepl 1985) and can reduce their body temperature to as low as 5°C for up to 14 days when kept at 3-5°C in the absence of food (Twente and Twente 1965). This species is commonly found in the mountains of

northern California and its hibernation physiology has been extensively studied (Snapp and Heller 1981). Black-tailed prairie dogs (*Cynomys ludovicianus*) are a larger species (approximately 35 cm in length) and inhabit the Colorado lowlands where they experience much milder climatic conditions (Tileston and Lechleitner 1966). This is reflected in the depth and duration of their hibernation bouts; they hibernate irregularly during winter months for up to 24 hours with a body temperature as low as 13°C (Fevold 1986; Harlow and Menkens 1985).

The size of a hibernator is a prominent factor in determining the respective hibernating 'ability' of the animal. This is a parameter that has received much attention in the literature (Nedergaard and Canon 1990). Indeed, the big brown bat *Eptesicus fuscus* is the most profound of hibernators and one of the smallest (Yacoe 1983). In contrast, the largest of all hibernating mammals is the brown bear which has very limited hibernation ability (it can only depress its metabolism to 40% of euthermic levels) despite its romantic role as the 'classical hibernator' (Nedergaard and Canon 1984). The reason for this is that the amount of fuel reserves that can be stored is inversely proportional to size, making hibernation less likely in larger animals.

Metabolic control over the depression of metabolism during torpor is poorly understood. Some attention has focused on a passive reduction in metabolism brought about by the reduction in body temperature. This is explained by the Q_{10} effect which is used to describe the change in rate of a chemical reaction in response to temperature. A Q_{10} of 2 is characteristic of chemical reactions and denotes a 2-fold decrease in the rate of a reaction for every 10°C decrease in temperature. For enzyme catalyzed reactions, Q_{10} values range from 2-3, and would predict an 8-27-fold reduction in metabolic rate

for a reduction in body temperature from 37 to 5°C. However, most hibernators experience a markedly greater reduction of metabolic rate than predicted from temperature effects (Wang 1987). One way this may occur is by an increased suppression of metabolic processes induced by hypercapnia, a result of apnoic (non-rhythmic, lowered) breathing during torpor. This effect may be further exaggerated by the relative change in the pKa of intra- and extracellular buffering agents with reduced body temperature. Another explanation advocates an active suppression and maintenance of metabolic rate. In support of this, the slow and deliberate entrance into hibernation relative to arousal purports a highly regulated process. Furthermore, metabolic arrest occurs prior to the reduction in body temperature which indicates an active rather than passive suppression of metabolic processes. This prompts the question: are there unique functional adaptations at the level of enzyme control that are characteristic of hibernating species? These adaptations would be responsible for both the reduction of overall metabolic rate, and the induction of functional changes in metabolic flux during torpor. With respect to the latter, this includes the increased use of triglycerides as a source of fuel relative to a decrease in the use of more limited fuel reserves such as protein and carbohydrate. This metabolic state may be accomplished by a variety of mechanisms which include : structure-function changes in enzymes relative to reduced temperature (Chapters 3 and 4 present study); changes in the concentrations of regulatory enzymes (Chapter 2 present study); post translational modification of key regulatory enzymes (Storey 1987a); reversible aggregation of enzymes (Srivastava and Bernhard 1986; Srere and Ovadi 1990) or their binding to subcellular structures (Arnold and Pette 1970); and changes in concentrations of enzyme inhibitors and activators. The present study will consider the contribution of the first two mechanisms in determining the overall metabolic state of hibernating species during bouts of torpor.

Methodology

A comparison of maximal enzyme activities between a euthermic and a hibernating animal is key for the determination of metabolic flux during torpor. This also allows for an evaluation of the contribution of this mode of enzyme control. *In vitro* determination of enzyme activities has long been employed for the determination of regulatory sites in metabolic pathways; whereas non-regulatory enzymes exhibit relatively high activity activities, regulatory enzymes must be rate limiting and typically of low activities (Newsholme and Crabtree 1986). Furthermore, changes in the potential for metabolic flux during hibernation can be estimated by comparison to maximal activities of enzymes during euthermia.

Unique functional adaptations in hibernating species can be explored in terms of structure - function relationships of enzymes. Structure and function are two intimately related characteristics of enzymes whereby the subtlest of enzyme deformity can have a large effect on its catalytic ability. During hibernation, both temperature and pH can play a role in altering the physical 'appearance' of an enzyme and will thus modulate activity. The extent of temperature effects can be evaluated by comparing structural and functional parameters between enzymes from hibernating and non-hibernating mammalian species. The present study will address the following parameters:

i) Energies of activation (determined from Arrhenius plots), which serve as indicators of the temperature effect on thermodynamic properties of the enzyme (i.e. activation energy and Q_{10} value), will be measured. Past studies suggest that enzymes of non-hibernating species may exhibit conformational transitions

with lowered temperature that are responsible for suppression of enzyme activity. This is shown by a break in the Arrhenius plot (Borgmann and Moon 1976). Hibernating species, on the other hand, require function over a range of temperatures and must maintain normal enzyme function.

ii) Assessment of pH effects on enzymes at different temperatures will be used to determine the effects of acidotic inhibition on enzymes during hibernation. With the use of imidazole buffer, one whose pKa mimics that of intracellular buffering agents, pH optima 'shifts' with temperature can be monitored to determine if this is a control mechanism for reducing activity via hypercapnia induced acidosis.

iii) Structural parameters of an enzyme can be determined from changes in the intrinsic fluorescence of tryptophan residues. Because both emission wavelength and fluorescence intensity are a function of the microenvironment, the effect of temperature and other structural modifiers can be monitored by their effect on enzyme fluorescence. This will be used to evaluate structural parameters of purified glycerol-3-phosphate dehydrogenase (G3PDH) from *C. ludivicianus* muscle relative to the enzyme from rabbit muscle.

iv) Kinetic parameters of enzymes are a function of the structure of an enzyme and can also be used to evaluate enzyme perturbation at specific sites (active site or allosteric site) induced by temperature. In the present study, Km values will be used as an indicator of functional changes in β -hydroxybutyrate dehydrogenase (β DH) and G3PDH with lowered temperature.

The present study will elucidate specific metabolic and enzyme adaptations in hibernating species in an attempt to evaluate their contribution to the process of hibernation. This will be done with consideration of enzyme activities in various pathways as indicators of changes in metabolic flux potential. In addition, unique structural and functional features of hibernator enzymes will be explored. Model systems used in this study are black-tailed prairie dogs (*Cynomys ludovicianus*), golden-mantled ground squirrels (*Spermophilus lateralis*) and two non-hibernating controls, laboratory rats and rabbits.

Table 1.1. Various forms of metabolic arrest in nature and the responsible environmental influence.

Condition	Example	Environmental influence	General reference(s)
hibernation	<i>S. lateralis</i> ; <i>S. richardsonii</i> ; <i>C. leucurus</i> ; <i>Myotis lucifugus</i> ; <i>Zapus hudsonius</i>	lack of food and water, cold weather	Wang 1989
daily torpor	<i>C. ludivicianus</i> ; <i>Peromyscus maniculatus</i> ; various small birds.	lack of food and water, cold weather	Lyman 1982; Wang 1989
aestivation	<i>Otala lactea</i> ; <i>Scaphiopus couchii</i> ; <i>lungfish</i>	lack of water, lack of food	Seymour 1973; Lahiri et al. 1970.
freeze tolerance	<i>Rana sylvatica</i> ; <i>Littorina littorea</i> ; <i>Trachemys scripta</i> (hatchlings); <i>Eurosta solidaginis</i>	freezing conditions	Storey and Storey 1992
anhydrobiosis	<i>Artemia salina</i>	dessication	Crowe and Clegg 1978
hypoxia/anoxia	<i>Littorina littorea</i> ; <i>Trachemys scripta</i> ; <i>Mytilus edulis</i>	lack of oxygen	Storey 1993

Chapter 2

Changes in metabolic flux potential during hibernation in the golden-mantled ground squirrel *Spermophilis lateralis*.

Introduction

The golden-mantled ground squirrel, *Spermophilus lateralis*, is a hibernator that can lower its body temperature to between 2-15°C for up to two weeks (Snapp and Heller 1981). These periods of torpor are characterised by the reduction of overall metabolic rate accompanied by the lowering of body temperature. Metabolic adjustments involve a general reduction of protein and carbohydrate metabolism relative to mobilization of triglyceride reserves. At the level of enzyme control this may be accomplished in a number of ways : temperature effects on enzyme structure and function (see Chapters 2 and 3), post translational modification of key regulatory enzymes (Storey 1987); acidotic inhibition of enzymes (Malan 1993; Malan 1980); reversible aggregation of enzymes (Srivastava and Bernhard 1986; Srere and Ovadi 1990) or binding to subcellular structures (Arnold and Pette 1970); changes in concentrations of effectors; and changes in the concentrations of regulatory enzymes (present chapter). This study will consider the role of the latter in determining overall metabolic flux and sites of regulation during hibernation in *S. lateralis*.

In vitro determination of enzyme activities has long been employed to provide insight into their potential flux *in vivo*. Accordingly, maximal activities can be used to identify key regulatory sites in addition to assessing changes in potential carbon flux throughout metabolic pathways. Non-regulatory enzymes exhibit relatively high activity, whereas regulatory enzymes must be rate limiting and are usually of low activity (Newsholme and Crabtree 1986). Enzyme maximal activities, however, serve only as an indicator of the potential for maximal substrate flux through any given pathway, the actual

flux at any given time being subject to the other modes of control previously mentioned. However, the utility of enzyme activity measurements is valid for two reasons; i) the subtlest of changes in enzyme concentration can induce catastrophic effects throughout the intricacies of metabolism; ii) changing enzyme concentrations via protein synthesis or degradation is an enormous burden on precious metabolic energy, a factor that suggests its importance, hence any changes found in the hypometabolic, hibernating state suggest their key importance.

The present study will determine the specific activities of 32 enzymes in 5 tissues of hibernating and euthermic *S. lateralis*. These will be used as indicators of metabolic flux in the pathways of glycolysis, fatty acid catabolism and synthesis, amino acid catabolism, the TCA cycle and gluconeogenesis.

Materials and Methods

Chemicals and animals

All chemicals were purchased from Sigma Chemical Company (St. Louis, Mo., U.S.A.), or from Boehringer Mannheim (Montreal, Quebec) and were of the highest purity available. Twenty-one *Spermophilus lateralis* were captured in the White Mountains of California during June 1992. All animals were individually housed in rat cages at the animal care facility of the University of California, Irvine and maintained at 22°C on a fall (10L:14D) photoperiod. At the end of an 8 week feeding period, half of the animals were induced to hibernate by placing their cages in a cold room at 4°C on the same photoperiod. After a 24 h habituation period, all food (but not water) was withheld. During the first nine days of fasting, the telemetered body temperature of each animal (telemeters were implanted during the initial 8 weeks) was measured every 12 h to determine the onset of hibernation. Those that did not enter torpor within this period were removed from the cold room. Those that did hibernate were maintained at 4°C for one month at which time all animals were sacrificed. *Ratus ratus* were obtained from the animal care facility at Carleton University. All animals were killed by decapitation and excised tissues were immediately immersed in liquid nitrogen and subsequently stored at -70°C.

Preparation of Homogenates

Unless otherwise stated, all extracts were prepared by homogenizing tissues (1:6 w/v, approximately 60 mg per preparation) in ice cold 20 mM imidazole, pH 7.5, containing 10% v/v glycerol, 100 mM NaF, 30 mM β -mercaptoethanol, 5 mM EDTA and 5 mM EGTA with a Diamed Pro 200 homogenizer set to full power followed by centrifugation in a Heraeus Biofuge 15 at 20 000 x g for 25 minutes. The supernatant fraction was collected and stored on ice prior to assay. Extracts for carnitine-palmitoyl transferase, carnitine octanoyl transferase and citrate synthase were also prepared as above, except that the pH of the extraction buffer was set to 8.1 and NaF and β -mercaptoethanol were omitted. Extracts for pyruvate dehydrogenase (PDH) assays were prepared as above in buffer containing 50 mM potassium phosphate, pH 7.8 containing 2 mM β -mercaptoethanol, 1 mM EDTA and 0.1% (w/v) Triton X-100; the samples were subsequently frozen at -20°C for one hour and thawed prior to assay of active PDH, a process that helps to break up mitochondrial membranes (S. Brooks, personal communication). Total PDH activity was determined in aliquots of crude homogenate incubated 1:1 (v/v) with 20 mM $MgCl_2$ and 2 mM $CaCl_2$ at timed intervals of 10 minutes up to 40 minutes with results then extrapolated to time = 0 to yield initial activity.

Partially purified arylamine transferase (for PDH assays) was prepared by homogenizing chicken livers (1:3 w/v, approximately 300 g per preparation) in ice cold 50 mM Tris, pH 8.0 containing 2 mM EDTA, 10 mM β -mercaptoethanol and a few crystals of PMSF. The homogenate was centrifuged at 8000 X g for 30 minutes at 5°C. Polyethyleneglycol 8000 was

then added slowly to the decanted supernatant with stirring to 20% w/v before a second centrifugation as above. The pH of the supernatant was adjusted to 8.0 and then it was applied to a DE-52 column equilibrated in 20 mM Tris buffer, pH 8.0 containing 10 mM β -mercaptoethanol. The partially purified enzyme was eluted with a gradient of 0 to 500 mM KCl in the same buffer. Active fractions were collected and stored in 40% v/v glycerol at -20°C until use.

Determination of enzyme activity

Unless otherwise stated, all assays were performed at 22.5°C using a Dynatech MR5000 microplate reader in a final volume of 0.250 ml plus added extract at an absorbance of 340 nm. Optimal assay conditions were determined for each enzyme using rat liver extracts and these conditions were used for assays in all *S. lateralis* tissues. Controls for non-specific activity (measured in the absence of the most specific substrate) were subtracted to yield net activity values. One units (IU) of activity is defined as the amount of enzyme that produced one μ mole of product per minute at 22°C. Before use, coupling enzymes were desalted by centrifugation through a small column of G-25 (Helmerhorst and Stokes 1980). Optimal assay conditions were as follows :

Glucokinase (GK; E.C. 2.7.1.2) : 100 mM tris buffer (pH 8.0), 1 mM EDTA, 2 mM $MgCl_2$, 100 mM glucose, 0.2 mM $NADP^+$, 1 mM ATP and 1 U glucose-6-phosphate dehydrogenase. Hexokinase activity was subtracted to yield net glucokinase activity.

Hexokinase (HK; E.C. 2.7.1.1) : 100 mM tris buffer (pH 8.0), 1 mM EDTA, 2 mM MgCl₂, 10 mM glucose, 0.2 mM NADP⁺, 1 mM ATP and 1 U glucose-6-phosphate dehydrogenase.

Phosphofructokinase (PFK; E.C. 2.7.1.11) 50 mM imidazole (pH 7.0), 50 mM KCl, 5 mM MgCl₂, 1 mM ATP, 1 mM fructose-6-phosphate, 0.15 mM NADH and 1 U each of aldolase, glycerol-3-phosphate dehydrogenase and triosephosphate isomerase.

Aldolase (ALD; E.C. 4.1.2.13) : 50 mM imidazole buffer (pH 7.0), 2 mM MgCl₂, 0.2 mM fructose 1,6-bisphosphate, 0.15 mM NADH, 1 U of triosephosphate isomerase and 2 U of glycerol-3-phosphate dehydrogenase.

Glycerol-3-phosphate dehydrogenase (G3PDH; E.C. 1.1.1.8) : 50 mM Tris buffer (pH 8.1), 30 mM glycerol-3-phosphate and 0.8 mM NAD⁺.

Pyruvate kinase (PK; E.C. 2.7.1.40) : 50 mM imidazole (pH 7.0), 50 mM KCl, 5 mM MgCl₂, 2 mM phosphoenolpyruvate, 0.15 mM NADH, 1 mM ADP, 0.2% (v/v) rotenone saturated ethanol and 2 U lactate dehydrogenase.

Lactate dehydrogenase (LDH; E.C. 1.1.1.27) : 50 mM imidazole buffer (pH 7.0), 2 mM MgCl₂, 2 mM pyruvate, 0.15 mM NADH.

Pyruvate dehydrogenase (PDH; E.C. 1.2.4.1) activities were measured as previously described (Elnageh and Gaitonde 1988) using a colorimetric assay with aminophenylazobenzene sulphonic acid (AABS) as the dye reagent.

Both active a and total (a + b) activities were monitored at 460 nm on a Gilford 250 spectrophotometer. Each assay contained 50 mM tris buffer, pH 7.8, 1 mM MgCl₂, 0.25 mM EDTA, 0.4% v/v Triton X-100, 10 mM pyruvate, 5 mM NAD⁺, 2 mM cocarboxylase, 1 mM coenzyme A and 1 U arylamine transferase in a volume of 1 ml. Nonspecific activity measured in the absence of pyruvate was subtracted for each determination.

5'-Nucleotidase (5NT; E.C. 3.1.3.5) : 20 mM imidazole buffer (pH 7.0), 500 mM MgSO₄, 3 mM AMP, 0.15 mM NADH, 2.5 mM 2-oxoglutarate and 2 U each of glutamate dehydrogenase and adenosine deaminase.

Adenylate kinase (ADK; E.C. 2.7.4.3) : 50 mM potassium phosphate buffer (pH 7.4), 500 mM MgSO₄, 200 mM KCl, 2 mM AMP, 0.15 mM NADH, 2 mM ATP, 2 mM phosphoenolpyruvate and 2U each of lactate dehydrogenase and pyruvate kinase.

ATP-citrate lyase (ATPCL; E.C. 4.1.3.8) : 200 mM tris/HCl buffer (pH 8.4), 10 mM β-mercaptoethanol, 0.2 mM CoA, 0.15 mM NADH, 10 mM MgCl₂, 20 mM potassium citrate, 10 mM ATP (in a 1:1 molar ratio with MgCl₂) and 1 U malate dehydrogenase.

Fatty acid synthetase (FAS) 100 mM potassium phosphate buffer (pH 7.0), 3 mM EDTA, 5 mM dithiothreitol, 0.05 mM malonyl-CoA, 0.032 mM acetyl-CoA, 0.3 mM NADPH and 0.2% (v/v) rotenone saturated ethanol.

Isocitrate dehydrogenase (NADP⁺ dependent) (NADPIDH; E.C. 1.1.1.42) : 50 mM imidazole buffer (pH 8.1), 5 mM MgCl₂, 6 mM isocitrate and 0.4 mM NADP⁺.

Glucose-6-phosphate dehydrogenase (G6PDH; E.C. 1.1.1.49) : 60 mM tris buffer (pH 7.5), 3.3 mM glucose-6-phosphate, 0.4 mM NADP⁺ and 6 mM MgCl₂.

6-Phosphogluconate dehydrogenase (6PG; E.C. 1.1.1.43) : 50 mM tris/HCl buffer (pH 7.0), 0.5 mM 6-phosphogluconate, 0.4 mM NADP⁺.

Malate dehydrogenase (NAD⁺ dependent) (NADMDH; E.C. 1.1.1.37) : 50 mM imidazole buffer, 10 mM MgCl₂, 10 mM oxaloacetate, 0.15 mM NADH.

Malate dehydrogenase (NADP⁺ dependent) (NADPMDH; E.C. 1.1.1.37) :

50 mM imidazole buffer, 10 mM MgCl₂, 6 mM oxaloacetate, 0.15 mM NADPH.

Malic enzyme (ME; E.C. 1.1.1.40) : 75 mM triethanolamine buffer (pH 7.4), 4 mM MnCl₂, 0.5 mM L-malate, 0.2 mM NADP⁺.

Carnitine octanoyltransferase (COT; E.C. 2.3.1.21) : 50 mM imidazole buffer (pH 8.1), 0.2% (v/v) Triton X-100, 5 mM L-carnitine, 50 μ M octanoyl-CoA and 0.1 mM 5,5' dithiobis-2-nitrobenzoic acid (DTNB). Enzyme activity was monitored at 410 nm.

Carnitine palmitoyltransferase (CPT; E.C. 2.3.1.21) : 50 mM imidazole buffer (pH 8.1), 0.2% (v/v) Triton X-100, 5 mM L-carnitine, 50 μ M palmitoyl-CoA and 0.1 mM DTNB. Enzyme activity was monitored at 410 nm.

3-Hydroxyacyl-CoA dehydrogenase (HOAD; E.C. 1.1.1.35) : 50 mM imidazole buffer (pH 8.0), 0.1 mM acetoacetyl-CoA and 0.1 mM NADH.

β -Hydroxybutyrate dehydrogenase (β DH; E.C. 1.1.1.30) : 50 mM imidazole buffer (pH 8.1), 20 mM β -hydroxybutyrate and 0.4 mM NAD⁺.

Glutamate dehydrogenase (GDH; E.C. 1.4.1.2) : 100 mM triethanolamine buffer (pH 8.0), 5 mM EDTA, 200 mM ammonium carbonate, 7.5 mM 2-oxoglutarate, 0.15 mM NADH and 1 mM ADP.

Branched chain ketoacid dehydrogenase (KADH) : 30 mM potassium phosphate buffer (pH 7.5), 2 mM MgCl₂, 1 mM dithiothreitol, 0.1% (v/v) Triton X-100, 2 mM ketoisovalerate, 0.2 mM NAD⁺, 0.4 mM cocarboxylase, 0.4 mM CoA and 10 U lipoamide dehydrogenase.

Serine dehydratase (SDH; E.C. 4.2.1.13) : 200 mM potassium phosphate buffer (pH 8.0), 2 mM EDTA, 100 mM L-serine, 0.11 mM pyridoxal phosphate, 0.15 mM NADH, 0.2% (v/v) rotenone saturated ethanol, and 1 U lactate dehydrogenase.

Aspartate aminotransferase (ASPAT; E.C. 2.6.1.1) : 500 mM imidazole buffer (pH 7.8), 30 mM β -mercaptoethanol, 15 mM 2-oxoglutarate, 250 mM L-aspartate, 0.11 mM pyridoxal phosphate, 0.15 mM NADH and 1 U of malate dehydrogenase (NAD⁺ dependent).

Alanine aminotransferase (ALAT; E.C. 2.6.1.2) : 500 mM imidazole buffer (pH 7.3), 30 mM β -mercaptoethanol, 15 mM 2-oxoglutarate, 500 mM L-alanine, 0.11 mM pyridoxal phosphate, 0.15 mM NADH and 1 U lactate dehydrogenase.

Fructose-1,6-bisphosphatase (FBPase; E.C. 3.1.3.11) : 25 mM imidazole buffer (pH 8.0), 5 mM MgSO₄, 0.2 mM NADP⁺, 2.8 mM fructose-1,6-bisphosphate and 1U each of glucose-6-phosphate dehydrogenase and phosphoglucose isomerase.

Phosphoenolpyruvate carboxykinase (PEPCK; E.C. 4.1.1.32) : 100 mM imidazole buffer (pH 6.6), 6 mM β -mercaptoethanol, 50 mM sodium bicarbonate, 1.2 mM phosphoenolpyruvate, 1.25 mM inosine diphosphate, 1 mM MnCl₂, 0.15 mM NADH and 2.5 U malate dehydrogenase (NAD⁺ dependent). All solutions were degassed prior to assay.

Isocitrate dehydrogenase (NAD⁺ dependent) (NADIDH; E.C. 1.1.1.41) : same as the NADP⁺ dependent except 0.4 mM NAD⁺ replaces NADP⁺ and 2 mM ADP was included in the assay mixture.

Ketoglutarate dehydrogenase (KGDH; E.C. 1.2.4.2) : 50 mM imidazole buffer (pH 7.5), 0.75 mM ketoglutarate, 0.4 mM NAD⁺, 2 mM cocarboxylase, 0.1 mM CoA and 0.2% (v/v) rotenone saturated ethanol.

Citrate synthase (CS; E.C. 4.1.3.7) : 50 mM imidazole buffer (pH 8.1), 0.2% (v/v) Triton X-100, 1 mM oxaloacetate, 0.1 mM acetyl-CoA and 0.1 mM DTNB. Enzyme activity was monitored at 410 nm.

Protein concentration was determined by the Bio-Rad assay kit (Coomassie blue G-250 binding method; Bradford 1976) with bovine serum albumin as the standard. Spectrophotometric quantification was performed at 595 nm using a Dynatech MR-5000 microplate reader in a final well volume of 310 μ l.

Enzyme activity assays were interpreted by use of the MPA program (Brooks 1994). All values were compared by two tailed Student t-tests. Significant differences were determined at the $P < 0.005$ and $P < 0.05$ levels.

Results

Table 2.1 is a compilation of homogenization factors for each tissues and the corresponding soluble protein concentration in mg per ml. Also shown is the conversion factor for the determination of mU/gram wet weight from U/mg soluble protein for the enzyme data of all other tables and figures. There were no significant differences in soluble protein content between hibernating and euthermic tissues.

Figures 2.1-2.5 show the hibernation induced changes in activities of enzymes in liver, kidney, heart, skeletal muscle and white adipose tissue (WAT), respectively. Enzyme activities that did not change during hibernation are listed in the corresponding Figure. Enzymes in the five figures are grouped into three sections as follows:

(a) glycolysis: α -glucokinase (GK), hexokinase (HK), phosphofructokinase (PFK), aldolase (ALD), glycerol-3-phosphate dehydrogenase (G3PDH), pyruvate kinase (PK), lactate dehydrogenase (LDH);

(b) Fatty acid catabolism and synthesis: 3-hydroxyacyl-CoA dehydrogenase (HOAD), β -hydroxybutyrate dehydrogenase (β BDH), carnitine-octanoyl transferase (COT), carnitine-palmitoyl transferase (CPT), ATP-citrate lyase (ATPCI), fatty acid synthetase (FAS), glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), (NADP⁺ dependent) malate dehydrogenase (NADPMDH), malic enzyme (ME), (NADP⁺ dependent) isocitrate dehydrogenase (NADPIDH); and

(c) amino acid catabolism, gluconeogenesis and TCA cycle: glutamate dehydrogenase (GDH), branched chain ketoacid dehydrogenase (KADH), serine dehydratase (SDH), aspartate aminotransferase (ASPAT), alanine aminotransferase (ALAAT), fructose-bisphosphatase (FBPase), phosphoenolpyruvate carboxykinase (PEPCK), (NAD⁺ dependent) isocitrate dehydrogenase (NADIDH), α -ketoglutarate dehydrogenase (KGDH), (NAD⁺ dependant) malate dehydrogenase (NADMDH) and citrate synthase (CS).

Among the enzymes of glycolysis, relatively few enzyme activities showed significant changes during hibernation. In liver, only LDH activity changed, but the effect was substantial, LDH increasing 2-fold in the tissue from the hibernator. Kidney demonstrated no change in enzyme activities associated with glycolysis during hibernation. In heart however, LDH activity decreased 5% during hibernation whereas aldolase activity rose by 30%. Skeletal muscle also showed reduced LDH activity during hibernation, dropping to 40% of the control euthermic values. Hexokinase activity was also reduced significantly in skeletal muscle during hibernation, falling to 80% of control. WAT showed a considerable reorganization during hibernation; both hexokinase and aldolase activities fell to 70% of control values whereas G3PDH activity increased 4-fold during hibernation.

PDH activity was detected only in kidney and heart extracts (Figure 2.6). Hibernation had no effect on total enzyme activity, but the percentage of the enzyme in the active form fell dramatically in both tissues during hibernation indicating this as a major locus in the control of glycolytic carbon

flow into the TCA cycle. In all of the other tissues tested, PDH activity was below the limits of detection, likely a result of low mitochondrial numbers.

The most prominent changes in enzyme activities associated with fatty acid metabolism were seen in the liver. Maximal activities of COT and CPT, which are rate limiting in β -oxidation both increased 3-fold during hibernation. ATPCL, an enzyme associated with fatty acid synthesis was suppressed during hibernation, however FAS activity increased by 10% relative to control levels. Two other enzymes associated with fatty acid synthesis showed significant changes in activity, G6PDH and NADPIDH, both of which decreased to 40% of control values during hibernation. Kidney demonstrated similar changes, with levels of G6PDH and NADPIDH decreasing 2-fold during hibernation. However, ME activity in this tissue increased 2-fold relative to control levels. Heart was another tissue in which enzymes utilizing NADP^+ as a cofactor decreased during hibernation. Both NADPMDH and NADPIDH activities decreased to 50% of control values during hibernation. In skeletal muscle, COT activity increased by 30% in hibernating tissues, whereas levels of G6PDH rose 3-fold. ME activity in this tissue decreased, to 80% of control levels.

Enzyme activities associated with amino acid metabolism generally showed no strong changes during hibernation. In the liver, GDH increased slightly (<5%) during hibernation, however decreases in activity were seen for ASPAT (10%) and ALAAT (25%) and SDH which was undetected in hibernating tissues. In kidney, GDH and ASPAT levels decreased 25% and ALAAT activity decreased 70% whereas KADH activity increased 2-fold. In heart extracts, a single enzyme activity changed, ASPAT, which increased

approximately 30%, whereas in WAT, this activity decreased 20% during hibernation. There were no changes in skeletal muscle activities associated with amino acid metabolism.

Gluconeogenic enzyme activities were represented by FBPase and PEPCK. In kidney of hibernators, there was a strong suppression of both activities, 10-fold for FBPase and 3-fold for PEPCK. Conversely, FBPase activity increased by approximately 30% in heart and muscle. During hibernation, there were no gluconeogenic enzyme changes in liver or WAT.

Four enzymes associated with TCA cycle flux were tested. These were CS, NADIDH, KGDH and NADMDH. The low activities of the enzymes KGDH and NADIDH relative to the other TCA cycle enzymes tested demonstrate their regulatory role. NADIDH increased in liver (3-fold), kidney (2-fold) and heart (2-fold) during hibernation, however in muscle, activity did not change. Conversely, KGDH increased in hibernator muscle extracts (being undetectable in controls), whereas KGDH activities were constant in all other tissues.

Discussion

Table 2.3 is a compilation of probable shifts in the importance of metabolic flux through different pathways in organs of hibernating relative to euthermic *S. lateralis* and provides for the majority of the observations noted herein. Increases or decreases in metabolic flux potential in a given pathway were determined by either a general increase or suppression of enzyme activities during hibernation. In pathways where no obvious trend was observed, the symbol '÷' was inserted.

Liver

The liver is one of the most metabolically active tissues and demonstrated the greatest changes in enzyme activity in this study. During hibernation, ketones generated from the catabolism of lipids are synthesized primarily in the liver and are then circulated to other tissues (see Chapter 3). The catabolism of lipids is regulated by the enzymes COT and CPT both of which increased in activity during hibernation. Not unexpectedly, there was a reduction in the production of NADPH during hibernation, indicating a disparity in biosynthetic processes. This was a common trend throughout all the tissues tested. There were no prominent changes in liver glycolytic enzyme activities with the exception of LDH. Previous studies showed that glycolytic flux is suppressed during hibernation by various other mechanisms, which include changes in the phosphorylation state of enzymes (for example PFK, PK in *Zapus hudsonius*; Storey 1987a; Storey 1987b), and changes in the concentrations of effectors (for example fructose 2,6-bisphosphate; Brooks and Storey 1992). This was reflected in the relatively few changes in enzyme maximal activities detected in this and other tissues tested.

Furthermore, PFK and PK maximal activities, the two major regulatory sites of glycolysis did not change during hibernation in any tissue. Changes in enzyme activities associated with amino acid catabolism demonstrated a decreased potential for use of protein as metabolic substrate during hibernation in liver in accordance with increased mobilization of lipid substrate.

Kidney

The kidney is another highly active tissue, however there were no changes in glycolytic enzyme activities during hibernation. Furthermore, there were no changes in the total activity of PDH, although a sharp suppression of the active α form demonstrated that this was a major locus in the control of carbohydrate flow into the TCA cycle during hibernation. Enzymes of fatty acid catabolism were unchanged, however enzyme activities involved in NADPH production for fatty acid biosynthesis were largely reduced during hibernation. In accordance with liver, amino acid catabolism was reduced and in addition, gluconeogenic enzyme activities were suppressed during hibernation.

Heart

Enzyme activities associated with glycolysis and fatty acid metabolism in the heart showed no obvious trends, however, maximal activities of gluconeogenic and TCA cycle enzymes increased during hibernation. ASPAT was the only enzyme activity in amino acid catabolism to change, and increased during hibernation. The reason for the latter is unclear, however for reasons mentioned earlier, the pathway of protein degradation is likely governed by substrate availability. PDH was also present in this tissues and

as was the case for kidney, the active α form of the enzyme was dramatically suppressed during hibernation.

Skeletal muscle

Activities of glycolytic enzymes in skeletal muscle generally decreased during hibernation, complemented by an increase in activities of β -oxidation enzymes. However, the potential for maximum flux through the TCA cycle, gluconeogenesis and the pentose phosphate shunt all increased during hibernation. It is likely that these pathways are actively maintained for rapid activation of shivering thermogenesis during arousal.

WAT

WAT is one of the least metabolically active tissues, due to its relatively low protein content. As a result, many enzymes were below the detection limits of the assays used in this study. The most prominent changes were observed with enzymes of glycolysis, where hexokinase and aldolase activities decreased, while G3PDH activity increased. This may indicate a trend towards inclusion of glycerol-3-phosphate derived from triglyceride catabolism into glycolysis relative to the use of glucose. Malic enzyme activity decreased during hibernation which may be the result of reduced fatty acid synthesis, and as well, levels of ASPAT decrease reducing the potential for amino acid catabolism.

There were several prominent trends throughout the tissues tested. Changes in glycolytic enzyme activities generally showed a trend towards a reduction in glycolytic potential during hibernation. However, this was not the case for liver, which only had changes in LDH activity. TCA cycle enzyme activities showed a general increase throughout the tissues tested, however,

this pathway is severely limited in its use of carbohydrate substrate in both kidney and heart by the strong suppression of PDH activity. As a result, an alternate source of carbon must be employed. The two enzymes COT and CPT generally increased in activity during hibernation, providing an increased potential flux for β -oxidation of fatty acids. CPT is strongly regulated by the effector malonyl-CoA, however this data suggests that a change in the concentration of the enzyme also plays a prominent role in regulating fatty acid oxidation. Another trend was evident for the enzymes (NADP⁺) IDH, (NADP⁺) MDH, malic enzyme, G6PDH and 6PGDH which are generally suppressed during hibernation. This indicates a decreased potential for production of NADPH and likely functions to limit biosynthetic processes during hibernation.

Table 2.1. Soluble protein concentrations of *S. lateralis* tissues in terms of mg/ml and the corresponding multiplication factor for conversion of mU/mg soluble protein to IU/gram wet weight of tissue. There was no difference in protein content between tissues from hibernating and euthermic animals. All data is presented as means $\pm$ SEM and are the mean of 8 separate preparations.

Tissue	extraction dilution	mg soluble protein per ml tissue extract	mU/gww : IU/gww conversion factor
liver	6	43.66 $\pm$ 3.12	0.26
heart	6	29.15 $\pm$ 1.37	0.17
kidney	6	26.84 $\pm$ 1.98	0.16
muscle	6	16.92 $\pm$ 1.46	0.10
WAT	6	4.55 $\pm$ 0.44	0.027

Table 2.2. Maximal enzyme activities that were maintained in *S. lateralis* tissues during hibernation. Enzyme maximal activities are classified as outlined in the Results section. All values are presented as means $\pm$ SEM and are the mean of 6-10 preparations.

liver	mU/mg soluble protein	kidney	mU/mg soluble protein
(a)		(a)	
HK	2.89 ± 0.15	HK	7.23 ± 0.51
Aldolase	21.3 ± 1.8	PFK	373.6 ± 38.9
PFK	118.9 ± 5.8	Aldolase	22.1 ± 2.2
G3PDH	14.2 ± 1.4	G3PDH	10.5 ± 0.8
PK	38.2 ± 1.6	LDH	2704.3 ± 116.9
		PK	39.6 ± 2.02
(b)		(b)	
HOAD	53.6 ± 12.2	HOAD	53.4 ± 15.5
β DH	26.7 ± 5.5	COT	13.5 ± 1.3
6PGDH	8.56 ± 2.48	CPT	0.75 ± 0.18
(NADP) MDH	30.7 ± 3.6	FAS	0.08 ± 0.03
ME	17.5 ± 1.4	6PGDH	6.77 ± 2.23
NADPIDH	4.11 ± 0.30	(NADP) MDH	89.0 ± 10.1
(c)		(c)	
KADH	2.36 ± 0.08	KGDH	2.27 ± 0.18
FBPase	23.3 ± 1.4	CS	74.9 ± 5.6
PEPCK	12.0 ± 0.8		
NADIDH	0.30 ± 0.05		
(NAD) MDH	9.17 ± 1.28		
CS	43.8 ± 2.3		

Table 2.2 continued,

heart	mU/mg soluble protein	skeletal muscle	mU/mg soluble protein
(a)		(a)	
HK	6.92 ± 0.30	PFK	973.7 ± 72.6
PFK	286.2 ± 13.4	Aldolase	141.0 ± 11.1
G3PDH	1.44 ± 0.13	G3PDH	7.67 ± 0.48
PK	281.1 ± 0.3	PK	215.9 ± 0.6
(b)		(b)	
HOAD	42.8 ± 17.3	HOAD	25.3 ± 23.7
COT	17.8 ± 2.2	CPT	2.25 ± 0.34
FAS	0.25 ± 0.06	6PGDH	3.94 ± 2.17
G6PDH	1.70 ± 0.25	(NADP) MDH	105.4 ± 6.2
6PGDH	2.16 ± 0.45	NADPIDH	37.9 ± 3.8
ME	1.42 ± 0.09		
(c)		(c)	
GDH	297.0 ± 19.9	GDH	3472.8 ± 132.9
KADH	1.70 ± 0.30	SDH	1.05 ± 0.11
ALAAT	13.76 ± 1.05	ASPAT	1737.9 ± 138.0
PEPCK	3.75 ± 0.24	ALAAT	590.5 ± 60.9
KGDH	1.84 ± 0.15	PEPCK	12.03 ± 0.83
CS	308.4 ± 28.6	NADIDH	0.87 ± 0.12
		CS	136.4 ± 20.8
WAT			
(a)			
PFK	1.72 ± 0.18		
PK	1.63 ± 0.10		
LDH	238.0 ± 34.8		
(b)			
G6PDH	8.26 ± 0.91		
(c)			
GDH	13.06 ± 1.69		
ALAAT	24.07 ± 2.98		
FBPase	2.78 ± 0.20		
CS	14.82 ± 3.36		

Table 2.3. Trends observed in metabolic pathways during hibernation relative to euthermia. Arrows denote estimated increases or decreases in flux potential determined from general trends observed with enzyme activities. Enzyme activities that were not detected in a particular pathway are denoted by nd while those denoted by the symbol '÷' demonstrated no obvious trends.

Metabolic pathway	liver	heart	kidney	muscle	WAT
glycolysis	↑↑	↑↑	÷	↓↓	÷
PDH	nd	↓↓	↓↓	nd	nd
fatty acid catabolism	↑↑	÷	÷	↑↑	nd
NADPH production (biosynthesis)	↓↓	↓↓	↓↓	÷	↓↓
protein catabolism	↓↓	↑↑	÷	÷	↓↓
gluconeogenesis	÷	↑↑	↓↓	↑↑	÷
TCA cycle	↑↑	↑↑	↑↑	↑↑	nd

Figures 2.1 to 2.5. Maximal enzyme activities in tissues of hibernating and euthermic *S. lateralis* expressed as mU/mg soluble protein. For each tissue, enzyme activities were placed in groups (a), (b) or (c) as outlined in the Results section and includes a list of enzyme activities that were not detected in either hibernating or euthermic tissues. For all graphs, open boxes represent euthermic tissue extracts; hatched boxes represent hibernating tissue extracts. Superscript a and b denote significant differences from control at the $P < 0.005$ and $P < 0.05$ levels respectively. nd denoted enzyme activities that were not detected. All results are the mean of 3-5 determinations on separate tissues preparations; error bars represent SEM.

Figure 2.1. Maximal enzyme activities in liver extracts. Activities that were not detected in either euthermic or hibernating extracts were GK and KGDH.

Figure 2.2. Relative enzyme activities in kidney extracts. Activities that were not detected in either euthermic or hibernating extracts were GK and β DH.

Figure 2.3. Relative enzyme activities in heart extracts. Activities that were not detected in either euthermic or hibernating extracts were GK, β DH, CPT, ATPCI and SDH.

specific activity (mU/mg protein)

Figure 2.4. Relative enzyme activities in muscle extracts. Activities that were not detected in either euthermic or hibernating extracts were GK, β DH, KADH and SDH.

Figure 2.5. Relative enzyme activities in WAT extracts. Activities that were not detected in either euthermic or hibernating extracts were GK, HOAD, β DH, COT, CPT, FAS, 6PGDH, NADPMDH, NADPIDH, KADH, SDH, PEPCK, NADIDH, KGDH, and NADMDH.

Figure 2.6. Percentage of pyruvate dehydrogenase activity in the active form in kidney and heart extracts of hibernating and euthermic *S. lateralis*. No active a activity was detected in kidney extracts from hibernating animals. Superscript a represents significant difference from control at the $P < 0.005$ level. Inset: total PDH activity (a+b) in units of U/gram wet weight in the same extracts. All results represent the mean of 3-5 tissue; error bars represent SEM.

Chapter 3

**Temperature effect on kinetic and
thermodynamic parameters of liver
 β -hydroxybutyrate dehydrogenase during
hibernation**

Introduction

It is well known that hibernation is a state of metabolic suppression, used by some mammals as an energy sparing strategy for winter survival in temperate climates. These animals 'fatten up' prior to entry into torpor and lipids provide the primary source of fuel during torpor (Dark and Ruby 1993). During hibernation, protein and glucose utilization is inhibited (Tashima et al 1970) by a coordinated shut down of glycolysis and protein degradation relative to an accelerated mobilization of triglyceride reserves. These are largely oxidized, but some portion is used to synthesize ketone bodies, predominantly at the liver, where they are circulated throughout the body as a source of metabolic fuel for other organs (Krulowicz 1985). The presence of ketone bodies in the blood has been shown to inhibit glucose uptake in many tissues during starvation relative to the increased oxidation of fatty acids in many tissues (Robinson and Williamson 1980). Furthermore, ketone bodies, themselves a product of hepatic β -oxidation, can be metabolised by many tissues (Robinson and Williamson 1980). During hibernation, levels of ketone bodies have been shown to increase (Krulowicz 1985), likely a result of increased acetyl-CoA availability resulting from a suppression of carbon entry into the TCA cycle (Chapter 2, present study).

β -hydroxybutyrate dehydrogenase (β DH; E.C. 1.1.1.30) is the enzyme responsible for the interconversion of the ketone bodies, acetoacetate (AA) and β -hydroxybutyrate (β OHB) : β -hydroxybutyrate + NAD^+ + H_2O $\longleftrightarrow$ acetoacetate + NADH + H^+ (Barman 1969). Both AA and β OHB are readily mobilized, however, β OHB is the prominent form of ketone exported from the liver (Krulowicz 1985). Although the reason why is unclear, this is likely a

compensatory mechanism to stabilize the NADH : NAD⁺ ratio of liver cells. As well, the presence of the reduced form helps to prevent acidification associated with buildup of the oxidized form as is also the case with lactate (reduced form of pyruvate) and malate (reduced form of oxaloacetate). β DH is located in the inner mitochondrial membrane and is a lipid dependent enzyme with an absolute requirement of phosphatidyl choline for function (Fleischer et al. 1983). Most studies of this enzyme have focused on lipid-enzyme interactions, as β DH is strongly modulated by phospholipid composition, however, there is a deficit of literature concerning the role of this enzyme during hibernation and the corresponding effects of temperature on the modulation of activity.

It is the purpose of the present study to compare the effects of temperature on the modulation of β DH activity in *C. ludivicianus*, *S. lateralis*, and rats. Whereas rats are homeotherms and must actively defend a constant high body temperature, the golden-mantled ground squirrel (*Spermophilus lateralis*) and the black-tailed prairie dogs (*Cynomys ludivicianus*) are two species of varying hibernation 'ability'. During periods of torpor, *S. lateralis* has been shown to reduce its body temperature to 5°C (Twente and Twente 1965) while *C. ludivicianus* can lower its body temperature daily to 13°C (Harlow and Menkins 1975). Using temperature as a probe, the determination of liver Km values, maximal activities, and pH behavior of hepatic β DH in these animals will provide a means of evaluating the effects of temperature and hibernation on the modulation of β DH activity.

Materials and Methods

Animals and Chemicals. The acquisition procedure for *Spermophilus lateralis* tissues is outlined in Chapter 2. Five male and 5 female black-tailed prairie dogs (*Cynomys ludovicianus*) were captured in Southeastern Wyoming during July 1993 and housed in standard rat cages at the University of Wyoming on a natural (fall) photoperiod at 22°C. Animals were fed Purina 5001 rodent diet. After 38 days, 6 of the 10 animals that were captured were sacrificed. The remaining 4 prairie dogs continued to fatten for an additional 36 days, at which point they were placed in their cages without food (but with water) at 5°C. After 6 weeks of hibernation, the four animals were sacrificed. *Ratus ratus* were obtained from the animal care facility at Carleton University. At sacrifice, animals were rapidly dissected and tissue samples were frozen in liquid nitrogen and then stored at -80°C.

Preparation of Samples. Frozen liver samples were homogenized (approx. 1:4 w:v) in ice-cold 50 mM phosphate buffer (pH 8.5), containing 15 mM β -mercaptoethanol (with a few crystals of phenylmethylsulfonylfluoride added) for 2 bursts of 15 sec with a Janke and Kunkel Ultraturex homogenizer at 70% full speed. Samples were then centrifuged for 20 minutes at 30,000 X g at 4°C in a Sorvall RC-5B refrigerated superspeed centrifuge. The supernatant fraction was subsequently passed through a small column (1 cm X 4 cm) of Sephadex G-25 by centrifugation on a benchtop centrifuge to remove low molecular weight species (Helmerhorst and Stokes 1980) and stored on ice prior to use.

Assay of enzymatic activity. Assays of enzymatic activity were carried out in both directions : AA + NADH (direction A) and β OHB + NAD^+ (direction B). For direction A, the pH of all buffers was set to pH 7.5, while in direction B, all buffers were set to pH 8.5. β DH activity was monitored by following either NADH oxidation or NAD^+ reduction at 340 nm using a Gilford 250 spectrophotometer with an attached refrigerated water bath for temperature control of cuvettes. One unit of activity is defined as the amount of enzyme required to oxidize 1.0 μ mole β OHB per minute. Optimal concentrations of substrates were as follows : 20 mM β OHB, 0.4 mM NAD^+ , 0.6 mM AA, and 0.15 mM NADH. The presence of non-specific utilization of NADH in the direction of AA reduction was effectively quenched with the addition of 1 μ l of rotenone saturated ethanol for each 1 ml of assay mixture. The pH values of all buffers were set to the appropriate pH at room temperature and were allowed to fluctuate with changing temperature. (for phosphate this corresponds to a 0.003 pH unit decrease per 1°C increase and a 0.028 pH unit decrease per 1°C increase for tris; Dawson et al 1987).

Arrhenius Plots. Arrhenius plots were constructed from maximal activity determinations for the direction of β OHB oxidation (as described above) for temperatures ranging from 45°C to 5°C in both phosphate and tris buffers.

Quantification of β OHB. Acid extracts of tissues were prepared by homogenizing frozen samples in 6% perchloric acid containing 1 mM EDTA (1:5 (w/v), approximately 100 mg per preparation) with a Diamed Pro 200 homogenizer set to full power followed by centrifugation in a Heraeus Biofuge 15 at 20 000 x g for 25 minutes. The supernatant fraction was collected and neutralized with 300 mM tris buffer containing 3 N KOH, 400 mM KCl and

centrifuged again (as above). Acid extracts were stored at -80°C prior to assay. βOHB tissue concentrations were determined by kit (Sigma Cat. # 310-A) which involved the enzymatic determination of βOHB concentration from the change in absorbance at 340 nm in a Dynatech MR-5000 microplate reader in a final well volume of 270 μl .

Calculations. K_m values were determined by fitting the data to the Hill equation through a nonlinear least-squares regression program (Brooks 1992). Statistical analyses were performed by two-tailed Students t-test with $P < 0.05$ as the lower limit for significant differences among results.

Results

Concentrations of the ketone body β OHB were determined in tissues of two hibernating species, *C. ludivicianus* and *S. lateralis* and a non-hibernating species, the rat (Table 3.1). In the rat, β OHB was undetectable in 3 out of the four tissues and displayed an extremely low value in heart extracts. However, in the hibernating species, there was a dramatic increase of this metabolite in all of the hibernating tissues tested. In *S. lateralis*, levels of β OHB rose by 30% in liver, 8-fold in muscle, 3-fold in heart, 11-fold in BAT of hibernators versus euthermic controls. Furthermore, β OHB was only detected in hibernating WAT extracts from hibernators and was not found in control extracts. In *C. ludivicianus*, levels of β OHB rose by 15-fold in liver and 3-fold in muscle of hibernators compared with controls, and β OHB was only detected in hibernating tissue extracts of heart and BAT. No β OHB was detected in *C. ludivicianus* WAT extracts. Furthermore, levels of the ketone in euthermic animals were in many cases significantly greater than in the corresponding rat tissue.

Maximal activities for liver β -hydroxybutyrate dehydrogenase (β DH) in the three species in the direction of β OHB oxidation are shown in Table 3.2. Activities were similar in rat and *S. lateralis* at 22°C but were lower for *C. ludivicianus*. When assayed at 5°C, the activity of β DH was reduced 6-fold in liver extracts from rat and *C. ludivicianus*, and about 10 fold in both hibernating and euthermic extracts from *S. lateralis* liver. There were no significant differences in β DH activities in *S. lateralis* liver extracts from euthermic versus hibernating animals indicating that the entrance into torpor does not alter enzyme activity.

K_m values for the liver enzyme from the three species were quantified at both 22°C and 5°C to determine the effect of lowered temperature on liver β DH K_m values. These are shown in Table 3.3. K_m values for acetoacetate were reduced in all species at 5°C, to 40% in rat and *C. ludivicianus* liver and 70% in *S. lateralis* liver of the values determined at 22°C. The same was true for the cosubstrate, NADH, where K_m values decreased at 5°C for both the rat and *S. lateralis* enzyme. In the opposite direction, K_m values for NAD⁺ were unaffected by assay temperature. Substrate K_m values were also determined for β OHB at a range of temperatures, shown in Figure 3.1. Temperature had no effect on the K_m values of the enzyme from either *S. lateralis* or *C. ludivicianus* liver and the K_m value for β OHB was the same for both hibernating and euthermic animals at all temperatures. However, K_m values for β OHB increased 5-fold at 5°C for rat β DH relative to those determined at temperatures greater than 17°C.

pH optima for β DH from all three species were determined to assess the possible role of hypercapnia induced acidosis during hibernation on the modulation of enzyme activity (Malan et al 1973). The pH behavior of β DH from all three species showed a broad optimum in the direction of AA reduction in the pH range of 6.5 - 7.2 whereas a sharper optimum at approximately pH 8.2 was seen in the reverse direction. In all of the tissues tested, the pH optimum did not change at the two assay temperatures in the directions of β OHB oxidation or AA oxidation (n=3).

Arrhenius plots for liver β DH from all three species (plots not shown) were used to calculate activation energies shown in Table 4. All plots were

linear over the temperature range tested, demonstrating the absence of temperature induced conformational transitions with temperature. Activation energies for β SDH were determined in tris and phosphate buffer and were slightly but not significantly higher for all species when performed in phosphate buffer.

Discussion

During hibernation, the increase in fatty acid oxidation promotes ketosis in hibernating mammals as demonstrated by the accumulation of β OHB, foremost in the liver where it is synthesized and as well to a lesser degree in other tissues such as the heart, kidney, brown adipose (BAT), white adipose (WAT) and muscle where it is consumed. This is clear from Table 3.1 where the levels of β OHB rose in all *C. ludovicianus* and *S. lateralis* tissues during hibernation. Ketosis has previously been documented with measurements of β OHB in the blood of *Spermophilus beldingi* during hibernation (Krilowicz 1985). Furthermore, levels of β OHB from euthermic tissues were generally higher than those in the non-hibernating rat indicating a proportionately greater use of fatty acids by the hibernating species. As a result, the functional integrity of β DH, which plays a prominent role in this process, must be maintained over the range of temperatures experienced by hibernating species relative to rats which maintain a relatively constant high body temperature.

Maximal activities of liver β DH from the three species were similar in rat and *S. lateralis* at 22°C whereas *C. ludovicianus*, a larger mammal had lower activity. At 5°C, β DH activity was suppressed in all animals, corresponding to Q_{10} values of 2.5, 2.3, and 3 calculated from enzyme activities measured at 22°C and 5°C for liver β DH from rat, *C. ludovicianus* and *S. lateralis*. As well, identical activities in between hibernating and euthermic liver extracts indicated that the entrance into torpor did not alter enzyme activity.

Hypercapnia induced acidosis is a mechanism that has received attention in the literature as a potential modulator of enzyme activity in hibernating species (Malan et al. 1985). A relative acid shift of the cytoplasm during hibernation could influence the activities of enzymes and pathways that are pH sensitive. pH optima were determined for liver β DH from the three species and were typical of dehydrogenases, with broad pH optima in the direction of AA reduction in the range of 6.5 - 7.2, while the oxidation reaction had a pH optima of 8.2 for the hibernator enzymes and about 8.1 for the rat enzyme. Literature values for β DH optima are identical in the direction of β OHB oxidation (Gotterer 1967). When tested at 5°C the pH optima were identical which suggests that temperature does not alter the pH behavior of liver β DH from the three species

Energies of activation were determined from Arrhenius plots performed in tris and phosphate buffers. This experiment was specifically designed to elucidate the contribution of acidotic inhibition arising from an acid shift of intracellular pH during hibernation arising from temperature induced pKa changes on histidine residues (contained in peptides and proteins). This has previously been implicated to account for inhibition of enzyme activity beyond that predicted by temperature effects alone (ie. Q10; Malan et al. 1973, 1985; Chapter 1, present study). The pH of all buffers is altered by temperature change. The major portion of intracellular buffering is provided by histidine whose alpha-imidazole groups pKa changes with temperature in a manner that results in a 0.018 pH unit increase for every 1°C decrease in temperature. This pKa change mimics the effect of temperature on the pKa of water, and the net result of buffering by α -imidazolium groups is that a constant charge state is preserved on proteins despite changing temperature.

This alaphastat regulation allows protein conformation and enzyme kinetic properties to be largely conserved for most proteins as temperature varies within the physiological range. Many mammalian enzymes generally function well within a narrow high temperature range that reflects the homeothermic environment of the cells and it has been proposed that two of the means of metabolic suppression that could be important in hibernation are 1) low temperature effects on enzymes (discussed in Chapters 1 and 4, present study); and 2) differential pH effects on enzymes at low temperatures since the respiratory acidosis that develops in hibernation means that the intracellular pH of the hibernating cell is somewhat lower than would be expected from the calculated pH increase that would occur due to alaphastat regulation (Malan 1973, 1985). Hence, for sensitive enzymes, this relative acidosis could change enzyme function at the hibernating body temperature. To test this hypothesis, β DH was assayed in 2 different buffers; phosphate which shows a small pH change with temperature (0.002 pH unit increase for 1°C decrease) and therefore provides a relative acidosis (compared with intracellular histidine buffering) for enzymes assayed at low temperatures; and tris (0.028 pH unit increase for 1°C decrease) which gives the opposite effect - a pH shift that is more basic than expected intracellularly. Any different behavior of β DH in these two buffers at low temperature would indicate, therefore, that enzyme function *in vivo* is influenced by the relative acidosis of the intracellular environment in tissues during hibernation. In all species, activation energies determined in phosphate buffer were slightly higher but not significantly different than those determined in tris buffer. This indicates that liver β DH from the 3 species is not modulated by temperature effects on intracellular pH and the reduction in activity is strictly a function of temperature. Furthermore, no breaks were observed in any Arrhenius plots

of β DH, demonstrating the absence of conformational changes induced by temperature.

K_m values for liver β DH indicated increased K_m values for AA in the three species with lowered temperature. This may be a factor for the role of β OHB synthesis for export from liver during hibernation. K_m values for β OHB tested in the temperature range of 5°C to 27°C were unchanged for liver β DH from the two hibernators, whereas they decreased 5-fold for the rat enzyme at 5°C relative to determinations at 17°C and above. Since K_m values are a measure of enzyme function, these results indicate a greater maintenance of functional integrity of the enzymes from both hibernating species over this range of temperatures relative to the enzyme from the non-hibernator.

During torpor, levels of the ketone body β OHB accumulate during hibernation in tissues from *S. lateralis* and *C. ludivicianus* to fuel tissues as part of a carbohydrate sparing strategy. This form of ketone body can be interconverted with acetoacetate by the enzyme β DH. In this study, the effects of temperature on this enzyme were determined in order to elucidate the effect of torpor on the function of this enzyme. From kinetic studies, it is clear that β DH from the two hibernating species maintains functional integrity over the range of temperatures between 5°C and 27°C better than the enzyme from the non-hibernator. Furthermore, the enzymes from the two hibernating species are not modulated by acidosis or temperature effects on the pK_a of intracellular buffering. The suppression of activity during bouts of torpor is solely a function of temperature reduction in activity, with corresponding Q₁₀ values of 2.6, 2.3 and 3 for the enzyme from rat, *C. ludivicianus* and *S. lateralis*.

Table 3.1. Concentrations of β OHB in tissues of rat, *C. ludivicianus* and *S. lateralis* expressed in μ moles/gram wet weight determined in PCA extracts of liver, skeletal muscle, heart, white adipose tissue (WAT) and brown adipose tissue (BAT) from the three species. All values are presented as means $\pm$ SEM and are the product of 3-4 separate determinations. All values determined in hibernating extracts are significantly higher than those determined in corresponding euthermic extracts ($P < 0.005$) except in WAT extracts which did not change during hibernation.

Animal	Tissue	hibernating	euthermic
Rat	Liver	-	0.00 ± 0.00
	Muscle	-	0.00 ± 0.00
	Heart	-	0.01 ± 0.00
	WAT	-	0.00 ± 0.00
<i>C. ludivicianus</i>	Liver	0.30 ± 0.05	0.02 ± 0.01
	Muscle	0.03 ± 0.01	0.01 ± 0.00
	Heart	0.03 ± 0.01	0.00 ± 0.00
	WAT	0.00 ± 0.00	0.00 ± 0.00
	BAT	0.10 ± 0.01	0.00 ± 0.00
<i>S. lateralis</i>	Liver	0.40 ± 0.01	0.28 ± 0.03
	Muscle	0.15 ± 0.04	0.02 ± 0.01
	Heart	0.13 ± 0.05	0.05 ± 0.01
	WAT	0.01 ± 0.01	0.00 ± 0.00
	BAT	0.45 ± 0.07	0.04 ± 0.01

Table 3.2. Activities of β DH (mU/gm wet wt) in rat liver, *S. lateralis* liver and *C. ludivicianus* liver at 22°C and at 5°C in the direction of β OHB oxidation. Assays were performed under optimal conditions as previously described. All values are presented as mean $\pm$ SEM (n). There was no significant difference in activity between β DH from hibernating versus euthermic *S. lateralis* and *C. ludivicianus* tissue and hence values were combined. All activities were significantly lower when performed at 5°C relative to 22°C. Q_{10} values calculated for the reduction in activity at 5° relative to 22°C are 2.5, 2.3, 3.0 for *S. lateralis*, *C. ludivicianus*, and rat, respectively.

Tissue	Temp. (°C)	Activity (mU/gm wet wt)
Rat liver	22°C	158.3 $\pm$ 13.1 (4)
	5°C	25.0 $\pm$ 2.06 (4)
<i>C. ludivicianus</i> liver	22°C	84.0 $\pm$ 6.6 (6)
	5°C	15.0 $\pm$ 2.2 (6)
<i>S. lateralis</i> liver	22°C	144.4 $\pm$ 18.4 (10)
	5°C	14.6 $\pm$ 1.87 (5)

Table 3.3. K_m values for β DH at 5°C and 22°C. All assays were conducted in phosphate buffer set to pH 7.5 at 22°C. Values are presented as mean $\pm$ SEM and are the product of 3-5 separate determinations for rat β DH and 8-10 separate determinations from the enzyme from both hibernators. There were no significant differences in K_m values between the enzyme from hibernating and euthermic liver and hence the results were combined. Superscript a and b refer to significant differences between the two temperatures at the $P < 0.005$ and $P < 0.05$ levels, respectively.

Tissue	Temp. (°C)	NAD (μ M)	AA (μ M)	NADH (μ M)
Rat liver	22°C	79 $\pm$ 3	96 $\pm$ 9	17 $\pm$ 3
	5°C	70 $\pm$ 8	40 $\pm$ 3 ^a	6.0 $\pm$ 2 ^b
<i>S. lateralis</i> liver	22°C	59 $\pm$ 10	64 $\pm$ 5	18 $\pm$ 0.4
	5°C	77 $\pm$ 5	46 $\pm$ 4 ^b	9.0 $\pm$ 2 ^b
<i>C. ludovicianus</i> liver	22°C	103 $\pm$ 10	82 $\pm$ 8	10 $\pm$ 0.7
	5°C	78 $\pm$ 11	31 $\pm$ 4 ^a	8.0 $\pm$ 0.9

Table 3.4. Activation energies (calculated from Arrhenius plots) for liver β DH from three species. Assays were performed in tris and phosphate buffers over the range of temperatures from 40°C to 4°C proceeding in the direction of β OHB oxidation. The pH of both buffers was set to pH 8.5 at 22°C and allowed to fluctuate with temperature. All numbers are presented as means $\pm$ SEM (n). There were no significant differences in activation energy between values determined in tris or phosphate buffers. As well, there was no significant difference between hibernating and euthermic tissues. Superscript a denotes significant differences at the $P < 0.005$ level from the corresponding rat liver value in the same buffer.

Tissue	Ea (kcal/mol) in tris buffer	Ea (kcal/mol) in phosphate buffer
Rat liver	9.64 ± 0.65 (6)	10.11 ± 0.07 (6)
<i>C. ludivicianus</i> liver	7.46 ± 0.43 (3) ^a	8.68 ± 0.37 (3) ^a
<i>S. lateralis</i> liver	11.89 ± 0.09 (3) ^a	12.95 ± 0.92 (3) ^a

Figure 3.1. Effect of temperature on Km values for β OHB for β DH from *C. ludivicianus*, *S. lateralis* and rat liver. All data points are the mean of 4 preparations for rat values and 8 preparations for hibernator values; error bars denote SEM. There were no significant differences in Km values between temperatures for *C. ludivicianus* and *S. lateralis*, whereas for the rat enzyme, significant differences from the corresponding value at 27°C ($P < 0.005$) are shown with an appended a.

Chapter 4

**A comparison of functional and structural
properties of glycerol-3-phosphate
dehydrogenase from *C. ludivicianus* and rabbit.**

Introduction

Many mammalian species have evolved the ability to lower their body temperature to limit the expenditure of energy in temperate climates. The black-tailed prairie dog (*Cynomys ludovicianus*) is a daily heterotherm that can lower its body temperature to 13°C during winter nights (Harlow and Menkens 1985). Typical of all hibernating mammals, the mobilization of triglycerides during bouts of torpor increases relative to the catabolism of carbohydrate and protein stores. The three forms of metabolic substrate derived from the catabolism of triglycerides are i) fatty acids which undergo β -oxidation to yield the majority of the energy; ii) ketone bodies produced from acetyl CoA; and iii) glycerol-3-phosphate and free glycerol derived from the breakdown of triacylglycerides and phosphoglycerides (Dark and Ruby 1993). Glycerol-3-phosphate dehydrogenase is responsible for the metabolism of glycerol-3-phosphate (G3P). Free glycerol is converted to glycerol-3-phosphate (G3P) by the action of glycerol kinase. G3P can then be converted to dihydroxyacetone phosphate (DHAP) by the action of glycerol-3-phosphate dehydrogenase (G3PDH) and can enter into glycolysis at the triose phosphate level (Lehninger 1975). Alternatively, G3P can participate in the glycerol phosphate shuttle for direct transfer of cytosolic reducing equivalents to the electron transport chain via mitochondrial glycerol-3-phosphate oxidase (flavin linked). During torpor, there is a surplus of G3P derived from the catabolism of triglycerides which can participate in both the glycerol-phosphate shuttle and glycolysis. Although glycolytic flux is inhibited during hibernation (Storey 1987), the arousing hibernator requires a source of gluconeogenic precursor, as glycogen stores dwindle during bouts of torpor (Tashima et al. 1970). Candidates include lactate and body protein, however,

glycerol derived from triglycerides can account for 60% of the replenishment of glycogen stores (Galster and Morrison 1975). As a result, there is a requirement for metabolic control at the locus of G3P metabolism in hibernating species.

Hibernation represents a major thermoregulatory adaptation in some species. Control over the lowering of metabolic rate has focused on passive metabolic control during the entrance into hibernation as a function of reduced body temperature (Snapp and Heller 1981) or acidotic inhibition resulting from hypercapnia during torpor (Malan 1973). More recent research has focused on the modulation of metabolic flux by more active forms of enzyme control. These include: post translational modification of key regulatory enzymes (Storey 1987a and 1987b); changes in the concentrations of regulatory enzymes; reversible aggregation of enzymes (Srivastava and Bernhard 1986; Srere and Ovadi 1990) or their binding to subcellular structures (Arnold and Pette 1970); changes in concentrations of enzyme inhibitors and activators. These mechanisms however, are not apparently factors that affect G3PDH. Temperature effects are prominent modulators of enzyme activity and are ubiquitous to all enzymes during bouts of torpor. The present study will determine temperature induced changes of G3PDH activity during hibernation by comparison of functional and structural adaptations of this enzyme in hibernating species. This will be accomplished by the determination of temperature effects on enzyme activity, pH behavior, K_m value, temperature induced conformational changes and stability differences.

Materials and Methods

The acquisition procedure for *C. ludivicianus* tissue is outlined in Chapter 3. Rabbits tissues were a kind gift from Mr. E. Fudd. All animals were sacrificed by decapitation. Excised tissues were immediately immersed in liquid nitrogen and subsequently stored at -70°C. All chemicals used in this study were obtained from Sigma Chemical Co. (St. Louis, Mo, U.S.A.) or from Boehringer Mannheim (Montreal, P.Q., Canada) and were of the highest purity available. Purified rabbit muscle α -glycero-3-phosphate dehydrogenase (G3PDH) was obtained from Sigma Chemical Co. (St. Louis, Mo, U.S.A.).

C. ludivicianus tissues (liver and muscle) were homogenized (1:3 w/v, approximately 1 gm per preparation) in ice-cold 50 mM phosphate buffer, pH 8.5, containing 15 mM β -mercaptoethanol, 1 mM EDTA (disodium salt) and 10% v/v glycerol using a Janke and Kunkel Ultraturax homogenizer at 70% full power. The supernatant was then centrifuged for 20 minutes at 27,000 g and desalted by centrifugation through a small G-25 Sephadex column (1 x 5cm, approximately 0.5 ml supernatant/column) pre-equilibrated in 10 mM potassium phosphate buffer, pH 8.0 (buffer A). The supernatant was then placed on a DE-52 Sephadex column equilibrated in the same buffer, and washed with buffer A until the absorbance at 280 nm was zero. The enzyme was eluted using a 0 to 1 M KCl gradient in buffer A. Active fractions were pooled and concentrated to 1 ml using dialysis tubing (exclusion 12,000 - 14,000) and polyethylene glycol (mol. wt. 8000). The concentrate was then injected onto a Varian TSK 4000 size exclusion column (7.5 mm x 60 cm; equipped with a guard column) equilibrated in 10 mM potassium phosphate buffer (pH 7.0) and 100 mM KCl and run on an HPLC. The column was

developed in the same buffer and active fractions were pooled and placed on a Matrix Red column equilibrated in 5 mM phosphate buffer containing 10 % (v/v) glycerol and 15 mM β -mercaptoethanol (buffer B). The column was washed with buffer B until the absorbance at 280 nm was zero and then the enzyme was eluted using a 0 to 1 M KCl gradient in buffer B and active fractions were pooled. The final preparation was stored in buffer B and was stable for at least two weeks with no loss of activity. Purity of the enzymes were confirmed by SDS-PAGE and by a single protein peak eluting on a Zorbax reverse phase column equilibrated in 0.15% TCA and eluted with a 5 - 60% acetonitrile gradient run on an HPLC. Purity of the rabbit enzyme obtained from Sigma was also confirmed as above.

Crude extracts used for activity measurements were prepared by homogenization of liver and muscle samples from both *C. ludivicianus* and rabbit (1:3, approximately 20 mg per preparation) in ice cold 50 mM imidazole, pH 7.5 containing 10% (v/v) glycerol, 1 mM EDTA and 15 mM β -mercaptoethanol. Homogenates were centrifuged as above and the supernatant stored on ice prior to assay. Enzyme activity was monitored in both directions of catalysis, by following either the reduction of NAD^+ or the oxidation of NADH at 340 nm using a Gilford 250 spectrophotometer with a water jacketed cell holder for temperature control. Standard assays were performed in 50 mM potassium phosphate buffer at pH 7.5 in the direction of DHAP reduction or in 50 mM imidazole buffer, pH 8.5, in the direction of G3P oxidation. Optimal concentrations of substrates were as follows: 0.1 mM NADH and 0.5 mM DHAP in the reduction direction and 10 mM glycerol-3-phosphate and 1 mM NAD^+ in the oxidation direction to a final cuvette volume of 1 ml. Assays were initiated with the addition of either G3P or DHAP

and demonstrated the absence of non-specific activities utilizing either NAD^+ or NADH. The pH of all buffers was set at pH 7.5 at 25°C and was allowed to fluctuate with temperature; for phosphate buffer this results in a 0.002 pH unit increase per 1°C and for imidazole, a pH unit increase of 0.017 per 1°C decrease in temperature (Dawson 1969). For assays to be run at 5°C, pH was determined at 22°C and compensation was made for the differences in buffer pKa with temperature. Determination of pH optima was performed in phosphate buffer under optimal conditions. K_m values were determined by fitting the data to the Hill equation through a nonlinear least-squares regression program designed by Dr. S. P. J. Brooks (1992).

Protein concentration was determined by the Bio-Rad assay kit (Coomassie blue G-250 binding method; Bradford 1976) with bovine serum albumin as the standard. Spectrophotometric quantification was performed utilizing a Dynatech MR5000 Microplate Reader to a final volume of 260 μl per microplate well.

The native molecular mass of G3PDH was determined by size exclusion HPLC as described in the purification procedure. Purified enzyme (0.50 ml) was injected and the eluant was collected in 1 ml fractions, assayed in the direction of G3P oxidation and compared to elution volumes of the following standards : ferritin, 450,000; phosphofructokinase, 360,000; pyruvate kinase, 237,000; catalase, 232,000; aldolase, 160,000; hexokinase, 102,000; and adenylate kinase, 41,000. The subunit molecular weight was determined by SDS-PAGE run on an 8% acrylamide gel and compared to commercially available standards. Standards used were : myosin, 200,000 kDa; β -galactosidase, 116,250 kDa; phosphorylase b, 97,400; serum

albumin, 66,200; ovalbumin, 45,000; carbonic anhydrase, 31,000; trypsin inhibitor, 21,500; lysozyme, 14,400; and aprotinin, 6,500.

Isoelectric focusing was performed by the method of Vesterberg (1971) using an LKB Products 8101 IEF column (110 ml) with pH 3.5 to 10 LKB ampholines in a sucrose density gradient with column development at 500 V for 16 h. Fractions were assayed in the direction of G3P oxidation.

Arrhenius plots ($\log V$ vs $1/K^\circ$) were constructed from measurements of enzyme activities at 9-12 temperatures ranging from 40°C to 5°C in 50 mM phosphate and imidazole buffers. Assays were conducted in the direction of G3P oxidation in phosphate and imidazole buffers which were set to pH 7.5 at 25°C and allowed to fluctuate with changing temperature.

Fluorescence measurements were carried out on a LS50 fluorometer with a water jacketed cell holder for temperature control. Excitation and emission maxima for G3PDH determined at 22°C were 280 and 340 nm, respectively. Temperature induced conformational measurements were performed on aliquots of the purified enzyme (approximately 1 μ g/ml in 20 mM potassium phosphate, pH 7.0). Emission spectra (excitation at 280 nm) were recorded in 5-nm wavelength increments and corrected by subtraction of the corresponding blank sample (minus enzyme).

For chemical denaturation studies, aliquots of purified enzyme (0.5 μ g/ml, in 20 mM potassium phosphate, pH 7.0) were incubated at different concentrations of guanidine hydrochloride for 60 min at 22°C. After incubation, the fluorescence emission spectrum was determined as above.

Measurement of activity loss with increasing concentration of guanidine hydrochloride were as above.

Thermal inactivation experiments were carried out by heating enzyme solutions (20 mU/ml in 20 mM potassium phosphate buffer, pH 7.0) at various temperatures in Baxter heating blocks. Aliquots were withdrawn at 15 min intervals for 60 mins and assayed for G3PDH activity at 22°C.

Results

Glycerol-3-phosphate dehydrogenase (G3PDH) from *C. ludivicianus* liver and skeletal muscle was purified to homogeneity as demonstrated by a single bands of G3PDH protein on an SDS-PAGE gel (Figure 4.1) and by single protein peaks eluting on reverse-phase HPLC. In addition, the retention time of the *C. ludivicianus* protein peak was identical to that of commercially purified rabbit muscle G3PDH (data not shown). Skeletal muscle G3PDH was purified 176 fold to a specific activity of 282 mU/mg protein, while liver G3PDH was purified 50 fold to a specific activity of 257 mU/mg protein. Table 4.1 illustrates the typical purification scheme for both liver (Table 4.1a) and muscle (Table 4.1b) G3PDH from euthermic *C. ludivicianus*. The enzyme was purified in three steps; first by ion exchange on DE-52 followed by size exclusion chromatography on an HPLC and finally by ion exchange on a Matrix gel red column that exhibits specificity towards dehydrogenases. Gel filtration chromatography on an HPLC size exclusion column yielded a native molecular weight of 77 ± 5 kDa ($n=3$) for G3PDH for both prairie dog tissues as well as rabbit muscle, larger than the value of 68 kDa previously reported for rabbit muscle G3PDH (White 1971). The subunit molecular weight of the purified enzyme was determined from SDS-PAGE (Figure 4.1) and was 40 ± 5 kDa ($n=2$). This indicates that the native enzyme was a dimer. Isoelectric focusing of crude homogenates of G3PDH from *C. ludivicianus* tissues demonstrated the presence of a single isoform with a corresponding pI of 6.4 for both muscle and liver. Previously reported values for rabbit kidney G3PDH were identical (White 1971).

pH optima for G3PDH from both *C. ludivicianus* tissues and rabbit muscle were typical of dehydrogenases, with a broad pH optima for the direction of DHAP reduction at 7.4 to 7.8 and a narrower pH optimum in the opposite direction in the range of 9.3 to 9.4. There were no observable differences in pH optima for G3PDH in the three tissues or between optima determined at 22°C vs 5°C for both temperatures. As well, Arrhenius plots constructed from Vmax measurements over the range of 40°C to 5°C were linear in all cases with calculated energies of activation of 10.93 ± 0.31 kcal/mol (n=6) when performed in imidazole buffer or 14.74 ± 0.37 kcal/mol (n=6) when performed in phosphate buffer.

Maximal activities G3PDH in both directions of catalysis were determined in crude extracts of liver and muscle from rabbit and *C. ludivicianus* and are shown in Table 4.2. There were no significant differences between activities measured in tissues taken from hibernating vs euthermic *C. ludivicianus* at 22°C and 5°C indicating that the entrance into torpor does not alter G3PDH activity. Maximal activities determined in both directions of catalysis were significantly higher in the liver extracts of *C. ludivicianus* compared with rabbits whereas the reverse was true for muscle extracts. Included in Table 4.2 are the activity ratios between the oxidative and reductive direction of catalysis determined at both 22°C and at 5°C. Activity ratios in all tissues increased approximately 60% with the decrease in temperature to 5°C except in rabbit liver which maintained the same activity ratio at both temperatures. This indicates that maximal activity of G3PDH from *C. ludivicianus* liver and muscle and rabbit muscle are modulated similarly by temperature change.

G3PDH K_m values for DHAP, NADH and NAD^+ were determined at 22°C and at 5°C for the purified enzyme from *C. ludivicianus* liver and muscle and rabbit muscle (Table 4.3). With lowered temperature, K_m values for DHAP increased in both *C. ludivicianus* tissues, but decreased in rabbit muscle. K_m values for NADH did not change with temperature, while those for NAD^+ increased for rabbit muscle and *C. ludivicianus* liver G3PDH but did not change for the *C. ludivicianus* muscle enzyme. K_m values for G3P were determined over a range of temperatures for the purified enzyme from both muscle tissues and are shown graphically in Figure 4.3. A comparison of G3PDH from the two species demonstrates a greater conservation of K_m values for G3P in the hibernating species throughout the range of temperatures tested.

Temperature induced conformational changes were monitored by changes in tryptophan fluorescence intensity at 340 nm. Fluorescence is a sensitive probe of localized protein motion and has been used extensively to monitor conformational changes induced by various structural modifiers such as temperature and chemical denaturants (Eftink 1994). The fluorescence emission intensity of muscle G3PDH from the two species is shown in Figure 4.3 for the temperature range of 5°C to 48°C. With decreasing temperature, the fluorescence signal increased linearly in both tissues. However, the increase with temperature was significantly greater for G3PDH from *C. ludivicianus* muscle relative to the rabbit muscle enzyme indicated by slopes of -0.235 ($r^2=0.99$) and -0.169 ($r^2=0.98$) respectively. This indicates a greater degree of conformational change with temperature in this species.

Thermal stability of the two enzymes incubated at 37°C was also monitored, shown in Figure 4.4. While the enzyme from *C. ludivicianus* muscle maintained activity over the course of 60 minutes, the activity of rabbit muscle G3PDH was strongly reduced within 15 mins and continued to decrease over time. However, at other temperatures tested, activities for muscle G3PDH from both species were either maintained over the course of 60 mins (5 to 27°C), or were both reduced to zero within 15 mins (45°C and above). The greater thermostability demonstrated by the hibernator enzyme was reflected by its chemical stability. With increasing concentration of the denaturant guanidine hydrochloride, the enzyme from the hibernating species maintained activity more so than the rabbit muscle enzyme (Figure 4.5 inset). Guanidine hydrochloride induced unfolding transitions were monitored by the fluorescence emission spectrum of muscle G3PDH from both species. Shown in Figure 4.5 is a plot of the wavelength of maximum emission intensity as a function of increasing guanidine hydrochloride concentration. Figure 4.5 demonstrates a greater wavelength shift in rabbit muscle G3PDH relative to the *C. ludivicianus* muscle enzyme.

Discussion

This study was undertaken to elucidate functional and structural adaptations of G3PDH in hibernating species by comparing the liver and muscle forms of the enzyme from *C. ludivicianus*, a hibernator, with the enzyme from rabbit, a non-hibernating control. In a variety of characteristics, the enzymes were virtually identical, exhibiting typical properties of mammalian G3PDH (White 1971). Arrhenius plots of G3PDH from *C. ludivicianus* muscle and liver and rabbit muscle showed no breaks and yielded identical energies of activation for all three sources. The pH optima in both directions of catalysis were also the same for all three enzymes at 5 and 22°C.

Maximal activities of G3PDH in muscle and liver of rabbit and *C. ludivicianus* were determined for both the oxidative and reductive direction of catalysis. From these measurements, the ratio of oxidative to reductive activity for G3PDH was compared between both species using temperature as a probe. In all tissues except rabbit liver, there was an increase in the relative activity of the reductive direction at 5°C compared to 22°C. This distinguishes rabbit liver G3PDH from the other enzymes. With respect to G3PDH from *C. ludivicianus*, temperature modulation of activity during torpor will result in an decreased relative activity towards DHAP formation and entry into glycolysis relative to a maintenance of the G3P pool.

Distinctions between rabbit muscle G3PDH and the enzyme from both *C. ludivicianus* tissues lie in a comparison of functional and structural parameters at 22°C and 5°C. Functional parameters studied for G3PDH were

K_m values, which provide an indication of how efficiently the enzyme is performing catalysis. This is a product of active site integrity and provides a means of comparing temperature effects between G3PDH from rabbit and *C. ludivicianus* muscle. *C. ludivicianus* liver and muscle G3PDH exhibited increased or equivalent K_m values for DHAP and NAD⁺ at 5°C relative to 22°C while K_m values for both substrates were decreased in the rabbit muscle enzyme at 5°C. This implies functional differences between these three enzymes. Furthermore, K_m values for G3P as a function of decreasing temperature over the range of 50 to 20°C increased 6-fold for the enzyme from rabbit muscle, while they increased 4-fold for the enzyme from *C. ludivicianus* muscle. These kinetic differences at a range of temperatures indicate that functional integrity of muscle G3PDH is better maintained in *C. ludivicianus* relative to rabbit.

Structure-function studies using temperature and chemical denaturants also indicate differences between muscle G3PDH from both species. Both of these agents induced conformational change within the enzyme which were monitored by decreases in activity or by changes in the fluorescence emission spectra. The latter has received application in monitoring conformational and unfolding transitions by changes in emission intensity and wavelength, respectively (Eftink 1994; Sacchetta et al. 1993).

Temperature induced conformational changes were monitored by fluorescence intensity changes over the temperature range of 5°C to 48°C and showed a greater degree of change for muscle G3PDH from *C. ludivicianus* relative to rabbit. This is an indicator of a structurally less rigid enzyme from *C. ludivicianus* relative to the rabbit enzyme. Furthermore, a

comparison with K_m values at this same range of temperatures indicates that the maintenance of functional integrity is independent of temperature induced conformational changes.

Enzyme stability is another means of characterisation which is an indicator of the structural integrity of an enzyme. Probes of enzyme stability include temperature and denaturants such as guanidine hydrochloride both of which can be monitored by activity measurements. Furthermore, chemically induced unfolding transitions can be monitored by the effect on the fluorescence emission spectrum which can be used to distinguish between enzymes on a structural basis (Sacchetta et al. 1993). Shown in Figure 4.5 are the effects of increasing guanidine hydrochloride on activity and the fluorescence spectrum of G3PDH. Unfolding patterns for the two muscle enzymes indicate a greater overall degree of unfolding shown by the greater wavelength shift experienced for G3PDH from rabbit relative to *C. ludivicianus*. This indicates structurally distinct enzymes. Furthermore, the maximal activity of muscle G3PDH from the hibernator was less sensitive to increasing guanidine hydrochloride concentration relative to the rabbit enzyme indicating a greater preservation of structural integrity of G3PDH from this species.

Thermostability of G3PDH is another parameter which serves to distinguish between muscle G3PDH from *C. ludivicianus* and rabbit muscle. Data in Figure 4.4 demonstrates a greater thermolability for rabbit muscle G3PDH relative to the enzyme from the hibernator.

The present study demonstrates that although G3PDH from three sources, rabbit muscle and *C. ludivicianus* liver and muscle share many characteristics, physical and functional parameters with respect to temperature indicated G3PDH from the hibernator is structurally less rigid but better maintains functional integrity at a range of temperatures, has a greater high temperature stability and is less sensitive to the presence of guanidine hydrochloride relative to the enzyme from rabbit.

Table 4.1. Purification protocol for G3PDH from *C. ludivicianus* liver (Table 4.1a) and *C. ludivicianus* muscle (Table 4.1b) Assay for activity of fractions was conducted in the direction of G3P oxidation.

(1a)

	Total Protein (mg)	Total Activity (U)	% Yield	Specific activity (U/mg)	Fold pure
Crude extract	246	1266	100	5.14	1
DE-52	49.0	997	79	20.4	4
HPLC size exclusion	1.20	212	17	177	34
Matrix Red column	0.50	129	10	257	50

(1b)

	Total Protein (mg)	Total Activity (U)	% Yield	Specific activity (U/ugm)	Fold pure
Crude extract	2110	3380	100	1.60	1
DE-52	240	1824	54	7.60	5
HPLC size exclusion	4.40	804	24	183	114
Metrex Red column	1.50	423	13	282	176

Table 4.2. Maximal activities and activity ratios of muscle G3PDH from rabbit and *C. ludivicianus* at 22 and 5°C. Assays were performed at optimal substrate concentrations in imidazole buffer, set to pH 7.5 at 22°C and allowed to fluctuate with temperature. Data is presented as means $\pm$ SEM. In all cases, there were no significant differences between hibernating or euthermic tissues. Values in each direction of catalysis are significantly different between tissue groups and species at the $P < 0.005$ level of significance. Superscript a denotes significant differences in ratios at 5°C from the corresponding activity ratio at 22°C.

Table 4.3. K_m values at two temperatures for the four substrates of purified G3PDH from *C. ludivicianus* liver and muscle and rabbit muscle. All assays were conducted at 22°C and 5°C, in 50 mM phosphate buffer at pH 7.5 in the direction of DHAP reduction and pH 8.5 for the determination of K_m values for NAD^+ . Data is presented as means $\pm$ SEM, $n = 3-5$ preparations.

Superscript a refers to significant differences from corresponding 22°C values at the $p > 0.005$.

Tissue	Temp	NAD^+ (mM)	DHAP (mM)	NADH (μ m)
<i>C. ludivicianus</i> liver				
	22°C	0.045 ± 0.003	0.352 ± 0.003	1.0 ± 0.2
	5°C	0.090 ± 0.001^a	0.259 ± 0.004^a	1.0 ± 0.1
muscle	22°C	0.143 ± 0.004	0.279 ± 0.004	3.0 ± 0.2
	5°C	0.155 ± 0.005	0.154 ± 0.004^a	1.0 ± 0.3
Rabbit muscle	22°C	0.144 ± 0.0033	0.404 ± 0.002	5.0 ± 0.5
	5°C	0.259 ± 0.004^a	0.504 ± 0.009^a	4.0 ± 0.2

Figure 4.1. SDS-PAGE of purified glycerol-3-phosphate dehydrogenase from *C. ludovicianus* liver (lane 2) and muscle (lane 3). Standards in lane 1 are listed in Materials and Methods.

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Figure 4.2. Effect of assay temperature on K_m values for G3P of purified skeletal muscle G3PDH from rabbit and *C. ludivicianus*. All points are mean $\pm$ SEM (3).

Figure 4.3. Fluorescence emission intensity at 340 nm as a function of temperature for *C. ludivicianus* and rabbit muscle G3PDH. Each point represents a single measurement. Slopes of both lines were -0.235 ($r^2=0.99$) for *C. ludivicianus* muscle and -0.169 ($r^2=0.98$) for rabbit muscle and were significantly different.

Figure 4.4. Thermolability curves of purified *C. ludivicianus* and rabbit muscle G3PDH induced by incubation of the enzyme at 37°C. All points are means $\pm$ SEM and are the mean of 4 preparations.

Figure 4.5. Unfolding transition curves of G3PDH induced by increasing concentrations of guanidine hydrochloride and monitored by λ_{max} displacement. Inset: corresponding loss of activity as a function of increasing guanidine hydrochloride concentration. All activity measurements are the mean of three determinations; error bars represent SEM. Data determined from fluorescence measurements is the product of a single determination.

Tissue	Maximal activity in <i>C. ludivicianus</i> tissues (U/gww)		Maximal activity in rabbit tissues (U/gww)	
	22°C	5°C	22°C	5°C
liver				
DHAP reduction	36.3 ± 0.07	9.22 ± 1.64	24.7 ± 0.09	4.37 ± 0.72
G3P oxidation	4.0 ± 0.01	0.63 ± 0.019	2.67 ± 0.02	0.46 ± 0.03
activity ratio (reduction/oxidation)	9.1 ± 0.03	14.7 ± 0.61 ^a	9.24 ± 0.06	9.6 ± 0.8
muscle				
DHAP reduction	5.02 ± 0.154	1.80 ± 0.19	8.28 ± 0.44	3.07 ± 0.17
G3P oxidation	0.55 ± 0.012	0.110 ± 0.004	1.10 ± 0.06	0.21 ± 0.004
activity ratio (reduction/oxidation)	9.1 ± 0.24	16.3 ± 0.74 ^a	7.5 ± 0.51	14.9 ± 0.37 ^a

Chapter 5

General discussion

The variety of mechanisms used by hibernating species to modulate enzyme activity during torpor can be classified as either active or passive forms of regulation. Active regulation involves the alteration of enzyme activity to increase or suppress flux in a particular metabolic pathway. This can occur by a variety of mechanisms (discussed in chapter 1); in this study, active control in *S. lateralis* was determined in terms of the alteration of enzyme maximal activities. Conversely, enzyme activities can be adapted to environmental effects which presents a form of passive control. In this study, this was determined by a comparison of functional and structural differences between enzymes from hibernating and non-hibernating species with respect to their responses to temperature change. These two mechanisms will be discussed.

Active control

The changes in metabolic flux potential presents an active form of metabolic control, which can be determined from maximal enzyme activities in the hibernating and euthermic states. In *S. lateralis*, glycolytic enzyme activities were generally reduced during hibernation in liver, kidney muscle and WAT, while in heart, their levels increased. However, activities of the enzymes PK and PFK, two important loci of glycolytic control, did not change. Previous studies have demonstrated that these enzymes may be regulated by allosteric effectors such as fructose 2,6-bisphosphate, an effector of PFK, which has previously been shown to increase in muscle and heart of *S. lateralis* during hibernation, but remain constant in liver and kidney (Brooks and Storey 1992). The data presented here suggests that glycolysis is not strongly regulated.

Decreased glycolytic flux characteristic during hibernation (Tashima et al. 1970) is likely a function of lowered glucose availability brought about by a reduction in glycogen catabolism (Brooks and Storey 1992, de la Roche and Storey, unpublished results).

Enzyme activities associated with NADPH production demonstrated a major trend during hibernation. All activities tested decreased or remained constant during hibernation indicating a decreased metabolic flux potential towards the generation of NADPH. The net result is a limit to all biosynthetic processes. In contrast, enzyme activities associated with protein catabolism demonstrated no uniform trends. Because levels of these enzymes are relatively high, their function is likely governed by substrate availability.

The TCA cycle is strongly regulated by activities of (NAD)IDH and KGDH whose activities either increased or did not change in all tissues except WAT. This indicates an increased TCA cycle flux potential during hibernation, likely governed by substrate availability, either via pyruvate dehydrogenase or anapleurotic pathways. Although increases in TCA cycle flux potential may have no relevance to the torpid animal, the arousal process involves an explosive flux of metabolic substrate which may require an increased TCA cycle capacity. In kidney and heart, the flux of glycolytic carbon into the TCA cycle is severely inhibited by the conversion of the active a form of PDH to the inactive b form by covalent modification.

Quantification of β -hydroxybutyrate (β OHB) in hibernating and euthermic tissues of *S. lateralis*, *C. ludivicianus*, and *R. ratus* demonstrated an increase in levels of β OHB during hibernation purporting a greater reliance on fatty acid

mobilization. The general mechanisms for this are unclear. Enzyme activities thought to regulate fatty acid metabolism are COT and CPT both of which increased during hibernation in most tissues. In a variety of metabolic states, CPT is regulated by concentrations of malonyl-CoA, however, the present study suggests that increases in enzyme activity play a prominent role in regulating fatty acid catabolism during hibernation.

The physiological state of *S. lateralis* during bouts of torpor is a function of substrate flux potential through biochemical pathways which in turn are a function of enzyme activities associated with these pathways. This study demonstrates that the change in enzyme activities in hibernating versus euthermic *S. lateralis* plays a prominent role in increasing the metabolic flux potential associated with fatty acid oxidation, and suppressing this flux in the pathways of glycolysis, biosynthesis and glycolytic entry into the TCA cycle in various tissues of *S. lateralis*.

Passive control

Passive control in terms of enzyme adaptation in heterothermic species is a more subtle form of metabolic control during torpor. Elucidation of these adaptations arises from a consideration of structural and functional differences between hibernating species relative to animals that do not hibernate.

Functional parameters of β -hydroxybutyrate dehydrogenase (β DH) were determined in extracts from *S. lateralis*, *C. ludivicianus* and rat. Characterisation of this enzyme on the basis of activation energy, maximal activity and pH behavior demonstrates that the suppression of maximal enzyme activity is solely

a function of the reduction in temperature in the three species. However, β DH was distinguishing between hibernating and non-hibernating species in terms of K_m values for β -hydroxybutyrate. Whereas, K_m values were maintained for β DH from both *S. lateralis* and *C. ludivicianus*, this kinetic parameter decreased throughout the range of temperatures for the rat enzyme.

Functional and structural adaptations of enzymes in hibernating species were further explored in the muscle enzyme glycerol-3-phosphate dehydrogenase (G3PDH). The muscle enzyme was purified from *C. ludivicianus*, a hibernator and compared to that from rabbits, a non-hibernating control. Characterisation of the purified enzyme involved isoelectric focusing, pH optima, activation energy and temperature modulation of maximal activity, which were the same in all three species. However, K_m values were again distinctive for the hibernating species, and better maintained at the temperature range of 5 to 40°C relative to the changes throughout this range experienced by G3PDH from the non-hibernator. Temperature induced conformational changes determined by enzyme fluorescence measurements indicate a greater degree of conformational change in muscle G3PDH from *C. ludivicianus* relative to the enzyme from rabbits. Stability was also used as a criterion for elucidating structural differences between G3PDH from the two animals. In the presence of both temperature and chemical denaturation, G3PDH from *C. ludivicianus* was more stable than the rabbit enzyme. This is likely a product of structural integrity, as enzyme fluorescence measurements in the presence of increasing concentrations of guanidine hydrochloride demonstrate a greater degree of unfolding transitions for G3PDH from rabbit. These results imply functional and structural adaptations of this enzyme in hibernating species.

In summary, metabolic adjustments to hibernation in *S. lateralis* are a function of both an active and a passive regulation. Active regulation involves the alteration of enzyme activities in order to route carbon in such a manner as to ensure the most efficient use of stored metabolic fuel. In addition, passive metabolic control is a result of adaptational differences in hibernator enzymes, for function at a range of temperatures.

Appendices

Appendix I. Steady state kinetics.

There are two basic parameters that describe enzyme action; these are V_{max} and K_m . V_{max} is the maximum activity of an enzyme at saturating concentrations of substrate, whereas, K_m describes the concentration which yields 1/2 of V_{max} . V_{max} is proportional to the total enzyme concentration, and is the product of the rate of catalysis and the concentration of the enzyme defined as follows:

$$V_{max} = k_{cat}[E]$$

where k_{cat} is the rate of catalysis, or the rate of dissociation of the enzyme-substrate (ES) complex (see below).

The other parameter, K_m , is independent of enzyme concentration. A simple single substrate and product enzyme catalysed reaction scheme is as follows:

In general, the rate of dissociation is generally more rapid than the conversion to product (ie. $k_{-1} \gg k_2$) and therefore, K_m is a function of the dissociation of the Michaelis complex:

$$K_m = \frac{k_2 + k_{-1}}{k_1}$$

In some cases, however, intermediate forms of the Michaelis complex along the reaction pathway are present in significant amounts, and will result in smaller dissociation constants than predicted above.

K_m values are an indicator of changes in the equilibrium dissociation constant when some other parameter (such as temperature) is altered, K_m values were used as an indicator of the functional integrity of an enzyme with decreased temperature. Furthermore, because K_m values are a function of enzyme-substrate dissociation, they are strongly altered by even subtle changes in the active site. As a result, this parameter was used as a sensitive probe of functional behavior and changes in active-site integrity with temperature and compared between enzymes from a hibernating and a non-hibernating species.

Appendix II. The effect of Alphastat modulation of enzyme activity during hibernation.

During hibernation, there are two factors which contribute to acidotic modulation of enzyme activity during hibernation. The first is the effect of changes in intracellular pH experience during hibernation which is a result of apnoic breathing and hypercapnia. The second is the effect of temperature on the pKa of intracellular histidine, the major intracellular buffer. This is known as the alphastat model and involves a basic shift of intracellular pKa arising from the effect of temperature on the histidine residues of protein. This allows for protein function and structure to be conserved with changes in temperature, a result of the shifts in enzyme pKa that mimic that of the cell. As a result, both an acid shift of intracellular pH and a basic shift of protein pKa result in a modulation of activity for pH sensitive enzymes. In this study, this was explored by determining energies of activation from Arrhenius plots for glycerol-3-phosphate dehydrogenase which is an indication of maximal enzyme activity changes over temperature. Arrhenius plots were performed in two buffers, tris, one whose pKa shifts strongly with temperature, and phosphate, one whose pKa shifts very little with temperature. These two buffers were specifically chosen to exaggerate a situation where changes in pKa with temperature are more and less prominent, respectively, than changes in intracellular pKa. In this experiment, there was no difference in activation energies determined in the two buffers demonstrating that the effect of alphastat regulation on activity during hibernation is not a factor in modulating enzyme activity.

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