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Properties of Enzymes form Mammalian Hibernators: Structure Function Relationships

By

Bradley John Thatcher, B.Sc., MSc.

A thesis submitted
To the Faculty of Graduate Studies and Research
In partial fulfillment of
The requirements for the degree of

Doctor of Philosophy

Department of Biology
Carleton University
Ottawa, Ontario
July, 1997

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The Faculty of Graduate Studies and Research
Acceptance of the thesis

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structure function relationships'**

submitted by
Bradley John Thatcher, B.Sc., M.Sc.

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

Chair, Department of Biology

Thesis Supervisor

External Examiner

Carleton University
1997

ABSTRACT

The examination of four enzymes (Glutathione S-transferase [GST], Glutathione reductase [GR], Aspartate aminotransferase [AspAT], and Glutamate dehydrogenase [GDH]) from two hibernators *Cynomys leucurus* and *Spermophilus richardsonii* were used to show three different responses to a hibernating life style. The production of a new (isozyme) of GST, α -class, in the liver and muscle tissues of *Cynomys leucurus*. N-terminal and amino acid compositions show strong homology α -class GSTs from a variety of species. The kinetic profile and structural studies suggest that this isozyme is similar to other α -class GSTs. PDM 18 showed a broader temperature optima and lower thermal stability compared to a comparable rabbit enzyme. A different form of GDH was found in the liver tissue of *Spermophilus richardsonii* in the hibernating animal. This new isozyme of GDH differed from the nonhibernating and bovine enzyme in both physical and kinetic parameters. There is a shift in the directional preference between the two GS forms of the enzyme in the presence of GTP, ATP, AMP. Further kinetic differences is also reflected in I_{50} , K_m , and K_a values between the two GS isozymes. The N-terminal sequence of the two GS isozymes is identical over the first 12 residues there are differences in the molecular weight by SEC, and retention time on reverse phase HPLC suggesting small differences in the polypeptide chain. The final observed case was that there were no substantial kinetic and minor structural changes between the enzyme from the hibernator and from a nonhibernator. This was seen with two enzymes from the study GR (from PD) and AspAT (from GS).

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$\lambda_{\max}$	Wavelength of maximal emission
AlaAT	Alanine aminotransferase
AspAT	Aspartate aminotransferase
BAT	Brown adipose tissue
cm ⁻¹	Wave numbers (1/wave length)
ddi-water	double deionized water (Barnsted 4 filter system)
Ea	Activation energy
EDTA	ethylenediaminetetraacetic acid
TCA	Tricarboxylic acid cycle
EGTA	ethyleneglycol-bis(β -aminoethyl ether) N,N,N',N'-tetraacetic acid
T _{1/2}	Protein half-life
E _{max}	Fluorescence emission intensity maxima (nm)
FAD	Flavin adenine dinucleotide
FT-IR	Fourier transform infrared spectroscopy
GDH	Glutamate dehydrogenase
GR	Glutathione reductase
GS	Ground squirrel
GSH	Glutathione (reduced)
I ₅₀	Inhibitor concentration that reduces activity by 50%
GSSG	Oxidize glutathione
GST	Glutathione S-transferase
GuHCl	Guanidine hydrochloride
HPLC	high performance liquid chromatography
I	fluorescence emission intensity
IEF	Isoelectric focusing
IR	Infra red spectroscopy
I ^{xxx}	Fluorescence emission intensity at XXX nm

K _m	Michaelis-Menten constant
α-KG	α-ketoglutarate
NADH / NAD ⁺	Nicotinamide adenine dinucleotide (reduced / oxidized)
NADPH / NADP ⁺	Nicotinamide adenine dinucleotide phosphate (reduced / oxidized)
NST	Nonshivering thermogenesis
PD	Prairie dog
PDL	Prairie dog liver
PDM	Prairie dog muscle
pI	Isoelectric point
PMSF	Phenylmethylsulphonyl fluoride
PP	pyridoxal phosphate
PVDF	polyvinylidene difluoride
RL	Rabbit liver
RM	Rabbit muscle
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SEM	standard error of the mean
ST	shivering thermogenesis
U	unit of enzyme activity (μmoles/min)

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HYPOTHESIS

Enzymes in hibernators must function at both euthermic and hibernating body temperatures, which frequently differ by about 30°C. Protein conformation and function are highly dependent on weak bond interactions, both hydrophilic and hydrophobic bonds being markedly influenced by temperature change. I hypothesize that hibernators will modify some of their proteins to compensate for the stress imposed on the animal by a hibernating lifestyle (as opposed to the virtually constant temperature experience of most proteins in mammalian organs), resulting in selected and specific changes to the structure and function of enzymes from mammalian hibernators have occurred.

GENERAL INTRODUCTION

Various mammalian species endure the prolonged cold temperatures and food scarcity of winter by hibernating. They enter extended periods of torpor in which their core body temperature falls to near ambient for days or weeks at a time; the animal then awakens with no obvious injury. Hibernation is a complicated and poorly understood physiological condition. Basic physiological measurements were conducted in the 1960's (Bartholomew and Hudson, 1962; Bruice and Butler, 1965). Hibernators allow their body temperature and metabolic rate to fall to levels that would kill most mammals, yet the hibernator suffers no apparent detrimental effects from prolonged bouts of torpor with core body temperatures of about 5°C or even lower (Bruice, 1965; French, 1988 ; Storey, 1989 ; Wang, 1989; Schmidt-Neilsen, 1983). The physiological changes that make up hibernation include strong metabolic rate depression during torpor; oxygen consumption by hibernating mammals may be as little as 1-4% for small animals like bats and mice, or 4-8% for larger mammals such as ground squirrels of the resting value at euthermic body temperature (Geiser, 1988). During hibernation many species rely solely on stored body fuel reserves to support energy metabolism over the entire winter (French, 1988; Wang, 1989). The length of each torpor bout can last between 15 to 20 days during midwinter for various small mammals (but is shorter at both the beginning and end of the season) with the overall hibernating season varying from 5 (marmots) to 10 (jumping mice) months (Schmidt-Neilsen, 1983; French, 1988; Wang, 1989). Bouts of torpor are punctuated by short periods of euthermia; these intervals last from 2 to 24 hours, the length being proportional to body mass, and are long enough to allow the animal to regain normal body temperature and metabolic rate (French, 1988). The net energy savings over the winter season that are produced by torpor are greatest for

mammals with very small body masses and can result in a 90% reduction in total energy expenditure, compared with the energy consumption that would be needed to remain euthermic over the winter (Schmidt-Neilsen, 1983; French, 1988; Wang, 1989). The waking process takes only a short time, and thus these animals must be able to function metabolically under either torpor or euthermic body temperatures with essentially the same biochemical systems (French, 1988; Storey, 1989; Wang, 1989).

There are several types of hibernation that animals use to avoid environmental stress. In the case of large mammals, such as bears, metabolic rate is reduced by only about 30 % and only a small reduction in body temperature occurs during torpor; the low surface area to volume ratio of bears apparently precludes their large body mass from cooling further. Some small mammals like the black-tailed prairie dog, *Cynomys ludovicianus* are facultative hibernators and hibernate only when environmental conditions become severe and not usually for a predictable or prolonged period of time (French, 1988; Wang, 1989; Marchand, 1991). Other species are known as profound hibernators and have a torpor cycle that is programmed to be seasonal and regular (Pehowich and Wang, 1983; French, 1988; Wang, 1989; Marchand, 1991). Hibernation usually starts in late summer or early fall and continues into the spring. The white-tailed prairie dog, *Cynomys leucurus* (Marchand, 1991), golden-mantled ground squirrel, *Spermophilus lateralis* (Barnes et al., 1987), and Richardson's ground squirrel, *Spermophilus richardsonii* (Pehowich and Wang, 1983) are all examples of profound hibernators. The body temperature of these species falls to below 8°C and the period of hibernation lasts for several months (interspersed with periodic arousals) (Pehowich and Wang, 1983; Barnes et al., 1987; Wang, 1989; Marchand, 1991). For these species

hibernation is a regular and necessary survival mechanism. Thus, they make good model systems for the study of protein function, structure, and evolution for life at two temperatures.

Hibernation involves a deep suppression of metabolic rate (Schmidt-Neilsen, 1983; French, 1988; Wang, 1989). This includes a reduction of body temperature to near ambient (or to about 5°C if ambient is subzero). During bouts of arousal, body temperature and metabolic rate are rapidly increased with heat generated *via* uncoupled lipid oxidation in the brown adipose tissue (Storey, 1989). Predictably, then, the enzymes of hibernators must be structured and/or regulated to allow coordinated metabolic functions to be sustained at both high (37°C) and low (5°C) body temperatures (Storey, 1987; French, 1988; Wang, 1989). Since the transition times between hibernation and arousal are brief and the intervals of euthermia between hibernation bouts are quite short, there is undoubtedly insufficient time for any large scale changes in protein complement (such as due to *de novo* protein synthesis or degradation) to serve high versus low temperature metabolism. Furthermore, the high energetic cost of such a scheme would be contrary to the main reason for hibernation, which is energy conservation (French, 1988; Wang, 1989). In the absence of major changes in protein complement of cells during transitions to or from the hibernating state, other strategies remain as potential mechanisms for controlling or readjusting metabolism during torpor. These include:

- enzyme/protein regulation by post-translational modification, e.g. reversible protein phosphorylation (Storey, 1989; Walsh et al., 1991)
- seasonal changes in protein expression to facilitate hibernation

- modification of the constitutive structure of selected (or perhaps even all) hibernator proteins so that they function effectively at both low and high temperatures (Hochachka and Somero, 1984; Jaenicke, 1991)
- modulation of protein half-life and/or anabolism/catabolism to ensure that there is enough enzyme to perform adequately during hibernation.

It is likely that a variety of strategies are used within a single animal species to modulate the activities of different enzymes, functional proteins, and metabolic pathways during hibernation (Storey, 1987; Storey, 1989; Walsh et al., 1991).

Studies of metabolic regulation and enzyme function at extreme temperatures have yielded some interesting results. Life has adapted to all manner of environmental extremes; temperature (both low and high), salinity, and pH are but a few examples. Some thermophilic bacteria produce stable enzymes that function at temperatures in excess of 70°C; surprisingly, these retain high sequence homology to enzymes from other organisms that are readily denatured at such high temperatures (Cannio et al., 1994). With the modification of as few as four amino acids the heat stability of a protein can be increased significantly (Hochachka, 1976; Somero and Low, 1976; Cannio et al., 1994). There are several possibilities that exist for the adaptation of proteins to function over wide thermal ranges. Each of these enzymes will then be compared to the hibernating form of the enzyme to determine if changes to the protein are occurring because of hibernation.

Several studies have approached the question of how enzymes from hibernators can work at high (25 or 37°C) versus low (0-5°C) temperatures by analyzing their kinetic

properties (Pehowich and Wang, 1983; Hochachka and Somero, 1984; Storey, 1987). In the bat (*Myotis lucifugus*), data suggests that there are hibernating and normothermic forms of lactate dehydrogenase (Storey, 1987; Storey, 1989). Changes to the isozyme patterns of pyruvate kinase in the muscle of hibernating bats have also been reported (Storey, 1987; Storey, 1989). Storey (1989) has shown that glycogen phosphorylase is present predominately as the less active b form in tissues from hibernating individuals and that phosphofructokinase and pyruvate kinase show kinetic changes during hibernation that may be the result of hibernation-induced reversible protein phosphorylation.

Kinetic changes in a protein can also reflect conformational changes at different temperatures. The structure of enzymes will affect their function and kinetic differences in enzymes can be linked to physical changes in the protein. Both the function and structure can be examined under a variety of conditions. Furthermore, it is reasonable to surmise that enzymes change for hibernation since only a few mammals can survive a 5°C body core temperature. A structural and kinetic approach to understanding proteins at low temperature will provide evidence for the mechanism(s) that are responsible for metabolic changes observed during hibernation. Structure/ function studies will also help elucidate some of the enzyme characteristics that hibernators require for survival, since the evidence is strong that some enzyme modification(s) are required for hibernation.

Studies of the enzymes of ectothermic animals that live at different near-constant temperatures have indicated that many enzymes are adapted to function optimally at an average body core temperature (Graves and Somero, 1982; Hochachka and Somero, 1984; Feller et al., 1994). These adaptations generally take the form of a modification of

enzymatic catalytic efficiency to compensate for reduced or elevated temperatures (Graves and Somero, 1982; Hochachka and Somero, 1984). For example, a study of the M₄-lactate dehydrogenases (M₄-LDH) of different species of barracuda of the genus *Sphyraena* showed that enzyme properties varied with latitude, and hence, with the mean sea water temperature that the animals experienced (Graves and Somero, 1982). In this case, there were clear differences in the kinetic properties and electrophoretic mobilities of barracuda M₄-LDH that correlated with variation in environmental temperature. The changes in electrophoretic mobilities suggested that a modification of the primary structure of the enzyme had occurred to compensate for living in colder water (Graves and Somero, 1982). This phenomenon is not restricted to this one genus as large variations in enzyme properties have also been observed for various other ectothermic species living under different temperature conditions (Hochachka and Somero, 1984). This result is not surprising since active lifestyles are carried out at all latitudes from the Antarctic to the tropics (Graves and Somero, 1982; Hochachka and Somero, 1984; Feller et al., 1994).

The case with homeothermic animals generally seems less problematic since core body temperature is maintained within a narrow range and, hence, systems are generally viewed as adapted to a single stable set of temperature conditions (Schmidt-Nielsen, 1983). One exception to this is the case of hibernation. During the winter season enzymes and functional proteins of hibernating mammals are required to function at two widely varying temperatures, the euthermic (generally 37-41°C depending on species) and hibernating (often 2-5°C) body temperatures. This is the duality of hibernation:

metabolism functioning at two set points, 37°C during arousal and at 5°C during torpor. Low temperature also puts other stresses on proteins in hibernating systems, including an alkaline pH shift of the cytoplasm, decreased strength of hydrophobic interactions, and increased strength of hydrophilic interactions (Douzou, 1977; Zubay, 1988). These could change both the intra- and inter-molecular interactions of a protein (Douzou, 1977; Zubay, 1988).

A modification of protein primary structure to change activity and stability is one solution to functioning at two temperatures. The sequence of a protein (primary structure) is the basis for its secondary, tertiary, and quaternary structures (Feeney et al., 1990). Modifications of catalytically essential, and even nonessential amino acids, can have profound effects on the higher order structure, and activity of an enzyme (Hatley and Franks, 1989; Malcolm et al., 1989; Wilks et al., 1989; Sanz et al., 1992; Cannio et al., 1994). Site-directed mutagenesis studies have shown that the substitution of a single amino acid can produce unpredictable results (Malcolm et al., 1989). Any changes to protein primary structure made by hibernating animals must account for several changes in the environment (Hochachka and Somero, 1984; Wang, 1989). The modifications made must allow for changes in catalytic efficiency observed at different temperatures (Hochachka and Somero, 1984). These modifications must also account for the "relaxation" of the protein with decreased temperature; as the temperature of a system is reduced there is a weakening of hydrophobic and Van der Waals interactions (Comstock, 1979). These forces between amino acids are important for the maintenance of higher order structure (Comstock, 1979). Some proteins are known to denature at temperatures

near 0°C but the effects of low temperature are normally reversible when the system is rewarmed (Comstock, 1979; Buzadzic et al., 1992). Enzymes from hibernators could be modified to preserve function and catalytic efficiency at two temperatures (Hochachka, 1976; Somero and Low, 1976; Traut, 1994). Organisms must be able to stabilize their protein structure and thus maintain catalytic activity to permit survival of the animal under both hibernating and euthermic conditions and during the rapid transition between the two states. Changes in intermolecular interaction could be achieved in three ways:

1. by changing some amino acids to alter the strength of secondary structure (Feeney et al., 1990)
2. by adding amino acids that modify β -sheets and α -helix structures to stabilize secondary structure (Cannio et al., 1994)
3. by changing the number of disulfide and/or ionic bridges.

The interactions between domains may also be modified to produce additional conformational stability but must not change the active site conformation which normally occurs at the interface between domains (Feeney et al., 1990). Quaternary structure is the final stage in the assembly of subunits into functional enzymes, and is dependent on the combination of all of the lower order structures. Subunit interactions are commonly held together by molecular forces that are most affected by chaotropic conditions (ionic strength, temperature, pH) (Comstock, 1979). Disturbances to the quaternary (4°) structure would affect the kinetic properties of proteins, thus providing a link between spectroscopic structural information and enzyme function. In most cases, the elimination

of 4° structure causes a loss of activity and significant changes in emission spectra of the protein (Comstock, 1979; Zubay, 1988; Hatley and Franks, 1989). The difficulty arises when one tries to determine if these changes are the result of design, or simply the result of temperature-induced conformational changes (Storey, 1989).

Studies of proteins have shown that temperature has a variety of structural effects on these molecules. Temperature is known to alter the quaternary structure of numerous enzymes which can result in disassociation of subunits, deactivation by denaturation (high temperature), loss of cofactors, changes in binding affinities, or changes in the conformation of binding sites (Comstock, 1979). Temperature change can also alter other chemical properties (thermodynamic and ionization parameters) of enzymes and result in changes to the catalytic function (Comstock, 1979).

The primary objective of this work is to gain some understanding of the interaction between protein structure and protein function in a unique mammalian system. The attempt will be made to link some of the kinetic changes in enzymes from hibernators to their structural characteristics at high and low temperatures. Structural and kinetic data will be combined to provide information on the adaptation of enzymes to the requirements of hibernation. These results can then be compared with the properties of enzymes from nonhibernators under the same conditions to sort out the effects of specific protein modification and adaptation from those of temperature alone. This should give information on aspects of the relationship between enzyme structure and function at low temperature in a well-developed system. I asked the following questions. Are enzymes from hibernating animals the same as those from related nonhibernating mammals? If they are different, then what changes in the protein can be linked to changes in function?

The choice of animal species to be used in the present study was made based on the need for a dedicated, or obligate, hibernator to ensure that hibernating tissue could be obtained and that the animal was well innately adapted for regular yearly hibernation cycles. The chosen species also had to be of a reasonably large animal (or readily available) to provide the amount of tissue necessary for enzyme purification for structural studies. These practical considerations are of vital important since some structural studies can require milligram quantities of highly purified protein and this could provide problems with small hibernators like bats. The use of an obligate hibernator should also increase the chances of finding enzymes that are produced specifically for hibernation. To this end, the animals chosen for use were the white-tailed prairie dog, *Cynomys leucurus* and the Richardson's ground squirrel, *Spermophilus richardsonii*. Both of these animals fit the practical experimental requirements. Both of these species start their annual hibernation cycle in the late summer or early fall and remain in hibernation for the entire winter, emerging only in the spring. The hibernation characteristics of these species should provide the best chance for finding enzymatic and structural differences between hibernating and nonhibernating animals.

The selection of enzymes to be studied was also based on several criteria. The enzyme must be in high concentration (in at least one tissue) to ensure that there is enough protein to accomplish the study. There should also be some indication that either the protein activity changes during hibernation or that the protein is different from the "normal" mammalian enzyme; this information can be gained from preliminary studies using enzyme scans from a variety of tissues and hibernators. The enzyme should also have a metabolic regulatory role. The enzymes examined were glutathione S-transferase

(GST), glutathione reductase (GR), aspartate aminotransferase (AspAT), and glutamate dehydrogenase (GDH).

The experimental rationale used was to examine the kinetic and structural characteristics of each enzyme to build an understanding of how hibernators have adapted their enzymes to deal with the temperature (or other metabolic) demands placed on them during hibernation. As the first step, a kinetic analysis of each enzyme was conducted in order to identify differences in function/properties between the enzyme from hibernating and nonhibernating mammalian species and the effect of temperature on these properties. In all cases the enzyme from a nonhibernating species was used as a benchmark for the typical mammalian enzyme that is designed to function within a very narrow temperature range. After analysis of kinetic data to show seasonal or hibernation specific changes in enzyme function/properties, the studies were followed by an examination of protein conformation and the effects of temperature on conformational change using fluorescence and Fourier transform infrared spectroscopy (FT-IR). This exposed conformational and stability differences between kinetically similar enzymes. The goal of this study is to characterize some of the differences in enzymes from hibernating animals that may be the result of low temperature adaptation.

CHAPTER 1:
Glutathione S-Transferase Purification and Kinetics

Introduction

The glutathione-S-transferases (GST; EC 2.5.1.18) are a complex group of enzymes with a broad spectrum of activities and substrates (Mannervik and Danielson, 1988; Rushmore and Pickett, 1993; Pickett and Lu, 1989; Ketterer et al., 1988; Singhal et al., 1992). All GSTs use reduced glutathione (GSH) as a substrate; GSH is a tripeptide (γ -Glu-Cys-Gly) with a reduced Cys sulphydryl (Cys-SH) (Mannervik and Danielson, 1988; Atkins et al., 1993). GSH reacts with electrophilic substrates with the Cys-SH to form a covalent bond through the sulphydryl. This reaction is believed to be the first step in the metabolism, excretion or biotransformation of both endogenous and xenobiotic electrophiles (Atkins et al., 1993; Dirr et al., 1994; Mannervik and Danielson, 1988). The purpose of the transformation of xenobiotics is thought to be to increase the solubility of hydrophobic electrophiles and/or to target them for further metabolism or excretion (Halliwell and Gutteridge, 1985). GSH is also complexed to proteins, and this is thought to have several functions including to prevent oxidative damage, neutralize possible free radicals, or repair damage caused by oxidation (Kumari et al., 1994; Gilliland, 1993). Glutathione is oxidized when it is used as a substrate by the enzymatic action of GST or glutathione peroxidase. The levels of GSH are typically held within strict limits in cells by de novo synthesis and the reduction of oxidized glutathione (GSSG) to reform GSH by glutathione reductase. Depending on tissue or organism type, the concentrations of GSH and GSSG are typically 0.1-5 mM and 6-200 μ M, respectively (Kosower and Kosower).

GSTs are found in all organisms that use aerobic metabolism from bacteria to mammals (Fahey, 1977). Most organisms have a variety of forms of GST which vary greatly from tissue to tissue, not only in the amount of each form, but also in the classes of GST represented, and their subunit composition (Ketterer et al., 1988). The changing GST profiles in response to xenobiotics, oxidative stress, and disease state show that the GST family of enzymes can be differentially induced and modified to suite specific substrates, thereby creating a specific enzyme to suit a particular need (McLellan et al., 1991; Tsuchida and Sato, 1992; Singhal et al., 1992). Cytosolic glutathione S-transferases are grouped into four gene classes designated α , μ , λ , and θ (Rozell et al., 1993). Each class of GST has different characteristics with respect to its preferred substrates, sequence homology, gene family, and kinetic properties (Pickett and Lu, 1989). The classes of GST can be identified kinetically using a combination of substrate preferences and inhibitor profiles. Each class of GST also shares sequence homology within the class, and to some degree with all other GSTs (Habig and Jakoby, 1981; Tahir et al., 1985). Furthermore, crystal data suggests that all GSTs have a similar folding topology; thus, 2° and 3° structures are similar in the crystal state. However, 4° structure can vary with the formation of both hetero- and homodimers in vivo, homodimers being dominant (Dirr et al., 1994).

Differences in GST profiles also occur between individuals as well as between the sexes and at different developmental stages. Changes in GST profiles are also observed in tissues as a response to a variety of stresses including disease, drug (toxicant) resistance and xenobiotic exposure (Tsuchida and Sato, 1992; Pickett and Lu, 1989;

Halliwell and Gutteridge, 1985). Glutathione S-transferases have a broad and overlapping spectrum of activities and are adaptable to deal with new substrates as the organism is exposed to them (McLellan et al., 1991). The diversity of GSTs causes complex mixtures of classes and isozymes to occur in most tissues with a few dominant species (Killick, 1991; McLellan et al., 1991). Evidence suggests that the amount of GST in a tissue is related to its metabolic activity and the exposure of the tissue to xenobiotics or stress. Furthermore, a systemic stress can affect GST activities and composition in one, several or all tissues of the animal (Mannervik, 1985; Singhal et al., 1992). Mammalian liver is a good example of a tissue in which GST occurs in a number of forms and with a number of functions (Mannervik, 1985). The important role of liver in detoxification and its high metabolic activity are the reasons for the large array of GSTs found in it. The diversity of GST classes, isozymes and functions in a tissue apparently reflects the activity, function and health of the tissue at the time of sampling (Mannervik, 1985; Ketterer et al., 1988).

The antioxidant activity of GST is the result of the lipid peroxidase activity of most GSTs and the action of Se-independent glutathione peroxidase (GPox) activity that will reduce H_2O_2 . Both of these enzymes use the reducing power of two GSH and produce one GSSG (Morrissey and O'Brien, 1980). Lipid peroxide reducing activity is seen with all GSTs, but activity towards another substrate, cumine hydroperoxide, is normally associated with only one class of GST in animals; in rats it is the α class and in rabbits the GST B (Morrissey and O'Brien, 1980). The antioxidant action of glutathione-S-transferase is believed to be important in the elimination of lipid peroxides from the

cell since no other repair mechanism for lipid peroxides has yet been found (Spaterna et al., 1993; Morrissey and O'Brien, 1980; Singhal et al., 1992). The role of GSTs in other antioxidant reactions is probably minor since other antioxidant enzymes (glutathione peroxidase, catalase, superoxide dismutase) specialize in the catabolism of specific reactive oxygen species like $\cdot\text{OH}$, H_2O_2 , and the superoxide anion O_2^- and are not known to react with lipid peroxides (Halliwell and Gutteridge, 1985).

The modification of proteins and the evolutionary modification of enzymes to suit specific environmental conditions is well documented (Somero, 1995; Feller et al., 1994). In this process one sees the modification of primary sequence resulting in changes to catalytic efficiency, structural stability, and to maintain the functionality of enzymes under various extreme conditions (Somero, 1995; Feller et al., 1994; Cannioe et al., 1994). Proteins are known to be sensitive to temperature (Somero, 1995) yet in the case of hibernating mammals, studies seems to show a reduced effect of temperature on enzyme kinetic parameters (Moon, 1978). Hibernation also introduces changes in blood pH and composition during torpor and wakening (McArthur et al., 1990; McArthur et al., 1992).

In the present examination of GSTs from hibernating and nonhibernating mammalian species, I report the biochemical characterization of the two major isozymes from prairie dog muscle and the identification of some of the liver isozymes, and compare these with the enzymes from rabbit organs.

Methods and Materials

Rabbit liver glutathione-S-transferase, reduced glutathione (GSH), hexyl-glutathione, and hexyl-glutathione agarose were purchased from Sigma Chemical Company (St. Louis, Mo.). Other substrates and inhibitors were purchased from Aldrich Chemical Company (St. Louis, Mo.) and were of the highest purity available.

Adult white-tailed prairie dogs (*Cynomys leucurus*) were captured in Southeastern Wyoming during July, 1993. The animals were housed individually in the animal care facility of the University of Wyoming. They were divided into 2 groups (n=12 each) that were allowed to feed and fatten until October; the two groups differed in the percentages of saturated versus polyunsaturated lipids in their diets (Frank, 1992). Six animals from each group were then sampled as controls, while the remaining 6 were induced to hibernate at 5°C. After 10 weeks, the hibernating animals were sacrificed after at least 4 days of continuous torpor in their final bout (mean torpor bout length is about 8 days in this species). Tissues were collected from euthermic animals in October, 1993, and from hibernating animals in February, 1994. Prairie dogs were sacrificed by decapitation and tissues were rapidly excised from the animal, frozen in liquid nitrogen, and then packed on dry ice for shipment to Carleton University; upon arrival, tissues were placed at -80°C for long term storage.

GST was purified from the liver and skeletal muscle of the white-tailed prairie dog as well as from rabbit skeletal muscle. Purified GST from both euthermic (control) and hibernating animals was prepared. Samples of frozen tissue were homogenized

(muscle 1:11 w:v, liver 1:6 w:v) in 40 mM potassium phosphate buffer pH 6.0 containing 1 mM EDTA, 1 mM EGTA, and 1 mM PMSF, followed by centrifugation at 26,000 x g for 25 min at 4°C in a Sorvall refrigerated superspeed centrifuge. The supernatant was removed and the pellet discarded. For muscle extracts, the supernatant was then loaded onto a 5 ml column of hexyl-glutathione agarose equilibrated in 40 mM phosphate buffer pH 6. The column was washed with this buffer and then with 40 mM phosphate pH 9.0 until absorbance at 280 nm of the eluant dropped to zero. GST isozymes were eluted with a minimum volume of 5 mM glutathione in 40 mM phosphate buffer, pH 9.0. The eluant was dialyzed against ddi-water, its pH was adjusted to 6.0, and then the sample was loaded onto a small column (2 ml) of hydroxylapatite (Bio-Rad) in order to separate GST isozymes. The column was washed with 10 ml of 20 mM phosphate buffer pH 6.0 and then the enzyme was eluted with a gradient of 20 to 50 mM phosphate in 30 ml with 1 ml fractions collected. The prairie dog muscle enzyme was eluted in two distinct peaks of activity which were designated PDM 18 and PDM 23. For liver extracts, GSTs were purified using hexyl glutathione affinity chromatography only, as described above. Commercial rabbit liver GST was checked for purity by SDS-PAGE and reverse phase HPLC and was then used without further purification.

HPLC Analysis: HPLC separations were performed on a Beckman HPLC system with a UV/VIS detector at either 225 or 280 nm. Reverse-phase separations were done on a Zorbax RP-300 C-8 (2.1 x 150 mm) column with a flow rate of 0.2 ml/min. Samples were loaded at 35% acetonitrile and 65% water containing 0.12% trifluoroacetic acid (TFA) and a linear gradient was run to 65% acetonitrile and 35% water with 0.12%

TFA over 25 min. An Altex TSK 2000 (4.5 x 300 mm) column was used for native molecular weight determination. This column was run isocratically in 50 mM potassium phosphate pH 7.0 containing 50 mM KCl at a flow rate of 0.715 ml/min. The SEC columns were calibrated using blue dextran (2000 kDa), thyroglobulin (670 kDa), ferritin (443 kDa), phosphofructokinase (360 kDa), pyruvate kinase (230 kDa), catalase (232 kDa), aldolase 160 kDa), glutathione reductase (124 kDa), hexokinase (102 kDa), adenylate kinase (68 kDa), and triosephosphate isomerase (53 kDa) as molecular weight standards.

Gel Electrophoresis: SDS-PAGE was conducted according to Laemmli (1970) using a Bio-Rad mini gel system (Protean 2). Samples were desalted by centrifugation through small columns (4 ml) of Sephadex G-10 followed by concentration using a Centricon 10 concentrator (Amicon). Samples were then mixed (1:1) with reducing buffer containing 50 mM dithiothreitol (DTT), 20% v/v glycerol, 1% w/v SDS, 50 mM Tris pH 8.8 and 0.04% w/v bromophenol blue. After boiling for 5 min, samples were loaded and run on 15% acrylamide gels with broad range molecular weight standards from Bio-Rad containing: ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), aprotinin (6.5 kDa). Blotting to PVDF (Immobilon-N, Millipore) was done in an Owl semi-dry blotter at 500 mA for 45 min with a buffer system consisting of 25 mM Tris, 192 mM glycine and 20% v/v methanol. Gels and blots were stained with Coomassie blue G-250 0.1% w/v in a 50% methanol-water tincture containing 5% acetic acid. The destaining solution was 50% methanol-water with 5% acetic acid. Gels and blots were then washed in ddi-water.

Amino Acid Composition and Protein Sequencing: Samples of purified enzyme were hydrolyzed in the gas phase (Picotag workstation, Waters) with constant boiling in 6 N HCl at 110°C for 20 to 22 hours under vacuum. Samples were then dissolved in 150 µl of sample buffer (Beckman) and an aliquot of 50 µl were was used for amino acid analysis on a Beckman 6300 amino acid analyzer according to manufacturers specifications.

Protein sequencing was done on either a Beckman 6000 Prosequencer or an Applied Biosystems 470 protein sequencer with an online 163 PTH analyzer. Samples were sequenced from PVDF blots and aqueous samples. Samples were loaded according to manufacture's specifications. Sequence homology was assessed using the NIH Blast program (National Institutes of Health on line sequence analysis program) and comparisons were made to the Swiss Protein database.

Enzyme Assay and Kinetic Analysis: Assays were conducted using a Gilford recording spectrophotometer with a constant temperature cell holder. The formation of a complex between 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione (GSH) was monitored by a increase in absorbance at 340 nm. The total volume in the cuvette was 1 ml containing 0.6 mM GSH, 0.1 mM CDNB, 1 mM EDTA, 50 mM phosphate buffer pH 7.0, and 1.9% v/v ethanol. CDNB (or other hydrophobic organic substrates) were dissolved in 95% ethanol and 20µl of substrate solution was added to the cuvette; the background reaction was then measured, followed by the addition of enzyme. One unit is defined as the activity of enzyme which produces 1 nmole of conjugate per minute. Glutathione S-transferase assays with substrates other that CDNB were done using the

protocols described by Habig and Jakoby (1981). Background reaction rate was subtracted from the total reaction to obtain rates for the enzyme-catalyzed reaction. Data were analyzed by a kinetics program (Brooks, 1992) using Michaelis-Menten kinetics. Significant differences were assessed using the Student's t-test with values of $P < 0.05$ or less considered significant.

Glutathione peroxidase activity of GST was assessed with cumene hydroperoxide as the substrate and monitored by the increase in absorbance at 340 nm. The assay mixture consisted of 1 mM EDTA, 10 mM GSH, 0.2 mM NADPH, 1 unit glutathione reductase, 2 mM NaN_3 , 1.2 mM cumene hydroperoxide (CuOOH) and 40 mM phosphate buffer pH 7.0 in a final volume of 1 ml. The enzyme sample was added to the cuvette after a background reaction rate was recorded; in all cases background rates were measured for each cuvette. One unit of enzyme activity is defined as the amount of enzyme, which will oxidize 1 μmole of NADPH per minute.

Results

Maximal activities of glutathione-S-transferase were measured in four tissues (liver, white adipose, skeletal muscle, and heart) of euthermic prairie dogs (Table 1-1). Total GSH utilization is the sum of the CDNB and CuOOH activities, which would account for the bulk of the enzymatic use of GSH. GST activity was highest in liver, followed by the heart and muscle, and adipose tissue. GST activity was also measured in tissues from hibernating animals but no significant difference in either CDNB or

CuOOH-linked maximal activities were found in any of the four tissues. Selenium-independent glutathione peroxidase (GPox) activity was measured with cumine hydroperoxide as the substrate and is used as a measure of the antioxidant activity of GST subunits (Table 1-1). The activity profile was similar to that seen with CDNB as the substrate. Thus, liver showed highest GSH oxidizing activity in the prairie dog, followed by the muscle and heart, and the lowest activity was in adipose tissue. Again, no significant changes in maximal activities were seen when tissue samples from hibernating individuals were assessed. The activity of the Se-independent glutathione peroxidase form of GST proved too unstable to work with, and when stored at 5°C there was a complete loss of this activity in less than 12 h post-homogenization.

Tissue-specific distribution and maximal activities of GST activity were also measured for comparison in tissues a nonhibernating mammal, the rabbit (Table 1-1). Overall, rabbit tissues showed substantially higher activities of GST than did the prairie dog. This was particularly striking for liver GST that was 22-fold higher in rabbit liver than in prairie dog.

Due to the high instability of the antioxidant, Se-independent GPox, activity of GST in prairie dog liver, only K_m values for cumine hydroperoxide were determined in crude extracts. K_m values were $235 \mu\text{M} \pm 2.74$ at 25°C and $79.4 \mu\text{M} \pm 1.82$ at 5°C ($n=3$).

Table 1-2 summarizes the purification of GST from liver and muscle of euthermic and hibernating prairie dogs. The liver enzyme was partially purified by 30-60 fold to a specific activity of 140-195 U/mg protein using the hexyl-glutathione affinity column but

due to low yields (14-22%) further purification was not undertaken. Thus, the liver preparation used for further study was a mixture of GST isozymes. The prairie dog muscle (PDM) enzyme was purified using two columns. The hexyl-glutathione affinity column gave a 56-64 fold purification with a substantial yield (37-51%). Application of the muscle enzyme to hydroxylapatite led to the separation of activity into two peaks of GST activity. These were subsequently called PDM 18 and PDM 23 based on their elution order from reverse phase HPLC, corresponding to activity peaks 2 and 1 respectively from hydroxyl appetite. GST purified from rabbit muscle resulted in a 120 fold purification using only the hexyl-glutathione affinity column. SDS-PAGE analysis of the purified enzymes is shown in Figure 1-1. The single bands seen for the muscle enzymes from both prairie dog (PDM 18, PDM 23) and rabbit indicate homogeneous enzyme preparations whereas the three bands detected for the prairie dog liver preparation suggest a mix of subunits or isozymes.

Size exclusion HPLC demonstrated that all of the enzymes eluted within a narrow range with an overall mean native molecular weight of 63 ± 3 kDa (Figure 1-2). There was a detectable difference in the weight between the two muscle subunits with PDM 18 being consistently smaller but this difference was not significant. However, there is a clear weight difference between the two muscle forms on SDS-PAGE (Figure 1-1B). SDS-PAGE reducing gels showed that the subunit sizes of all enzymes examined was between 23-25 kD (Figure 1-1A). This indicates that the enzyme was, in all cases, a dimer. The subunit size was estimated as 25.3 ± 2 kDa for PDM 23 ± 2 and 24.8 ± 2 kDa for PDM 18 (Figure 1-1B).

Table 1-1. Maximal activities of GST in tissues of euthermic prairie dogs and rabbits using either CDNB or cumine hydroperoxide as the substrate. CuOH activity measures both Se-independent and Se-dependent GPox activity of the enzyme. Frozen tissue samples were homogenized 1:10 (w/v) for muscle and heart or 1:5 (w/v) for adipose and liver, kidney and brain, then centrifuged, and enzyme activities were measured using aliquots of the crude supernatant.

Prairie dog tissue	CDNB activity mU/mg	Cumine hydroperoxide activity mU/mg
Muscle	8.59 $\pm$ 1.2	21.4 $\pm$ 4.7
Liver	21.6 $\pm$ 3.8	38.5 $\pm$ 3.4
Adipose	1.86 $\pm$ 0.9	1.8 $\pm$ 0.78
Heart	9.09 $\pm$ 2	16.4 $\pm$ 1.8

Data are milliunits/g fresh weight means $\pm$ SEM, n=3 separate pieces of tissue.

Table 1-2. Purification of glutathione-S-transferase from muscle and liver of hibernating and euthermic *C. leucurus*. Activity is measured with CDNB as the substrate. Data is from the third purification run. In the final purification step used for muscle, the two isozymes are separated and the numbers 1 and 2 represent the final purification parameters for isozyme PDM 1 and PDM 2, respectively.

Prairie dog tissue	Protein mg/ml	Total Activity μ moles/min	Specific Activity μ moles/min/mg	Fold Purification	Yield
Hibernating					
Liver crude (cytosol)	202	4859	2.396		
Hexyl-glutathione	0.497	696	140	59.9	14.3
Muscle crude (cytosol)	240	9488	3.95		
Hexyl-glutathione	0.138	3472	40	10.12	30.5
Hydroxyapatite 1	14.79	1006	21.43	5.4	10.6
Hydroxyapatite 2	12.4	2892	28	7.1	30.4
Euthermic					
Liver crude (cytosol)	325	5961	5.21		
Hexyl-glutathione	0.69	1347	154	29.5	22.6
Muscle crude (cytosol)	281	7358	2.54		
Hexyl-glutathione	0.256	3721	35.8	14	50.6
Hydroxyapatite 1	15.3	2014	22.04	8.68	27.4
Hydroxyapatite 2	13.4	1827	27.3	10.7	24.8

Figure 1-1 (A). PVDF blot of a SDS-PAGE separation of GST (15% acrylamide) purified from rabbit and prairie dog liver and muscle. Samples in lanes A, C, and D show enzyme preparations after hexyl-glutathione affinity chromatography. Samples E and F were further purified by hydroxylapatite chromatography. Lane B contains commercial rabbit liver GST after desalting using a Centricon 10 (Amicon). Lanes are: (A) Rabbit muscle, (B) Rabbit liver (commercial), (C) Prairie dog muscle, (D) Prairie dog liver, (F) Prairie dog muscle 1, (E) Prairie dog muscle 2. Staining was with Coomassie blue; lanes on both sides show Bio-Rad wide range protein standards.

(B). The scan of a SDS-PAGE gel (18% acrylamide) showing the molecular weight difference between the two GST isozymes from prairie dog muscle. The third lane contains Bio-Rad wide range protein standards. The gel was stained with Coomassie blue.

Figure 1-2. Size exclusion HPLC of native GSTs from prairie dog liver and muscle and rabbit liver. Samples were run after the final stage of purification as described in the Methods and Materials. Chromatograms were scanned and the injection marks were lined up to allow direct comparison of native molecular weights.

Further analysis of the enzymes was conducted using reverse-phase HPLC. When the partially purified muscle enzyme preparation, from the hexyl-glutathione step, was applied to the column, the elution profile showed two peaks, one at 18 and one at 23 minutes. These correlated, respectively, with peaks 2 and 1 from hydroxylapatite. When each of the final purified hydroxylapatite peaks was run on HPLC, the two enzymes, PDM 18 and 23, were shown to be homodimers as only a single peak (indicating a single subunit type) was eluted. The reverse phase chromatogram for purified PDM 23 showed one peak with a retention time of 23 min (chromatogram not shown) whereas the chromatogram of PDM 18 also contained a single peak with a retention time of 18 min (chromatogram not shown). These peaks can be seen in the reverse phase chromatograms that compare extracts from muscle of hibernating versus euthermic prairie dogs (Figure 1-3). The ratios of the two isoforms differed substantially in the euthermic versus hibernating animals. Both forms, PDM 18 and PDM 23, were observed in muscle samples from hibernating animals in a ratio of approximately 1:1. However, in euthermic muscle PDM 18, although detectable, was present only in low amounts, generally comprising about 1%, and not more than 3%, of the total GST. A comparable reverse phase HPLC analysis of liver GST (after hexyl-glutathione chromatography) is shown in Figure 1-4. The liver sample contained a mixture of 6 different GST isoforms, with elution times of 14, 19, 21, 23, 24, and 26 minutes. All peaks were run on SDS-PAGE to check molecular weight. The SDS-PAGE, reverse phase HPLC and binding to hexyl-glutathione resin all suggested that these proteins were GSTs. A comparison of the

subunit profile from hibernating versus euthermic animals did not show any change in the types of GST subunits present but did show a clear change in the proportions of different subunits. Liver GST from hibernating individuals, compared with euthermic animals, showed a major increase in the relative amount of the subunit eluting at 14 min which rose from less than 1% of total enzyme in euthermic extracts to 20% of the total in hibernating animals (Figure 1-4). The subunit eluting at 19 min also increased in amount during hibernation from 9.4% for the euthermic enzyme to 12% during hibernation. By contrast, the amounts of subunits eluting at 20 and 22 min decreased during hibernation from 27% to 18% and 30% to 24%, respectively. Notably, however, the proportions of these latter two subunits remained close to 1:1 in both states. The proportions of subunits at 24 and 26 min were unchanged by physiological state. All of the reported changes in subunit compositions were determined by relative peak areas as calculated by an integrator and standardized. Calculated values are data from several chromatograms from every purified preparation that was done ($n=7$), each initial homogenate being made from pooled tissue taken from several animals. Chromatographic data, especially numerical calculations from reverse phase HPLC, were based on chromatograms from every preparation to produce an average or typical result rather than that representing single animal or purification. In the case of enzyme kinetics, all of the data was gathered from a single large purification of the enzyme using tissue sampled from a large number of animals to reduce the variation, and provide a more complete picture of changes than could be observed with tissue from a single animal..

Figure 1-3. Representative reverse phase HPLC separation of GST subunits from prairie dog muscle after hexyl glutathione affinity chromatography. Included are the samples from muscle of hibernating (top) and euthermic (bottom) animals. Similar results were obtained for all muscle samples.

The effect of assay pH on the activity of purified GSTs is shown in Figure 1-5A for assays conducted at 25°C and in Figure 1-5B for assays at 5°C. At 25°C, the pH optima of all GST forms was within a narrow range from 6.55 to 7. Rabbit muscle and liver and PDM 18 GST had pH optima at pH 7.0, whereas PDM 23 and prairie dog liver GST showed slightly more acidic optima at pH 6.5. Rabbit muscle GST showed a broad pH versus activity curve, whereas all other enzymes had sharp optima. When assayed at 5°C, all enzymes showed a general alkaline shift in pH optimum as well as a great broadening of the optimum activity range (except for PDM 18) (Figure 1-5B). PDM 18 showed a pH optimum of 7.44 whereas activity for all other enzymes showed a broad plateau between pH 6.55 and 8.55.

K_m values for GSH and CDNB were determined at both high (25°C) and low (5°C) temperatures (Table 1-3). A change in assay temperature differentially affected the K_m GSH of liver versus muscle isoforms. Liver GST from both species showed a strong increase in K_m value (reduced substrate affinity) at 5°C, K_m GSH rising 3.6- and 7.4-fold for prairie dog and rabbit liver GST, respectively. By contrast, low temperature increased affinity for GSH of the muscle isoforms, K_m GSH decreasing significantly at 5°C to 82, 66, and 41% of the corresponding 25°C value for PDM 18, PDM 23, and rabbit muscle GST, respectively. K_m CDNB of the muscle forms also decreased significantly at 5°C, falling to 20, 18, and 45% of the corresponding values at 25°C for the three enzymes, respectively. Rabbit liver K_m CDNB also decreased at 5°C to 21% of the 25°C value whereas K_m CDNB of the prairie dog liver enzyme was oppositely affected and increased by 2.9-fold at low temperature. K_m values for both substrates of

the prairie dog muscle enzymes differed substantially from each other, PDM 23 having considerably greater affinity for both substrates. K_m values of either enzyme resembled those of rabbit muscle GST and similarly the K_m values of prairie dog and rabbit liver enzymes were also very different. This shows considerable interspecies variation in enzyme properties.

One of the distinguishing characteristics of different GST isozymes is their ability to use different substrates. Table 1-4 shows the maximal activities of prairie dog and rabbit GSTs using alternative substrates. Compared with CDNB-dependent activity prairie dog liver GST showed a very restricted substrate profile using only CDNB and cumine hydroperoxide of the six substrates tested. The other four enzymes all showed activity with nitroquinilin oxide (NQO). However, the ratios of CDNB: NQO activities were very different between enzymes, being 100:24.5 for rabbit liver, 100:14.7 for rabbit muscle, 100:392 for PDM 18 but only 100:0.5 for PDM 23. PDM 18 and rabbit liver GST also showed low activities with trans-4-phenyl-3-buten-2-one (TPBO) and rabbit liver and muscle GST shared the use of nitrophenyl acetate as a substrate. PDM 23 was the only enzyme that utilized 1,2-epoxy-3-(p-nitrophenoxy)propane as a substrate and maximal activity with this substrate was slightly higher than with CDNB. Furthermore, all GSTs were also tested with three other putative substrates (p-nitrobenzylchloride, ethacrynic acid, and 1,2 dichloro-4-nitrobenzene) but none of the enzymes showed any activity with these compounds.

Table 1-3. Substrate affinities (K_m) for CDNB and GSH of purified glutathione S-transferases from prairie dog and rabbit tissues at both 25°C and 5°C.

Substrate Tissue	Temperature (°C)	GSH K_m (μ M)	CDNB K_m (μ M)
Rabbit liver	25	29.6 ± 0.32	43.8 ± 3.2
	5	$219 \pm 9.3^*$	$9.3 \pm 0.025^*$
Rabbit muscle	25	171 ± 3.7	30.7 ± 0.24
	5	$70.4 \pm 5.1^*$	13.9 ± 0.23^a
C. leucurus liver	25	109 ± 5.14	149 ± 8.6
	5	$391 \pm 11.3^*$	$430 \pm 20.3^*$
C. leucurus muscle 1	25	104 ± 1.7	395 ± 16.3
	5	85.6 ± 4.1^a	$80.5 \pm 3.1^*$
C. leucurus muscle 2	25	30.8 ± 1.82	87 ± 2.8
	5	20.4 ± 0.98^a	$15.7 \pm 0.57^*$

Data are means $\pm$ SEM, $n = 3$ determinations on a single enzyme preparation. ^a - Significantly different from the corresponding value at 25°C via the Student's t-test, $P < 0.001$; * - $P < 0.05$.

Table 1-4. Substrate specificity of prairie dog and rabbit glutathione S-transferases assayed at 25°C. Enzymes were assayed with constant enzyme concentration for comparative purposes.

Tissue Substrate	Rabbit Liver Vmax (mU)	Rabbit muscle Vmax (mU)	PDM 1 Vmax (mU)	PDM 2 Vmax (mU)	Prairie dog liver Vmax (mU)
Nitrophenyl acetate	1.14 ± 0.012 ^a	0.236 ± 0.0012 ^a	NA	NA	NA
1-Chloro 3,4-dinitrobenzene	23.6 ± 0.18*	11.2 ± 0.65*	6.89 ± 0.2*	2.37 ± 0.047*	9.58 ± 0.36*
Cumline hydroperoxide	NA	NA	NA	NA	2.8 ± 0.05
Nitroquiniln oxide	5.78 ± 0.13*	1.65 ± 0.021*	27 ± 2*	0.0117 ± 0.0026*	NA
trans-4-Phenyl-3-buten-2-one	0.026 ± 0.0001 ^a	NA	0.063 ± 0.0008 ^a	NA	NA
1,2-Epoxy-3-(p-nitrophenoxy)propane	NA	NA	NA	2.69 ± 0.2	NA

NA indicates no measurable activity. Data are means ± SEM, n=3. Significantly different Vmax values for a substrate were determined by the Student's t-test. (a) indicates a difference between enzymes for the same substrate P < 0.001, (*), differences are significant at P < 0.05.

GST classes can be distinguished by their susceptibility to inhibition by different compounds (Tahir et al., 1985) and Table 1-5 shows the effects of three commonly used inhibitors on the prairie dog and rabbit muscle enzymes. I_{50} values for *C. leucurus* liver GST, assayed at 25°C, showed that Rose Bengal was the most effective inhibitor followed by Cibacron blue and hexylglutathione. Furthermore, this was the only GST tested that showed its greatest inhibition with Rose Bengal. PDM 18 and PDM 23 were most strongly inhibited at 25°C by Cibacron blue, followed by Rose Bengal, and showed considerably lower sensitivity to hexyl-glutathione. The I_{50} values for PDM 18 were all significantly different from each other ($P < 0.005$); the same was true for PDM 23 between temperatures. I_{50} values for each inhibitor of PDM 18 and 23 were also significantly different from each other ($P < 0.001$). Rabbit liver GST was inhibited strongly by Rose Bengal and Cibacron blue at 25°C (I_{50} values not significantly different) and less effectively by hexyl glutathione (I_{50} over 100-fold higher than for the other compounds, $P < 0.001$) (Table 1-5). Rabbit muscle GST was extremely sensitive to Cibacron blue at 25°C, showed a higher I_{50} value for Rose Bengal and again a very high I_{50} for hexyl-glutathione. At 5°C inhibitor effects changed substantially for all enzymes (Table 1-5). The I_{50} of all inhibitors changed significantly with temperature, as did the relative effectiveness of the three on each enzyme, but their rank order (strongest to weakest inhibitor) did not change. Except for *C. leucurus* liver GST, the rank order of inhibitors still followed the pattern Cibacron Blue > Rose Bengal > Hexylglutathione.

Effects of temperature on the prairie dog versus rabbit enzymes were further assessed using Arrhenius plots ($\log V$ vs. $1/^\circ K$) constructed from assays of V_{max} enzyme

activity with CDNB as the substrate at 15 temperatures over the range from 0 to 34°C. In all cases, Arrhenius plots yielded straight lines with no breaks over the full range of temperatures tested (plots not shown). Calculated activation energies (E_a) for the enzymes were determined from separate evaluations of E_a from each replicate (Table 1-6). All of the GSTs tested had similar E_a values although the activation energies for the rabbit muscle and the prairie dog liver (PDL) sample were significantly different. This difference is not of great importance since the PDL sample is a mixture of isozymes and the rabbit muscle enzyme is a single pure isozyme. The calculated activation energies are consistent with a Q_{10} value close to two, which is typical for most enzymes.

The two forms of GST purified from prairie dog muscle were both homogeneous as determined by SDS-PAGE and reverse-phase HPLC. Both of the GST isozymes were homodimers since only one GST subunit was observed for each preparation using reverse phase HPLC. These homogeneous homodimers were therefore suitable for sequence analysis. The N-terminal sequence of PDM 18 was PPYTVYFPVRGR (which shows homology to the π class of GSTs). The closest matches from the Swiss Protein database were to rat π class subunit and Yf mouse (both showed homology over the first 12 amino acids). PDM 23 GST was found to be N-terminally blocked and hence no sequence could be obtained. Amino acid composition was also determined for both prairie dog muscle enzymes, rabbit liver, rabbit muscle, and 4 of the major subunit types of liver GST isolated from reverse phase HPLC (Table 1-7).

Table 1-5. Inhibition constants (I_{50} values) for inhibitors of glutathione S-transferase from prairie dog and rabbit liver and muscle at two assay temperatures, 5 and 25°C. Assays were conducted using suboptimal GSH (0.3 mM) but otherwise the same conditions as in the standard assay with CDNB as the substrate.

Inhibitor Tissue	Temperature (°C)	Rose bengal I_{50} (μM)	Cibricron blue I_{50} (μM)	Hexyl Glutathione I_{50} (μM)
Rabbit liver	25	$1.47 \pm 0.46^*$	$1.14 \pm 0.15^*$	$165 \pm 18^*$
	5	$3.6 \pm 0.12^*$	$0.332 \pm 0.02^*$	$322 \pm 12.5^*$
Rabbit muscle	25	$4.6 \pm 0.125^*$	< 0.16	$307 \pm 10^*$
	5	$1.97 \pm 0.25^*$	1.59 ± 0.14	$273 \pm 6.7^*$
C. leucurus liver	25	$3.23 \pm 0.37^*$	$20.9 \pm 1.06^*$	$306 \pm 14^*$
	5	$1.79 \pm 0.26^*$	$3.01 \pm 0.75^*$	$150 \pm 0.28^*$
C. leucurus muscle 1	25	$0.462 \pm 0.01^*$	0.122 ± 0.004	4.57 ± 0.96^a
	5	$1.35 \pm 0.012^*$	< 0.16	5.87 ± 0.45^a
C. leucurus muscle 2	25	0.98 ± 0.07^a	$0.798.046$	$18.1 \pm 0.33^*$
	5	1.35 ± 0.02^a	< 0.16	$338 \pm 8.2^*$

The differences between the I_{50} values for these compounds were significant at the (a) $P < 0.005$ level between different enzymes, (*) $P > 0.05$ significance is for the differences between temperatures 25 and 5°C for the same enzyme and inhibitor.

Table 1-6. Activation energies for glutathione S-transferase from prairie dog and rabbit tissues. Data are calculated from Arrhenius plots (log V max versus $1/^\circ\text{K}$) of enzyme maximal activities measured at 15 temperatures between 0 and 34°C .

Tissue	Ea (kcal/mole)
Rabbit liver	10.1 ± 0.049
Rabbit muscle	8.36 ± 0.08
C. leucurus liver	13.8 ± 0.16
C. leucurus muscle ¹⁸	11.6 ± 0.049
C. leucurus muscle ²³	9.83 ± 0.025

Values are in kilocalories per mole, means $\pm$ SEM, $n = 3$. Activation energies and Ea values were calculated with the aid of computer software (Brooks, 1992).

Table 1-7. Amino acid analysis of glutathione-S-transferase subunits purified from *C. leucurus* muscle (PDM) and liver (PDL), and rabbit muscle (RM), as well as the commercial enzyme from rabbit liver (RL). Subunits of prairie dog enzymes were separated by reverse phase HPLC followed by SDS-PAGE (blotted to PVDF) for hydrolysis. Values are percentages of the total amino acids. NA indicates that the amino acid was not observed on the chromatogram; values in parentheses indicate unreliable numbers due to decomposition of the amino acid during hydrolysis. Data are n = 1.

Amino Acid	PDM 18	PDM 23	PDL 20	PDL 21	PDL 24	PDL 26	RL	RM
Asx	9.4	9.0	10.6	9.8	10.7	5.9	10.8	11.5
Thr	5.3	3.9	4.5	4.1	3.4	2.4	4.2	4.8
Ser	12.9	11.2	3.9	3.8	4.1	3.4	3.4	5.9
Glx	12.9	13.1	13.2	13.9	13.0	25.8	14.1	12.0
Gly	30.3	20.2	6.0	6.3	5.4	15.8	6.3	7.2
Ala	9.3	7.9	4.8	5.7	6.7	4.4	6.4	5.3
Cys	NA	NA	[0.67]	[1.4]	[0.002]	[6.7]	[0.4]	[0.4]
Val	0.4	0.3	3.5	4.0	5.3	4.1	5.4	4.2
Met	0.0	0.5	1.2	1.1	1.7	0.8	2.8	1.9
Ile	4.1	4.6	4.7	4.8	5.2	3.4	5.4	5.4
Leu	6.2	7.6	12.4	11.9	14.2	8.6	12.8	13.8
Tyr	NA	NA	2.9	2.3	1.7	0.8	NA	0.2
Phe	NA	3.7	5.8	5.6	4.1	0.3	5.4	6.4
His	2.7	2.5	2.0	2.0	1.6	1.1	1.6	1.5
Lys	3.5	9.0	10.6	11.3	12.3	7.1	9.6	7.2
Arg	NA	4.8	7.0	6.5	5.0	3.7	4.9	5.8
Pro	3.1	5.8	6.0	5.5	5.2	3.2	6.4	6.3

Figure 1-4. Representative reverse phase HPLC separation of GST subunits from prairie dog liver after hexyl glutathione affinity chromatography. Included are the samples from muscle of hibernating (bottom) and euthermic (top) animals. Similar results were obtained for all liver samples; this 7 peak pattern held up in multiple trials as did the ratio of peak heights in hibernating vs euthermic similar on multiple trials.

Figure 1-5. The pH dependence of glutathione S-transferase activity from rabbit and prairie dog muscle and liver at 25°C (A) and 5°C (B). Activity is expressed relative to the maximum activity at the pH optimum, which was set to 100. Errors are given as a percentage of the value and the error in the measurement of cuvette pH values was ± 0.03 pH units. Data are means $\pm$ SEM n=3 determinations of separate preparations of enzyme.

A **B**

The composition of the two prairie dog muscle enzymes differed distinctly, particularly in their contents of glycine, lysine, phenylalanine and arginine. However, both differed distinctly from the rabbit muscle enzyme, which showed substantially different amounts of serine, glycine, valine, methionine, leucine, and phenylalanine than the prairie dog enzymes. Although no method could be found that adequately separated the mixture of liver GST isozymes to produce sufficient active enzyme for kinetic analysis of the isoforms, the multiple subunits of liver GST isozymes could be separated on denaturing reverse phase HPLC. This allowed the preparation of sufficient amounts of the different subunit types for characterization by N-terminal sequencing and amino acid composition. Amino acid compositions of four subunits (named based on the retention time on reverse phase HPLC) are shown in Table 1-7. PDL 20, PDL 21, and PDL 24 were all very similar to one another with minor variations in amino acid composition. However, PDL 26 was distinctly different, varying considerably from the other three in the amounts of 11 amino acids with particularly major differences in the content of glutamate/glutamine, glycine, tyrosine and phenylalanine.

The four major prairie dog liver subunits from euthermic animals (as seen in Figure 1-4) were also subjected to N-terminal sequence analysis (GST subunits that eluted with the same retention times in samples from hibernating animals were assumed to have the same sequences). The N-terminal sequences were (_ indicates that amino acid identification was not possible in the cycle):

liver 21 (PDL 21)	_MRLGYWDIRGLTNPIRLLLEYT (C-terminal)
liver 22 (PDL 22)	_MRLGYWDIRGLT

liver 24 (PDL 24) _MRLGYWDIR

liver 26 (PDL 26) VLEYLDLLS

PDL 21, 22 and 24 clearly have homologous N-termini whereas PDL 26 is very different; this correlates with the differences noted above in the amino acid composition of PDL 26 compared with the others. However, despite N-terminal homology, both the reverse phase elution profile and the amino acid composition indicate that there are important, if small, differences between these subunits that confirm that they are distinct subunits but with a common conserved N-terminal sequence. By verifying the size by SDS-PAGE, it was determined that none of the sequences were from protein fragments and the sample had not been degraded. Based on their N-terminal sequences and comparisons with the sequences in the Swiss Protein database, the liver subunits 21, 22, and 24 were identified as belonging to the GST class μ whereas subunit 26 was a class θ enzyme.

N-terminal sequences and amino acid composition were also obtained for the rabbit muscle and liver GST subunits (liver being the commercial preparation). These preparations were confirmed to be homogeneous by reverse phase HPLC and SDS-PAGE prior to sequencing and in both cases only one subunit was present in each preparation.

The N-terminal sequences were:

rabbit muscle PMTLGYWDIRGLA (C-terminal end)

rabbit liver PVTLGY_DVRGLAL

Based on these sequences, both would be GST class μ subunits. Both of these enzymes are μ class by N-terminal sequence homology but are clearly not the same

subunit type since they have different retention times on reverse-phase HPLC and vary both in N-terminal sequences and amino acid composition.

Discussion

The changes to GSTs during hibernation are reflected in the changes to the subunit profile seen in Figures 1-3 and 4. The muscle tissue from *C. leucurus* showed a significant increase in the amount of the PDM 18 isozyme in the hibernating animals. This form of GST can be observed in the euthermic muscle sample but the amount was very small, even though these animals were at the same point in their yearly cycle with the same feeding and lighting conditions prior to separation of the two groups. The hibernating animals were then allowed to hibernate and the euthermic animals were sacrificed. The production of the subunit at 18.8 minutes (Figure 1-3) seems to occur in response to hibernation and is not the result of diet or exposure to environmental factors. The same conditions also apply to the GST subunits from liver. The changes in the liver subunit profile are more complicated because of the larger number of subunits isolated from this tissue and its metabolic complexity. Hibernation changes in the liver profile affect every subunit that appears on the chromatogram. The most noticeable change was the large increase in the subunit at 14 minutes in the hibernating sample (Figure 1-4). There was also a redistribution of the proportions of the other subunits in the liver sample. The relative amounts of the subunits at 20 and 21 minutes 1:1 does not seem to change with hibernation; but their total amount relative to the other subunits did

decrease. The samples run on reverse phase HPLC were subjected to the same purification (as stated in the Methods and Materials) and thus, the subunits examined all have affinity for hexyl glutathione and are eluted from this resin. Therefore, there should be no effect from the purification on the relative amounts of subunits found in either tissue.

The analysis of the subunits from the prairie dog tissues by amino acid composition and N-terminal sequencing allowed the classification of most of the subunits. The liver subunits were separated by reverse phase HPLC to homogeneous proteins, which were sequenced. The N-termini were then compared to the Swiss protein database using Blast to determine the closest homology matches. The liver subunits at 21, 22 and 24 minutes (Figure 1-4) were all identified as GST type μ with the closest matches being to Yb1 mouse and Y1 Chinese hamster with 95% homology. The subunit at 26 minutes was GST type θ and showed 100% homology to rat Yvs-Yvs, GT1 chicken, and θ human. N-terminal sequencing of the μ GSTs all showed the same N-terminal sequence with the same blank first cycle _MRLGYWDIRGLTNPIRLLLEYT (N-terminal of subunit at 21 min). However, the amino acid composition data showed that each of the μ subunits differed in some details (Table 1-7).

The two classes of GST subunits found in prairie dog liver, θ and μ , result from two distinct gene products. GST class θ enzymes are primarily responsible for the metabolism of halogenated hydrocarbons (e.g. chloro- and dichloro-methane). This class of GST is highly conserved in phylogeny with the enzyme in both plants and mammals showing up to 80% sequence homology. As with all other θ class GST enzymes, PDL 26

contained the highly conserved serine 9 which is believed to be vital in the catalytic activation of GSH at the active site (Board et al., 1995, Tan et al., 1996). GST class θ does not share great sequence and conformational homology with the other GST classes μ , π , α which all have much more in common in terms of sequence and conformational traits (Board et al., 1995).

Prairie dog muscle contained two GST isozymes. The N-terminal sequence of PDM 18 showed it to be homologous with π class GSTs from mouse (Yf), Chinese hamster, and rat (GST 7-7) with 93% homology over 15 amino acids. As expected for π -class GSTs there was a tyrosine at position 7 from the N-terminal and another Tyr 3 which would be close to the active site (Ji et al., 1996). Identification of PDL 14 could not be confirmed by N-terminal sequencing because the protein was blocked and no sequence was obtained. Substrate and I_{50} values were also of no use in identifying this GST since the holoenzyme was not purified; only the purified subunit was available from reverse phase HPLC. Kinetic identification is only meaningful for GSTs in the purified state since there are so many forms of the enzyme with overlapping activities. Indeed, a π class enzyme was purified from the muscle by hexyl-glutathione (Bogaards et al., 1992).

The identification of PDM 23 poses a slightly more complex problem. The retention time on reverse phase suggests a μ subunit. This enzyme species typically elutes between π and θ subunits and mixed with α subunits (Bogaards et al., 1992). The retention time of 23 min is in the range of the μ class subunits seen in the liver (Figure 1-6 and 7). Furthermore, the substrate profile for PDM 23 showed activity toward TPBO

which is a substrate that is often associated with μ class enzymes (Table 1-4) (Mannervik and Danielson, 1988). The inhibitor profiles are of less help since α and μ class GSTs share various inhibitor characteristics. Overall, the results suggest that the PDM 23 is probably class μ .

Identification of PDL 14 can only be implied based on its retention time on reverse phase; this enzyme had the shortest retention time and since this is a characteristic of class π GST it is probable that PDL 14 belongs to this class. The μ class GSTs from the liver retained a strong sequence homology with rat and human μ class enzymes. As expected these GSTs contain a tyrosine close to the N-terminus which is believed to activate GSH in a similar manner to Ser-9 in class θ (Ji et al., 1996). Activation of GSH is thought to occur through the free hydroxyl in each of these amino acids (Board et al., 1995). The active tyrosine is at position 6 in all three μ class GSTs that were sequenced: PDL 21, 22, and 24. The N-terminal sequence from all three of these GSTs is identical showing a high degree of conserved sequence homology within the μ class GST from prairie dog liver.

Kinetic examination of the GSTs from the prairie dog demonstrated that there were no remarkable kinetic differences between the enzymes from any of the animals tested. That is to say that the kinetic parameters did not fall outside the normal range that is observed in GSTs from nonhibernating mammals like the rat or human. There were also no changes in GST maximal activity in the tissues from hibernating versus euthermic animals. Increases in the activities of antioxidant enzymes have been observed in intrascapular brown adipose (IBAT) during hibernation in other species (Buzadzic et al.,

1990). However, changes in GST maximal activities in liver and muscle of prairie dogs are clearly not necessary during hibernation although whether this is a tissue-specific or species-specific phenomenon cannot be determined from the present data. There is, however, a clear change in subunit composition of the enzyme in the hibernating animal. This change occurred in both muscle and liver with the appearance of a new subunit in each case, which increased in hibernation (Figure 1-3 & 4). In the case of euthermic animals the isozyme PDM 18 could be found in low levels in the awake animal, but became a significant component of the muscle GST during hibernation. There were also variations in the subunit composition of prairie dog liver GST during hibernation which indicates that there are hibernation specific changes that occur in the isozyme composition of GSTs but that these do not alter the overall maximal activity of the enzyme in either tissue.

Liver GST from hibernating and euthermic prairie dogs showed differences in subunit composition, but the overall kinetic parameters of the liver GST fraction did not show changes resulting from hibernation. The substrate profile was the same for both liver samples, euthermic and hibernating (data not shown). Furthermore, the K_m values for CDNB and GSH did not change significantly in this fraction even though the reverse phase HPLC data demonstrated substantial changes in the subunit composition of liver GSTs during hibernation, including the dramatic increase in PDL 14. The treatment of the *C. leucurus* liver enzyme as a single form of GST is not far from accurate. The composition of the hexyl-GSH purified euthermic sample demonstrated that 90% of the liver GST is class μ with a 10% θ contamination.

Commercial GST from rabbit liver was used as a control; this preparation contained one isozyme, a homodimer. In the case of *C. leucurus* muscle and rabbit liver and muscle GST single isozymes were also studied. This rabbit liver GST shows significant similarities to other rabbit liver μ -class GST (Primiano and Novak, 1993). The substrate specificity and I_{50} data give information on the subunit composition (Tahir et al., 1985). Furthermore, kinetics at high (25°C) and low (5°C) temperatures were also done to characterize GST. The reductions observed in the K_m for GSH and CDNB in the prairie dog tissues at 5°C indicate that there is an increase in the affinity of the enzyme for substrate with decreasing temperature. The increased substrate affinity suggests that less of the substrate may be present in the cell during hibernation or, alternatively, the change in the muscle K_m may allow a reduction to be made in cellular GST content during hibernation. The reactions of CDNB and the other electrophilic substrates are very energetically favorable and can proceed spontaneously at room temperature. At low temperature, the rate of the reaction with CDNB is close to the limits of detection. At 5°C GST proved to be an effective catalyst for the reaction of CDNB and GSH *in vitro*. At low temperature, the catalytic action of GST is vital for the electrophilic reaction to occur *in vitro*. At temperatures above 37°C the spontaneous reaction rate makes the measurement of the enzyme-catalyzed reaction indistinguishable from the background reaction rate. The increase in the K_m values in the liver with temperature decrease could indicate a reduction in the need for GST activity or an intentional reduction in xenobiotic metabolism. The kinetic data was comparable to the literature values for the subunits

identified (Buzadzic et al., 1990; Pickett and Lu, 1989; Ketterer et al., 1988); thus hibernation did not seem to result in the production of a highly unusual form of GST.

GST kinetic properties were significantly affected by assay temperature; K_m values for GSH and CDNB were differentially affected by low temperature (Table 1-3). PDM and rabbit muscle GST showed a decrease in K_m for GSH with decreasing temperature whereas the PDL and RL isozymes showed a K_m increase. The PDL GSTs were the only isozymes that increased their K_m value for CDNB at low temperature. Temperature also had a significant effect on the I_{50} values for inhibitors (Table 1-4) although the changes in I_{50} values do not show a relationship to the changes in K_m values for GSH or CDNB. There was a substantial increase in the I_{50} for hexyl-glutathione for RL and PDM 23 which mimics a substrate-product complex (Dirr et al., 1994) with modest changes in the other I_{50} values.

In summary, hibernation causes predictable and significant changes to the GST profile in *C. leucurus* muscle and liver. There was a dramatic change in the GST subunit profiles of the liver and muscle tissue of *C. leucurus*, during hibernation. There is the production of a new subunit in the muscle, PDM 18, and the dramatic increase in the amount of the RL 14 subunit. These changes to the subunit profiles do not appear to affect the total GST activity in the tissue, since there was no change in total amount of GST activity in the muscle and liver of *C. leucurus* during hibernation. Purified muscle GST isozymes were kinetically characterized and compared to GSTs from the liver and muscle of a rabbit, this showed that the GSTs from muscle were functionally similar to other GSTs. Furthermore, the kinetic properties of PDM 18 fall within the expected

range for π -class GSTs. N-terminal sequencing and amino acid compositions provided identifications for three μ -class and one θ -class GSTs in the liver of the prairie dog and a π -class enzyme in the muscle. The muscle π -class GST is produced in tissue from hibernating animals (PDM 18), the closest homology matches are to Yf (mouse) and GST 7-7 (rat). The liver tissue also displays an increase in a subunit during hibernation; unfortunately, identification of the class or kinetic properties of this isozyme could not be determined. Hibernation appears to be a stress that causes changes in the GST profile of muscle and liver tissues of the PD. Hibernation affects every tissue in the animal somehow, often with profound changes in many systems. This puts stress on every tissue and it appears that the glutathione S-transferase enzymes respond to the stress of hibernation. The hibernation specific GST changes may be made to cope with metabolic byproducts, xenobiotics, lipid peroxidation or any number of other functions preformed by GST.

CHAPTER 2:

Glutathione S-Transferase Structural Studies

Introduction

Spectroscopic studies on proteins have been used extensively to give insight into protein structure. The use of infra-red, fluorescence, UV/visible and nuclear magnetic resonance spectroscopy and circular dichromism have given valuable insights into the behavior and conformation of proteins in solution (Creighton, 1989). Enhancements in technology for both detection and data manipulation have allowed investigators to see small changes in proteins in solution and even *in vivo*. Complicated protein spectra can now be simplified and enhanced to the point where even a single amino acid in a protein can be observed and characterized (Creighton, 1989; Goormaghtigh et al., 1994). Spectroscopy gives the opportunity to examine protein structure in a variable environment with the option to examine functional parameters under identical conditions. Unlike x-ray crystallography which requires proteins in a crystal state which is unnatural and rigid, the use of spectroscopy allows for the examination of proteins in solution where their conformation and flexibility can be examined in a more realistic environment and a functional conformation. Spectroscopic measurements can be used to examine and characterize changes in protein conformation resulting from interactions with other molecules as well as physical and/or environmental changes. Spectroscopic determinations of protein structure must be based on some arbitrary starting point with a well-defined set of conditions. In the majority of cases protein spectra are affected by any factor that can perturb molecular conformation (ionic strength, temperature, pressure, solvent, and 1° structure).

Protein fluorescence has been used for the measurement of conformational changes in a protein. The sensitivity of fluorometry and the strength of tyrosine emissions has made this a useful tool for the examination of protein-DNA, protein-protein and protein-small molecule interactions. Furthermore, fluorescence has been used to study the effects of numerous physical/environmental conditions on protein conformation including temperature, pressure, and solvents. If the protein can be monitored by fluorescence, then there are events where specific spectral changes can be linked to specific physical changes in the protein like subunit separation, denaturation, and ligand-binding. The emission profile of a protein is affected by the exposure of the fluorophore to an aqueous environment.

There are three amino acids that fluoresce in solution: Phe, Trp, and Tyr. These amino acids absorb light in the near UV range (257, 278, and 274 nm, respectively) and emit light in an aqueous solution in the visible range at 280, 305, and 350 nm, respectively. The emission sensitivity of Phe (8 is the fluorescence sensitivity factor) and Tyr (200) are weak and thus Trp (1100) fluorescence normally dominates the protein spectrum. Furthermore, Tyr undergoes a non-radiative energy transfer to Trp and thus, Tyr emissions are totally masked if there is Trp in the protein. This effect does however, increase the emission intensity for the Trp spectra. Typically protein λ_{max} values range from 310 to 340 nm indicating that the tryptophans are in hydrophobic environment for native proteins. Trp fluorescence is nearer the blue end of the spectrum in the compact native state (Creighton, 1989). Trp fluorescence changes on exposure to water; the more aqueous the environment the larger the red shift of the maximum emission wavelength

(λ_{max}) and the lower the emission intensity (I). In a hydrophobic environment, the λ_{max} for Trp in a protein can shift as low as 300 nm. The concentration of the three fluorescent amino acids in a protein is statistically 3/20 and for the dominant emitting species (W) the concentration drops to 1/20. With a small number of fluorophores in a protein, the contribution of each Trp makes a significant contribution to the overall emission spectrum. As the protein conformation changes, the local environment of the structural elements in the protein can change, thereby potentially affecting each Trp. As the various Trp residues are exposed to or shielded from water in a protein there is a shift in the λ_{max} and/or I.

Temperature also has a significant influence on the fluorescence spectra of a protein. As temperature increases there is usually a decrease in the I of emission at the λ_{max} . The reduction in I generally occurs at a rate of 1% per 1°C for proteins. Temperature change also causes structural changes in the protein, which affect both λ_{max} and intensity. Low temperature (non-freezing) stabilizes the conformation of a protein and strengthens hydrophobic interactions. With an increase in temperature, there is a weakening of hydrophobic interactions and more energy is made available to the system, promoting denaturation. At higher temperatures, there is an elimination of protein structure (denaturation) and proteins start to aggregate and precipitate from solution resulting in large changes to the protein structure.

Denaturation of proteins, by chemical or physical means, tends to occur in stages. It has also been observed that protein structure denatures in the opposite manner to its formation, which is to say that 4° structure is the first to go, followed by 3° and 2°

structural elements. The general effectiveness of several denaturants have been examined and are generally rated from strongest to weakest as: guanidine hydrochloride > urea > sodium dodecyl sulfate > high temperature > pH extremes > high hydrostatic pressure (Creighton, 1989). The loss of major structural elements, subunit separation or 3° folding produces significant changes to the Trp emission spectra. This type of structural change in a protein is easily characterized by changes in the fluorescence spectra. These types of events cause large changes in the protein's internal environment resulting in sharp changes to the λ_{max} and/or I over a narrow range of conditions.

Fourier transform infra-red (FTIR) spectroscopy, as its name implies, uses infra-red light to look at the bond energies and motions of dissimilar atoms. Fourier transform additions of numerous spectra to intensify weak signals and reduce noise has made the use of IR spectroscopy a good tool for the examination of protein structure. In proteins there are a variety of functional groups which can produce strong IR absorbances including carbonyl, amides, amines, hydroxyl, and carbon-hydrogen bonds, all of which are IR active. Proteins, like other organic molecules with functionalities, have complex characteristic IR spectra. Unlike small organics where the small molecular weights (100-1000 Da) allow for high concentrations, proteins are large (14-300 kDa) and thus the concentrations of both proteins and their functionalities, which can be observed, are low. The only functionality that is concentrated enough and relatively free of interference from other compounds for routine FT-IR is the amide linkage. From examination of the amide bond, information on the secondary structure (α -helices, β -sheets and random coil) can be identified and characterized. The regularity and strength of the protein secondary

structure locks a number of amino acids in a rigid conformation with identical bond angles and very similar bond energies. Producing the three main divisions of the amide peak from proteins are α -helices, β -sheets and random coils, which can be distinguished by FT-IR spectroscopy. The bond energy variations in the helices and sheets are caused by amino acid functional group interactions. Most of the information from the IR spectra of a protein is derived from the absorbances caused by the helices and sheets because they are identifiable and can be related to X-ray structures and molecular calculations to determine the corresponding structural domain for each peak. A reduction of these structures and an increase in random coil is a good indicator of terminal (elimination of 2° structure) protein denaturation. The intensity and characteristics of the IR absorption spectra can also monitor the overall conformation of the protein.

The bond energies of the amide I region of the IR spectrum (approximately 1620-1680 cm^{-1}) show characteristic absorption that are attributable to structural elements. The contribution of each amide bond may be as small as 1/10000 of the total absorbance in the amide I region. When several of these bonds are located in specific structures like helices and sheets, they affect the shape and location of the amide I region of a specific protein. This occurs due to the consistency of bond angles and energies in a rigid structure like a sheet or helix. It is generally observed that α -helix structures appear as peaks closest to 1620 cm^{-1} , the next structures are the β -sheets which appear nearer 1640 cm^{-1} and the random coil occupies the region closest to 1680 cm^{-1} . As a protein denatures there is a destruction of the sheets and helices to form random coils. The FTIR spectra at different points during the denaturation will show some of the order of unfolding, and the

relative stability of the 2° structural elements. Investigators have also been able to examine changes in protein conformation caused by the binding of a substrate, the effects of temperature, and photochemical events (Mantele, 1993; Surewicz et al, 1992; Zuber et al, 1992).

X-ray crystallography studies on α , μ , and π -class glutathione transferase (GST) from a variety of animals show that the mammalian enzyme is comprised of two domains (I and II). The total 2° structural content consists of about 48-59 % α -helix and 8-10% β -strand structure. The domains are linked by an oligopeptide of 6 amino acids (Dirr et al., 1984). Sequence homology between GSTs can range from 97% within a class to 20% between classes with some highly conserved sequences, most notably at or near the active site. The crystal structures of all cytosolic GSTs examined to date show similar spatial characteristics (Dirr et al., 1984).

Figure 2-1. Representative drawing of the observed structure of GST from X-ray crystal data.

The N and C-terminal amino acids of the enzyme lie on the indicated axis.

Domain I is the smaller of the two and contains the N-terminal region of the protein.

Domain II contains the C-terminal and forms the boundary of the central spiral. The central spiral is the black circle, which is also the active site.

The subunit interface is class-specific for all GSTs, that is to say that a μ subunit will only dimerize with another μ class subunit (Dirr et al., 1984). Furthermore, there are also class-specific structural elements (Dirr et al., 1984), which allow classification of GSTs by structure but do not alter the overall conformation of the protein. The N-terminal domain (I) is smaller than the C-terminal domain (II) and is comprised of an α/β type core with a 4-stranded β -sheet flanked on one side by two α -helices (Dirr et al., 1984). This gives a topographical arrangement of $\beta\alpha\beta\alpha\beta\alpha$; GST class does not affect this arrangement. Domain II is predominantly an α -type structure containing 5 α -helices forming a right handed spiral (Dirr et al., 1984). It is also known that 14% of the water accessible subunit surface becomes buried at the subunit interface with the formation of the dimer.

Fluorescence spectroscopy can provide information on the relative stability of proteins and conformational changes in the protein. FT-IR gives information on the type and number of 2° structural elements in a protein and shows any changes in the amide bond energy from the β -sheets, α -helices, and random coils they contain under similar experimental conditions (Zuber et al. 1992). The information on the state of the 2° structure can then be used to fine tune and expand the fluorescence data. The purpose of the present study is to determine whether the hibernation specific GST from *C. leucurus*

(prairie dog) differs structurally or in stability from the enzyme in a nonhibernating species (rabbit). A spectral comparison of the hibernation specific GST PDM 18 with a rabbit liver GST was made to examine the effects of temperature on these two proteins.

Methods and Materials

Rabbit liver glutathione-S-transferase, reduced glutathione (GSH), hexyl-glutathione, and hexyl-glutathione agarose were purchased from Sigma Chemical Company (St. Louis, Mo.). High purity guanidine-HCl was purchased from Boehringer Mannheim (Montreal, PQ).

Details of collection and experimentation with prairie dogs as well as the purification of GST from muscle tissue and the assay of enzyme activity were described in Chapter 1. Both the prairie dog and the rabbit GST were subjected to a single run on the SEC HPLC to remove any stabilizers in the commercial (bottled) rabbit liver enzyme and to ensure that both enzymes were in the same buffer and at the same pH prior to the fluorescence study.

HPLC separations were performed on a Beckman HPLC system with a UV/VIS detector at either 225 or 280 nm. Reverse-phase separations were done on a Zorbax RP-300 C-8 (2.1 x 150 mm) column with a flow rate of 0.2 ml/min. Samples were loaded at 35% acetonitrile and 65% water containing 0.12% trifluoroacetic acid (TFA) and a linear gradient was run to 65% acetonitrile and 35% water with 0.12% TFA over 25 min. An Altex TSK 2000 (4.5 x 300 mm) linked to a TSK 3000 (4.5 x 300 mm) column was used

for native molecular weight determination. This column was run isocratically in 50 mM potassium phosphate pH 7.0 containing 50 mM KCl at a flow rate of 0.715 ml/min. The SEC columns were calibrated using blue dextran (2000 kDa), thyroglobulin (670 kDa), ferritin (443 kDa), phosphofructokinase (360 kDa), pyruvate kinase (230 kDa), catalase (232 kDa), aldolase 160 kDa), glutathione reductase (124 kDa), hexokinase (102 kDa), adenylate kinase (68 kDa), and triosephosphate isomerase (53 kDa) as molecular weight standards.

Gel Electrophoresis: SDS-PAGE was conducted according to Laemmli (1970) using a Bio-Rad mini gel system (Protean 2). Samples were desalted and concentrated using a Centricon 10 concentrator (Amicon). Samples were then mixed (1:1 v/v) with reducing buffer containing 50 mM dithiothreitol (DTT), 20% v/v glycerol, 1% w/v SDS, 50 mM Tris pH 8.8 and 0.04% w/v bromophenol blue. After boiling for 5 min, samples were loaded and run on 15% acrylamide gels with broad range molecular weight standards from Bio-Rad containing: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). Blotting to PVDF (Imobilon-N, Millipore) was done in an Owl semi-dry blotter at 500 mA for 45 min with a buffer system consisting of 25 mM Tris, 192 mM glycine and 20% v/v methanol. Gels and blots were stained with Coomassie blue G-205 0.1 % w/v in a 50% methanol-water tincture containing 5% acetic acid. The destaining solution was 50% methanol water with 5% acetic acid. Gels and blots were then washed in ddi-water.

Protein Sequencing: Protein sequencing was done on either a Beckman 6000 Prosequencer or an Applied Biosystems 470 protein sequencer with an online 163 PTH analyzer. Samples were sequenced from PVDF blots and aqueous samples. Samples were loaded according to manufacturer's specifications. Sequence homology was assessed using the NIH Blast program (National Institutes of Health on line sequence analysis program) and comparisons were made to the Swiss Protein database.

Fluorescence: Samples of purified GST (10 μ g for PDM 18 and 5 μ g of the RL isozyme) were placed in a quartz cuvette containing 20 mM phosphate buffer pH 7.5, and 1 mM EDTA; total volume was 3 ml. At the start of each session, the fluorometer was calibrated for emission response and wavelength according to the manufacturer's specifications. A temperature-controlled cell holder was used and the sample was continuously stirred between fluorescence readings. Between spectra the lamp was turned off to prevent damage to the protein from constant UV light. All spectra were recorded three times (with the same sample) and an overlay of the spectra was used to visually verify the consistency and accuracy of each spectrum. One of the three spectra was randomly selected (using a random number generator) for experimental use. This procedure was used for all samples under all conditions. For temperature studies, temperature was increased slowly (0.5°C/ min) and samples were equilibrated for 15 min at each temperature prior to recording spectra. For denaturation studies, aliquots of guanidine hydrochloride (GuHCl) were added to the enzyme in the cuvette and the mixture was stirred for 30 min before recording spectra. Fluorescence emission was recorded using a Perkin Elmer LS50B fluorometer, scanning between 300 and 400 nm

with a slit width of 2.5 nm and a scan rate of 50 nm min⁻¹; excitation was at 280 nm. Background spectra were corrected for buffer and water emissions and baseline noise before each determination of relative intensity (I) and emission maximum (λ_{max}).

FT-IR: The purified GST was lyophilized and redissolved in fresh (unopened) D₂O. The solvent replacement cycle was repeated 3 more times to ensure that almost all of the water was replaced by D₂O. The sample was then dissolved in 3 mM phosphate buffer (in D₂O) pH 7.5 to a final protein concentration of 1 mg/ml. An 8 μ l aliquot of this solution was then applied to a CaF₂ window with a 0.12 mm spacer and sandwiched in the sample holder. Spectra were run on a BIO-RAD FST-40 IR. For each spectrum, 256 scans were collected with a resolution of 2 cm⁻¹. Spectra were subtracted from a background scan. The chamber was allowed to purge with dry nitrogen gas for at least 30 min after opening the sample chamber. Samples were left in the cell between readings and the sample chamber was constantly purged with dry N₂ during the course of the experiment. Temperature was maintained by using a water-jacketed IR cell connected to a water bath and temperature was measured by a thermocouple located in the cell holder. Samples were run starting with the lowest temperature and ramped up at 0.5°C per min. The cell temperature was allowed to stabilize for 10 min before a spectrum was started.

Results

The homogeneity of the proteins from rabbit liver and *Cynomys leucurus* muscle were verified by HPLC and SDS-PAGE prior to use. The specific activity (74 U/mg), as

well as the reverse phase HPLC and SDS-PAGE separations (Figure 2-2 A and B) all confirmed the presence of a single subunit. This protein eluted from reverse phase HPLC as a single peak with a retention time of 17.5 min and on SDS-PAGE showed a molecular weight of 25 ± 2 kDa which indicated that the sample was PDM 18. The identities of the samples were further confirmed by N-terminal sequencing from a PVDF blot. The sequences were PPYTVYFPVRGR for prairie dog, consistent with PDM 18, and PVTGLY_DVRGLAL for the rabbit liver GST (Chapter 1). The SDS-PAGE of rabbit GST can be observed in Figure 2-2A and this was the same protein preparation as used in Chapter 1 (new bottles). From this information it was clear that both of these enzymes were of adequate purity and were properly identified for use in spectroscopic studies.

Thermal denaturation of GST was monitored by fluorescence emission spectroscopy. Initial scans of rabbit liver and PDM 18 GSTs at 0°C were compared (Figure 2-3 I). The spectra shown have been normalized to allow differences between the PD and rabbit enzymes to be best observed. PDM 18 GST had a λ_{max} at 343.84 nm whereas that of the rabbit enzyme was at 340.92 nm indicating a slightly more hydrophobic environment for tryptophan residues in the rabbit GST. The PD enzyme showed more emission near 300 nm than the rabbit enzyme but otherwise the curves were very similar.

The fluorescence emissions for the GSTs at various temperatures show the effects of increasing temperature and thermal denaturation (Figure 2-3 II and III). The PDM spectra showed a change in both the λ_{max} and intensity with heating (Figure 2-3 II). Over the temperature range tested the E_{max} reached 20.8 at 0°C and the λ_{max} had two

minima (341 nm) at 9 and 22°C. The relationship between λ_{max} and temperature showed shifts in the λ_{max} but no definite trends, either red or blue shifting of the spectra, with increasing temperature. The B spectrum recorded at 37°C shows a shift in the λ_{max} to 342.5 from 344 nm suggesting a tightening of the structure. There is also only a small loss of intensity when compared to the rabbit GST at the same temperature (Figure 2-3 II and III). The thermally denatured spectrum of this PDM 18 (Figure 2-3 II T) showed substantial perturbation of the protein structure with substantial changes in I^{340} and both λ_{max} and values. The shifts in the λ_{max} are not large between 0 and 58°C but they are significant. The severe deterioration of this curve both in shape and intensity compared to the chemically denatured GST suggested that the concentration of protein in solution was decreasing. At high temperature this was observed and from the appearance of the solution in the cell (precipitation, clouding was observed), protein coagulation was suspected. The limited λ_{max} shift of the thermally denatured GST compared with 6.0 M GuHCl shows that the structure of PDM 18 is not fully unfolded by heat.

The major differences between the rabbit liver and prairie dog GST were observed in the spectra at 37°C (Figure 2-3 III A, B) where a reduction in the I^{340} and no shift of λ_{max} were observed as a function of temperature. The rabbit liver GST (Figure 2-3 III) had several differences from the PD enzyme with increasing temperature. The reduction in I^{340} was larger for the RL enzyme but no substantial net shift in the λ_{max} occurred between 0 and 37°C for the RL GST (Figure 2-3). The intensity reduction observed in the RL enzyme was expected (1% decrease per 1°C is typical). However, PDM showed a much smaller effect on the intensity up to 37°C. The effect of thermal denaturation for RL GST shows

Figure 2-2. (A) Analysis of the purity of GSTs from prairie dog muscle and rabbit liver by SDS-PAGE (15% acrylamide gels). Lane A, contains molecular weight standards; lane B, 2 μ g of the PDM 18 GST; lane C, 10 μ g of PDM 18; and lane D, 7 μ g of rabbit liver GST.

(B) Reverse phase HPLC chromatogram of purified PDM 18 GST and the rabbit liver GST with detection at 280 nm

A

B

Figure 2-3. (I) Normalized fluorescence spectra of PDM 18 and rabbit liver GST.

The blank-corrected spectra were normalized and smoothed (using a 7 point average) using software from Perkin Elmer. (II) Fluorescence spectra of PDM 18 GST under several different conditions. A, shows the emission spectrum at 0°C; B, the spectrum at 37°C; T, thermally denatured at 56°C. (III) Fluorescence spectra of rabbit liver GST under the same conditions as for the prairie dog enzyme. A, shows the emission spectrum at 0°C; B, the spectrum at 37°C; C, Chemically denatured; T, thermally denatured at 56°C.

substantial aggregation and precipitation of the protein at high temperatures. Furthermore there was an incomplete denaturation of GST by heat (T); since precipitation occurred both PDM and RL GST were clearly not completely unfolded in solution (Figure 2-3 III)..

The effect of temperature on the I^{340} of the PD and RL GST can be seen in Figure 2-4. Temperature effects on the I^{340} of PDM 18 were to some degree unusual (Figure 2-4A). The decreasing slope of the intensity curve from 0 to 25.1°C was typical of the effects of increasing temperature on the fluorescence intensity which is typically a slow reduction in intensity (1%/°C) as temperature increases. However, the PDM 18 then showed a dramatic and sustained increase in I at 25.2°C. Furthermore, the optimal temperature range of PDM 18 activity occurred between 27 and 35°C, which was slightly after the increase in I for the enzyme (Figure 2-4A). The enzyme activity with CDNB becomes difficult to distinguish from the background at temperatures above 40°C, because the spontaneous reaction of substrates is very fast, but by this temperature the enzyme activity was definitely on the decline (Figure 2-4) and was completely undetectable within a few degrees higher. The increase in fluorescence intensity over the range of 25.2°C to 42°C indicated a substantial alteration in the protein structure over the narrow temperature range of 0.1°C (Figure 2-5A); this coincided with a large jump in the enzymatic activity. The sharp decline in the I^{340} between 42 and 44°C (PDM 18) was also indicative of a significant conformational change, which was preceded by a substantial loss of enzyme activity. The degeneration observed in the spectra at high temperatures, which included flattening, large loss of I , and the irregularity of the spectrum suggest a

conformational change and/or a change in protein concentration. Furthermore, the lack of I^{340} variation above 44°C suggests that the protein conformation was no longer affected by temperature. There was also a reduction in the slope of the thermal denaturation curve from 0.18 (0-25°C) to 0.07 I/°C, a change of over 50% for the temperature range from 25.2 to 42°C. The RL GST provided a different temperature intensity profile (Figure 2-4B). The rabbit liver enzyme showed a more conventional trend with a continuous decrease in I^{340} with increasing temperature. This curve showed two different events in the protein with the brief flattening of the curve between 2 and 10°C and then again at 24 to 28°C followed by sharp decline in the I^{340} both preceding and following these changes in the slope (Figure 2-4B). The activity of RL GST showed a sharp temperature optimum, which peaked at 35°C followed by a sharp drop in activity.

The effect of GuHCl on the solvated molecular weight was studied by HPLC SEC at the same denaturant concentrations used for fluorometry. The native molecular weight of PDM 18 was determined to be 52 ± 2 kDa by this method (Figure 2-6).

The protein showed a decrease in molecular weight with increasing guanidine-HCl concentration and the SEC chromatography showed that a minimal molecular weight (23 ± 2 kDa) was reached at 2.5 M GuHCl. With further increases in guanidine-HCl, the molecular weight of PDM 18 was stable up to 6.0 M GuHCl. The rabbit enzyme had a stable molecular weight up to 2.5 M GuHCl but at higher levels there was a sharp decline in the molecular weight from 50 ± 2 kDa (native) to 24 ± 2 kDa after which there was no further change in the elution profile up to 6.0 M GuHCl.

Figure 2-4. The changes in I^{340} and enzyme activity of GST from (A) prairie dog muscle and (B) rabbit liver as a function of increasing temperature. The enzyme was assayed from an aliquot of 10 μ l from the fluorometric sample and assayed at a temperature $\pm 2^\circ\text{C}$ relative to the cell temperature. The assay conditions were done at optimal substrate concentrations.

Figure 2-5. Fluorescence scans of GST from prairie dog muscle (I) or rabbit liver (II) after denaturation using different concentrations of guanidine hydrochloride. Spectra were acquired at 20°C as described in the Methods and Materials. Concentrations of GuHCl used with PDM were as follows (I): A, 0 M; B, 2.0 M; C, 2.5 M; D, 3.0 M; and E, 6 M GuHCl. GuHCl concentrations used with rabbit liver GST were as follows (II): A, 0.0 M; B, 2.0 M; C, 3.0 M; and D, 6.0 M GuHCl.

Figure 2-6. A compilation of the SEC HPLC runs for the denaturation of PDM 18 using guanidine hydrochloride. Samples of PD GST were denatured by the addition of the indicated concentration and left to stand for 2 hours prior to injection. The SEC column was equilibrated with the appropriate concentration of GuHCl in the standard HPLC SEC buffer listed in the methods and materials.

Changes in the emission spectrum presented in Figure 2-7 show some of the spectral changes observed in GST during chemical denaturation. The PD sample displayed a constant and gradual transition from native to denatured states with one abrupt change (Figure 2-7 A). If the denaturation of PD GST occurred in two stages there would be a separation of the subunits and unfolding of the subunits. The red shift of the λ_{max} occurred with very little loss of I (less than 5%) up to 2.0 M GuHCl. A sudden change in I was then observed followed by modest changes in both values up to 6.0 M GuHCl. The spectra for denaturation of the rabbit enzyme show a trend of slightly decreasing λ_{max} and increasing I values reaching a minimum at 1.0 and a maximum at 2.0 M GuHCl, respectively (Figure 2-7 B). After the peak values a steady increase in the λ_{max} and a decrease in I were observed at higher GuHCl concentrations. The spectra of rabbit GST did not have any abrupt changes to λ_{max} or I over the concentrations of GuHCl tested.

The denaturation of prairie dog GST by GuHCl showed a typical chemical denaturation profile with respect to changes in the λ_{max} (Figure 2-7 A). This denaturation showed a red shift in the λ_{max} of 15 nm between 0.0 to 6.0 M GuHCl. The intensity profile for the chemical denaturation remained constant up 1.5 M GuHCl. This correlates well with a sharp decline of 35% in the intensity between 2.0 and 2.5 M GuHCl (Figure 2-8A), which was just after the drop in enzyme activity (Figure 2-8A) and just before subunit separation as determined by SEC (Figure 2-6). At low concentrations of GuHCl (0.0 to 1.5 M) there were only minor fluctuations in the intensity and λ_{max} . This trend resumed from 4.0 to 6.0 M and hence, the major changes occurred between 1.5 and

3 M GuHCl (Figure 2-7). It was noted that the most significant decrease in the I^{340} (35%) occurred over the same concentration range as the subunit separation observed by SEC (Figure 2-7) and the decline in activity (Figure 2-8) between 2.0 and 2.5 M GuHCl. Rabbit liver GST displayed some of the same trends as PD GST when denatured by GuHCl (Figure 2-8B). The effect of GuHCl on $\lambda_{\max}$ showed a profile very similar to that seen with the PD enzyme. There was an increase (29%) in the emission intensity of the enzyme at GuHCl concentrations up to 2 M but then a decrease in I^{340} began. The rapid shift in the $\lambda_{\max}$ for this enzyme started near 1.0 M GuHCl, but even the lowest concentrations of GuHCl had an effect on the conformation (Figure 2-7B). The most significant changes in $\lambda_{\max}$ occurred over the next 1.5 M GuHCl and this was followed by a stabilization that occurred at GuHCl concentrations over 3.5 M.

The FT-IR spectra of PDM 18 and RL GST revealed only one shift in the spectra at high temperature. Up to this point (63°C), the spectra of the two GSTs were almost identical. The FT-IR spectra indicated substantial α -helix and smaller amounts of β -sheet and random coil structures in the amide I region of the spectra. The amide II and III were not useful for analysis and so were ignored. The initial absorbance maxima for the PDM 18 and RL GSTs at 2°C were 1632 cm⁻¹ and 1635 cm⁻¹ respectively. At 64.2°C the absorption max for the RL GST began to shift towards higher cm⁻¹. The PDM 18 followed a few tenths of a degree later at 65.5°C (Figure 2-9). There was a progressive increase in the rate of spectral shift up to 68°C for the PDM and 67.1°C for the RL GST. After this critical temperature the change was abrupt and was followed by stabilization at temperatures over 72.3 and 70.6°C for the PDM and RL respectively (Figure 2-9). The

absorption maximum for each of the GST PDM and RL stabilized at 1664 and 1659, respectively. The spectra demonstrated a consistent reduction in absorbance with an increase in temperature from 2°C.

Figure 2-7. Effects of GuHCl on the λ_{max} and enzyme activity of (A) prairie dog PDM 18 and (B) rabbit liver GST. Enzyme assay was done on the sample after florescent measurements were recorded 35 min after addition of enzyme to GuHCl. Samples were equilibrated at 20°C and spectra were also recorded at this temperature. Cuvettes for the enzymatic assay contained the same final concentration of GuHCl as the corresponding flourometric sample.

Figure 2-8. The effects of GuHCl on the emission intensity of GST from (A) prairie dog muscle (PDM 18) and rabbit liver. Spectra were recorded at 20°C after equilibration of 35 min at 20°C.

Figure 2-9. A plot of the shift in infra red absorbance maximum with increasing temperature for the PDM 18 and the RL GST enzymes.

Discussion

Analysis of the proteins showed that the two forms of GST examined were the same isozymes that were kinetically characterized in Chapter 1. The reverse phase retention, SDS-PAGE mobility (Figure 2-2) and the N-terminal sequence confirmed the identity of the two GSTs as PDM 18 (π -class GST) and RL μ -class enzyme. The GST PDM 18 was the subunit produced in the prairie dog muscle in response to hibernation and was identified as class π by N-terminal sequence homology. The π -class GSTs show a preference for ethacrynic acid and other α,β unsaturated carbonyls (Mannervik and Danielson, 1988). Pi class GSTs do not tend to have the large numbers of isozymes in an animal or tissue as the more abundant α and μ class GSTs. For example, only one π -class isozyme has been found in the following animals: rat (7-7), mouse (N 3-3) and human (π) (Mannervik and Danielson, 1988). The GST class- μ family of isozymes is typically found in abundance in hepatic tissue of rats (6 isozymes) and humans (2 isozymes) (Mannervik and Danielson, 1988). Mu-class GST isozymes are also found in skeletal muscle and testicular tissue (Hussey and Hayes, 1993). Mu-class GSTs are best known for their preference for epoxide containing substrates (Mannervik and Danielson, 1988).

There are several relevant differences and similarities between the Mu and the Pi class GST from a number of organisms. The π -class GSTs have two highly conserved W residues near the N-terminal end of the protein at position 29 and 37. Furthermore, Trp-37 is a G-site residue, which is part of the α -2 structural element (Dirr et al., 1994). The

μ -class GSTs have four highly conserved Trp at positions 8, 46, 148 and 216. Two of these Trp are in secondary structural elements W-8 (β -1) and W-46 (α -2) and are in the protein G-site (Dirr et al., 1994). Trp-148 is positioned between α -5 and α -6. The final Trp-216 is located very close to the C-terminal end of the protein. The C-terminal region of π and the μ class GST showed a high degree of conformational flexibility. The location of the W on this flexible tail may partially explain the variation in the intensity with temperature, as C-terminal movement in the μ -class GST is bound to cause variations in the fluorescence of the protein. As noted above there are 2 N-terminal Trp in π and μ class GSTs. Furthermore, one of these Trp (37 π and 46 μ) is located in the same structural element α -2, which is part of a hydrophobic sequence. The two N-terminal Trp in the μ class GST are part of hydrophobic sequences and structures and thus could account for the lower λ_{max} of the rabbit GST in solution (Figure 2-2 I).

The PD and the RL GST did show differences during denaturation by both temperature and chemical means (Figure 2-5 and 8). During the thermal denaturation of GST from PD muscle and the rabbit liver, there were distinct differences (Figure 2-4). The PD-GST showed a sharp increase in the I_{340} , suggesting a significant change in the protein conformation. This change was reflected in the rabbit enzyme as a leveling of the slope (Figure 2-5). Both of the curves changed during heating and at the same temperature as the peak activity. The sharp drop in activity that occurred near 40°C is an indication that GST was being thermally denatured. The sharp drop in the I that occurred between 44 and 45°C suggests a significant structural change. This fluorescence change in the PDM 18 precedes the first observable signs of precipitation in the sample cell.

There was evidence that at high temperatures (above 48°C) the protein precipitated from solution causing clouding of the sample. This raises the question as to whether GST subunits separate during thermal denaturation or if they undergo aggregation and precipitate from solution. The sharp drop in the I_{340} that occurred near 44°C (Figure 2-5A) may be an indication of precipitation or the separation of the subunits of PDM 18. There was also a change in the rate at which the protein is affected by temperature, which stabilized and showed no further effect of temperature up to 56°C. It was also noted that the PD GST had a broader temperature profile, which climbed more rapidly from 15°C than the RL enzyme (Figure 2-5). The large I change near 29°C seemed to be connected with a structural change that was beneficial to the enzymatic activity. The increase in activity (45%) over the temperature range may indicate control of the PDM 18 GST by a temperature-linked adaptation.

The slope above 25.2°C was less than the slope below 25.1°C. This indicates that the change that occurred between 25.1 and 25.2 showed some level of thermal stabilization of the resulting structure. This was followed by another rapid structural change that occurred between 43 and 45°C resulting in a 50% reduction in intensity over 2°C and the complete thermal denaturation of the PDM GST. Since there was no further change in the I^{340} (Figure 2-4), there would be no further changes in the W environment. The rabbit enzyme showed smaller temperature effects than the PD, and contained many similar characteristics with PDM 18. There was only a slight stabilization of the conformation between 26.2 and 31.7°C. This was followed by a resumption of the same I^{340} vs. temperature profile seen from 5 to 26°C until complete thermal denaturation

occurred at 40°C (Figure 2-4). GST is known to denature in a two step process involving the separation of subunits, followed by denaturation of each of the subunits with urea or GuHCl (Aceto et al., 1992). The denaturation of other GSTs shows the same trend that is seen with the rabbit enzyme - a smooth change in the fluorescence profile over the entire denaturation range.

CD measurements also indicate that the spectrum of GST is an addition of the spectra of the two monomers (Matuyama et al., 1985). This suggests that no major structural modifications that alter the 2° structure of the monomers occur with dimer formation and there is equal contribution from each of the subunits in the spectrum of the active enzyme. Fluorescence spectra of a π -class GST denatured with urea showed a similar denaturation profile to the GSTs we examined. Since both of the GST examined are homodimers, denaturation would be expected to occur in two steps: the separation of the monomers followed by the denaturation of the monomers.

The denaturation of PDM 18 and RL showed a typical denaturation curve for GuHCl (Figure 2-7) with results that were comparable to other GSTs (Matuyama et al., 1985). This denaturation profile is common to proteins from a variety of sources in the presence of chemical denaturants like GuHCl and urea (Matuyama et al., 1985; Omar and Beauregard, 1994; Omar and Beauregard, 1995; Rendon et al., 1995). Compared to the GST human π -class enzyme (Aceto et al., 1992) more GuHCl stability is seen in the RL and PDM. The increase in λ_{max} does not start until 1.5 M GuHCl, which is much higher than the human GST- π (Aceto et al., 1992) (Figure 2-7A). In addition, the denaturation is not complete until 4.0 M GuHCl which is 1.5 M higher than the human

enzyme and 0.5 M higher than the RL enzyme. The rabbit liver GST showed a similar denaturation profile to the PD enzyme (Figure 2-5B). Furthermore, the activity for both of these GSTs is present at 2 M GuHCl for the PD and 1.5 M for the RL, whereas the human is inactivated at 1 M (Aceto et al., 1992).

The PDM and the RL GSTs had the same initial λ_{max} (341 nm) which is 9 nm higher than the human π -class (Aceto et al., 1992), suggesting a more open structure since the position of these residues is highly conserved. It should also be noted that these λ_{max} values are close considering the evolutionary distances between the three species: human, rabbit, and prairie dog. The information from crystal structures and other spectroscopic studies suggests that all cytosolic GSTs (α , μ , π) are very similar in overall conformation and structure with only minor class specific differences.

The FT-IR spectra yielded data on the 2° structure with increasing temperature. The spectra did not show a change that could be associated with the separation of subunits. The GSTs that we examined were comprised of homodimers and should have similar IR profiles as monomers and dimers. With no dramatic change in the IR spectra suggesting the separation of the subunits during thermal denaturation, it is reasonable to suggest that subunit separation is not occurring as a result of increasing temperature. Studies of the 2° structures with CD showed that the spectrum of the holoenzyme is the sum of the spectra for the subunits (Matuyama et al., 1985). Furthermore, when the GST was a homodimer there were only minor changes in the CD spectra during subunit separation (Matuyama et al., 1985). It was determined that the 2° structure in the case of PDM 18 and RL GST that the secondary structural elements (helices and sheets) remain

intact after inactivation and the major fluorescent changes had occurred. This follows the expected pattern for thermal denaturation of the protein. The problem of the mechanism for thermal denaturation is still unclear. The question as to subunit separation has not been resolved. The fluorescent spectrum showed several changes with temperature but none that could be positively linked to subunit separation during thermal denaturation as was seen for chemical denaturation. The precipitation of the protein at high temperatures may indicate that the subunits do not separate but simply refold or aggregate reducing solubility and precipitate from solution. The damage to the protein structure from high temperature seems irreversible since the resulting precipitate was only soluble in SDS-PAGE buffer with boiling. This data suggest that denaturation of PDM and RL GST with GuHCl involves a two step process. The first step is the separation of the subunits with only minor changes in the subunit conformation, followed by the destruction of the 2° structure at higher concentrations of denaturant.

CHAPTER 3:
Purification and Characterization of Glutathione
Reductase

Introduction

Glutathione reductase (GR; EC 1.6.4.2) is a dimeric flavo enzyme; each subunit contains a flavin mononucleotide (FMN) and a binding site for NADPH. The active site is located near the C-terminus at the interface of the two subunits. Thus, only the dimer has catalytic function. GR catalyses the reductive dissociation of the glutathione dimer (GSSG) to form glutathione monomers (GSH). This reaction requires a reducing equivalent in the form of NADPH and releases two molecules of reduced GSH and a NADP^+ . A redox active pair of Cys located near amino acid 49 in the mouse enzyme (Thieme, et al., 1981; Krauth-Siegel, et al., 1982) is believed to be involved in electron transfer. The only other disulfides found in the mouse and human enzymes are involved in holding the two subunits together. Good homology exists between glutathione reductase from bacteria, plants, and animals with respect to conformation and sequence. The fact that the active site is located between domains probably requires the retention of conformation (and hence sequence) to ensure the size and shape of the active site.

Reduced glutathione has been implicated in a variety of cellular roles (Spaterna, 1993). The concentration of GSH in mammalian cells is maintained at a high value, 0.1-5 mM, compared with GSSG which is typically 6-200 μM , and maintenance of the GSH/GSSG ratio is the function of glutathione reductase (Kosower and Kosower). Oxidative damage occurs in the cell at a relatively constant rate; based on oxygen concentration and metabolic use of O_2 , the damage from reactive oxygen species can take various forms including lipid peroxide formation, cysteine oxidation, and oxygen radical reactions with DNA and proteins. GR transfers reducing equivalents from NADPH to

GSSG and keeps the concentration of GSH high and GSSG low. In vivo glutathione reductase maintains an equilibrium that favors the formation of GSH, and compensates for the reaction rate of GPox.

Glutathione reductase maintains the GSSG/GSH equilibrium and is, thus, tied into both antioxidant and toxicant metabolism. Antioxidants are part of the cell's general housekeeping system and participate in the repair of DNA and proteins, reduction of lipid peroxides, and metabolism of oxygen radicals. During periods of arousal in hibernating mammals, when body temperature is warming and O₂ consumption by mitochondria is increasing rapidly, there is a corresponding rise in the generation of reactive oxygen species. At the start of the rewarming process the brown adipose tissue starts generating heat which is followed, after body temperature has risen somewhat, by shivering thermogenesis (ST) in the skeletal muscles. Heart rate and blood flow increase as a result of the rising temperature. During the warming process there is also a switch from strictly lipid oxidation to lipid and carbohydrate catabolism to provide the energy for ST. The rapid increases in metabolism and blood flow and O₂ concentration will put stress on the antioxidant system.

Protein fluorescence is a good tool for examining small changes to protein structure. This technique uses the sensitivity of tryptophan (Trp) and to a lesser degree tyrosine (Tyr) fluorescence. The emission spectrum of Trp is sensitive to an aqueous environment, any changes in the exposure of the Trp to water is reflected in a change in the emission spectrum. Trp fluorescence shows a decrease in intensity (I) and a shift of the emission maximum (λ_{max}) on exposure to an aqueous environment. In the case of GR,

there are at least 4 Trp and 20 Tyr per holoenzyme and the emission spectra should contain components of both of these amino acids. The secondary structure of GR is comprised primarily of α -helix and strand structures. This should provide a hydrophobic core for the protein. The active site region of the enzyme contains mostly hydrophobic amino acids. Glutathione reductase contains three different binding sites for NADPH, FMN, and GSSG; its function is to move reducing equivalents from NADPH via an intermediate protein disulfide and FAD onto GSSG. This requires specific distances and orientation of reactive groups to facilitate the transfer. Furthermore, the active site shape and size is dependent on the conformation of each subunit at the subunit interface, which forms the active site. With no internal structural disulfides, it is possible to denature the subunits completely without reduction and the intra-subunit disulfide bond removes the complexity of subunit dissociation and its effect on the emission spectra in a non-reducing environment.

Enzymes can be adapted to function under a variety of conditions. The adaptation of animals and their systems to a lifestyle is an ongoing process. Temperature is just one factor that can affect the kinetics and the structure of a protein. An enzyme is adapted to fit its role in the metabolism of an organism, which is influenced by a number of factors. There is a wide selection of animals adapting, changing, and modifying enzymes in response to temperature (Borgmann and Moon, 1976; Moon, 1978; Moon, 1978; Bauman, 1990; Denlinger, Lee et al., 1992). The adaptation of enzymes in hibernating mammals should occur to accommodate the changes in metabolism that occur during hibernation as well as temperature variation. Glutathione reductase is a component in the

glutathione system, which is involved in stress response. The questions that I investigated are:

a) does liver GR changed for/during hibernation in the prairie dog (*Cynomys leucurus*)?

b) how does the prairie dog liver enzyme compare with the enzyme from a nonhibernating mammalian (bovine) species?

Adaptations of prairie dog liver (PDL) GR are bound to occur and will effect the kinetics and the structure of the protein. The effects of temperature on enzyme activity and structure can be studied *in vitro* by examining kinetic parameters and fluorescence spectroscopy. Hibernation specific adaptations will be evident by differences in GR from hibernating and euthermic liver samples. The PDL GR will also be compared with the liver enzyme from a nonhibernating species, the bovine.

Methods and Materials

Twelve adult white-tailed prairie dogs were captured in southeastern Wyoming during the summer of 1993. The animals were housed individually in standard rat cages at the University of Wyoming and maintained at $T_a = 22^\circ\text{C}$ on a natural (fall) photoperiod. The animals were fed a modified version of Purina 5001 rodent diet, which contained additional fat (fat content - 15%). After 37 days of feeding and fattening, 8 prairie dogs were sacrificed. Tissue samples were immediately removed, frozen in liquid nitrogen, and stored at -80°C for later analysis. The remaining 4 animals were allowed to continue feeding and fattening for an additional 36 days. At the end of this period (7 November, 1993), the animals in their cages were transferred to an environmental

chamber at 5°C to induce hibernation. Within 7 days, all 4 animals began hibernating and they continued to hibernate at $T_a = 5^\circ\text{C}$ for 10 weeks. All 4 animals were then sacrificed while they were torpid and their tissues were excised and stored as described above. Glutathione reductase from bovine liver was purchased from Sigma Chemical Co.

Samples of frozen tissues were homogenized 1:5 w/v in 50 mM phosphate buffer, pH 7.5, containing 5 mM EGTA, 5 mM EDTA and 1 mM PMSF and then centrifuged at $26,000 \times g$ for 25 min at 4°C. The supernatant was removed and loaded onto an 8 ml column of DEAE-Sephadex equilibrated with the homogenizing buffer. Enzyme activity passed unbound through the column and the eluant was collected and dialyzed against 10 mM Tris, pH 8.5. The sample was then loaded onto a fresh 5 ml column of DEAE-Sephadex equilibrated with 15 mM Tris, pH 8.5. The enzyme bound to this column and was then eluted with a 0 to 1 M KCl gradient. Active fractions were pooled and dialyzed against 5 mM Tris, pH 7.5. The dialysate was then loaded on to a column of Red agarose (10 ml of resin) and the column was eluted with a 0 to 1 M KCl gradient buffered with 10 mM Tris, pH 7.5. Active fractions were pooled, desalted by passage through a small column of Sephadex G-10, and then concentrated by dialysis against solid polyethylene glycol (PEG) 8000. The sample was then applied to an ADP-agarose column equilibrated with 10 mM Tris pH 7.5 and eluted with a 0 to 1 M NH_4Cl gradient in Tris buffer. The final purified enzyme was stored at 5°C in Tris with 100 mM $(\text{NH}_4)_2\text{SO}_4$ after concentration and diafiltration with a Centricon 10 (Amicon). Under these conditions, the sample was stable for at least 3 weeks. Protein concentrations were determined using Bio-Rad protein assay with BSA as the standard.

Kinetic analysis was carried out using a Gilford spectrophotometer with an attached chart recorder and water bath for temperature control of the cuvettes. NADPH consumption was monitored at 340 nm in a sample volume of 1 ml; assay conditions for maximal activity were 1 mM EDTA, 5 mM GSSG, 0.2 mM NADPH, and 50 mM phosphate buffer pH 7.5. Reactions were started by the rapid addition of enzyme. For temperature experiments, cuvette temperature was measured a micro-thermistor attached to a YSI telethermometer prior to addition of the enzyme. Kinetic and statistical calculations used software by Brooks (1992).

Fluorescence samples of purified glutathione reductase (7 μ g in all cases) were placed in a quartz cuvette containing 20 mM phosphate buffer pH 7.5 and 1 mM EDTA; total volume was 3 ml. At the start of each session, the fluorometer was calibrated for emission response and wavelength according to manufacturer specifications. A temperature-controlled cell holder was used and the sample was continuously stirred between readings. Between spectra, the lamp was turned off to prevent damage to the protein from constant UV light. All spectra were recorded three times (with the same sample) and an overlay of the spectra was used to visually verify the consistency and accuracy of each spectrum. One of these three spectra was then randomly selected (using a random number generator) for experimental use. This procedure was used for all samples under all conditions. For temperature studies, temperature was increased slowly (0.5°C/ min) and samples were equilibrated for 15 min at each temperature prior to recording spectra. For denaturation studies, aliquots of guanidine hydrochloride (GuHCl) were added to the enzyme in the cuvette and the mixture was stirred for 30 min before

recording the spectrum. Fluorescence emission was recorded using a Perkin Elmer LS50B fluorometer, scanning between 300 and 400 nm with a slit width of 2.5 nm and a scan rate of 50 nm min⁻¹; excitation was at 280 nm. All spectra were corrected for buffer and water emissions and baseline noise before determination of relative intensity (I) and emission maximum (λ_{max}).

HPLC separations were performed on a Beckman HPLC system with a UV/VIS detector at either 225 or 280 nm. Reverse-phase separations were done on a Zorbax RP-300 C-8 (2.1 x 150 mm) column with a flow rate of 0.2 ml/min. Samples were loaded at 35% acetonitrile and 65% water containing 0.12% trifluoroacetic acid (TFA) and a linear gradient was run to 65% acetonitrile and 35% water with 0.12% TFA over 25 min. An Altex TSK 2000 (4.5 x 300 mm) column was used for native molecular weight determination by size exclusion chromatography (SEC). The column was run isocratically in 50 mM phosphate pH 7.0 with 50 mM KCl at a flow rate of 0.715 ml/min. The SEC column was calibrated using blue dextran (2000 kDa), thyroglobulin (670 kDa), ferritin (443 kDa), phosphofructokinase (360 kDa), pyruvate kinase (230 kDa), catalase (232 kDa), aldolase (160 kDa), glutathione reductase (124 kDa), hexokinase (102 kDa), adenylate kinase (68 kDa), and triosephosphate isomerase (53 kDa) as molecular weight standards.

SDS-polyacrylamide gel electrophoresis (PAGE) was conducted by the method of Laemmli (1970) in a Bio-Rad mini-Protean 2 system. The 12% acrylamide reducing gels were run at 42 mA (constant current) per gel. Samples were loaded in 50 mM Tris (pH 8.8) containing 15 mM DTT, 20% v/v glycerol, 1% w/v SDS, and 0.04% w/v

bromophenol blue. Bio-Rad broad range standards were used: myosin (200 kDa), β -galactosidase (116.25 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). Electroblothing was done using an Owl semi-dry blotter at 500 mA constant current for 45 min in a Tris-glycine (25 mM Tris, 192 mM glycine) buffer system containing 20% v/v methanol. Electroblothing of samples from gels on to polyvinylidene difluoride (PVDF) membranes (Immobilon-N, Millipore) was done for sequencing and amino acid analysis (Matsudaira, 1987). Blots and gels were stained with 0.1 % w/v Coomassie brilliant blue R-250 (Bio-Rad) in 50% methanol and 5% acetic acid and destained in 50% methanol and 5% acetic acid. Gels were photographed and stored in 7% v/v glycerol. PVDF blots were air dried and stored in plastic wrap.

Isoelectric focusing (IEF) was performed in 110 ml glass columns (LKB) using a 0 to 46 % w/v sucrose gradient and 1.32 % w/v Ampholines (pH 4.5 to 6.5). Samples (2 ml total volume) were loaded in the center of the gradient and columns were run for 12 h at 5°C at a constant 500 V. After focusing, the columns were slowly drained into 39 fractions. Extracts were prepared by homogenizing 0.5 g samples of liver from euthermic or hibernating animals in double distilled water 1:10 w/v followed by centrifugation as above; the mixed sample was composed of 0.25 g of liver from each condition. The supernatant was desalted by passage through a small column of Sephadex G-25 and then loaded on to the IEF column. All three columns were run at the same time using the same stock solutions; 39 fractions (120 drop) were collected from the column and the pH

of each fraction at 20°C was recorded. A 0.5 ml sample from each fraction was then removed, passed through Sephadex G-25 equilibrated in standard homogenization buffer (as above) to remove sucrose and ampholines, and then assayed for GR activity.

Results

Glutathione reductase from the liver of *C. leucurus* was purified using a series of ion exchange and affinity chromatography steps (Table 3-1). The enzyme from liver of both euthermic and hibernating animals was prepared with similar yield (35-47%), fold purification (4850-4990 fold) and final specific activity (177-206 U/mg) for both. Both enzymes were homogeneous by SDS-PAGE and reverse phase HPLC (Figure 3-1). Furthermore, neither technique could resolve any difference between the two enzymes; both showed equivalent migration distance and elution time for the two enzymes. Subunit molecular weight of prairie dog GR was also determined from SDS-PAGE to be 54 kDa $\pm$ 2 (Figure 3-1). This is slightly larger than bovine GR, which has a weight of 52 kDa $\pm$ 2 (Figure 3-1). The native molecular weight of GR was estimated from size exclusion chromatography on HPLC; the value for the prairie dog enzyme was determined to be 105 kDa $\pm$ 2, compared with a value of 108 kDa $\pm$ 2 for bovine GR on the same column (retention times were 54.32 and 56.36 minutes, respectively). Liver GR from both euthermic and hibernating animals eluted at the same time and a sample prepared by mixing the two enzymes showed only a single sharp peak with a retention time of 54.53 minutes. By contrast, a mixed sample containing bovine and prairie dog GR eluted as a broad peak from 54-56 min. The isoelectric point (pI) of GR was

Table 3-1. A typical purification scheme for glutathione reductase from prairie dog liver.

	Total protein mg	Total activity U	Specific activity U/mg	Fold purification	Yield (%)
Euthermic					
Crude	5690	204	0.0358	0	100
DEAE 1	28.2	182	6.44	180	90
DEAE 2	3.96	145	36.6	1020	71
RED agarose	0.931	108	116	3250	53
ADP agarose	0.542	96	177	4990	47
Hibernating					
Crude	4060	173	0.0426	0	100
DEAE 1	27.3	151	5.52	200	87
DEAE 2	1.97	110	55.6	1300	64
RED agarose	0.472	78	165	3876	45
ADP agarose	0.296	61	206	4850	35

Figure 3- 1. SDS-Polyacrylamide gel electrophoresis of purified glutathione reductase from prairie dog (hibernating and euthermic) and bovine liver. Lanes 1, 6 and 7, are molecular weight standards; lane 2, euthermic prairie dog GR; lane 3, hibernating GR; lane 4, mixed sample of hibernating and euthermic enzymes; lane 5, bovine GR.

determined using crude extracts of liver from euthermic and hibernating prairie dogs as well as an extract prepared by mixing tissue from both conditions. Activity eluted from IEF in a single, sharp peak with maximal activity in fraction 34 of 39 in all cases; the pH of this fraction was 6.24 for the euthermic extract, pH 5.95 for the hibernator and 6.12 for the mixed samples. The IEF data indicate a single form of GR present in prairie dog liver with a mean pI of 6.10 ± 0.10 (the mean of the above 3 values).

The effect of assay pH on GR activity is shown in Figure 3-2. At 37°C, both enzymes showed sharp pH optima at pH 7.5 for prairie dog and pH 8.0 for bovine GR. At the lower assay temperature (5°C), however, the pH curves for both enzymes became substantially broader. The optimum for bovine GR shifted to pH 6.5 to 7.0 whereas optimal activity occurred over a wide range, pH 6.5 to 8.5, for the prairie dog enzyme. Indeed, the activity of prairie dog GR, assayed at 5°C, did not drop below 20% of the maximum over the entire range tested (pH 5 to 9.5).

The effect of assay temperature on the V_{max} activity of bovine and prairie dog GR is shown in Figure 3-3. In both cases, the temperature optimum of the enzymes was 37°C and the two enzymes showed parallel changes in activity with decreasing temperature. Prairie dog GR maintained a small but consistently higher relative activity than the bovine enzyme over the temperature range from 2.5 to 34°C.

Figure 3-4 presents Arrhenius plots of the effect of temperature on the maximal activity of GR from prairie dog and bovine liver. Both enzymes showed a distinct break in the slope of the relationship at 34°C. The break in the Arrhenius plot also occurs just prior to the rapid increase in the V_{max} activity (Figure 3-3). Over the temperature range

from 2.5°C to 34°C, the slopes for both enzymes were parallel with activation energies of 17.8 ± 0.02 kcal/mol for the prairie dog enzyme and 18.9 ± 0.10 kcal/mol for bovine GR (Table 3-2). The difference in activation energy between the PD and the bovine enzymes was not significant, but the break in the Arrhenius plot occurs at the same temperature. The V_{max} and related kinetic parameters share a number of similarities between the PDL and the bovine GR and share a number of similarities with changing temperature (Figure 3-3 and 4).

Table 3-3 shows substrate affinities for GSSG and NADPH for purified euthermic prairie dog and bovine GR. Affinity of the two enzymes for GSSG differed substantially, K_m GSSG of the bovine enzyme being 4.5-fold higher at 37°C ($P < 0.005$) and, 19-fold higher at 5°C than the corresponding value for the prairie dog enzyme. K_m GSSG of both enzymes also changed with assay temperature and fell significantly at 5°C. The effect of 5°C assay temperature was much more pronounced on the prairie dog enzyme (K_m at 5°C was only 11% of the 37°C value) compared with bovine GR (K_m fell to 45 % of the 37°C value). K_m values for NADPH did not differ between species when assayed at 37°C but at 5°C, the K_m NADPH of prairie dog GR had changed very little (increased by 30 %) whereas that of the bovine enzyme increased 2-fold, compared with 37°C. Both enzymes could also use NADH as the nucleotide cofactor to a limited extent, the activity ratio NADH/NADPH being 0.033 for prairie dog and 0.013 for bovine GR. Kinetic constants were also compared between GR purified from euthermic versus hibernating animals but these values differed by less than 8% ($n=3$) and were not significantly

Table 3-2 Activation energies for glutathione reductase for the prairie dog and bovine enzymes

Animal	Temperature range (K)	Activation energy Ea (kcal/mol)
Prairie Dog	275-300	18.9 $\pm$ 0.021
	300-325	26.1 $\pm$ 0.125
Bovine	275-300	17.8 $\pm$ 0.102
	300-325	27.4 $\pm$ 0.251

Data are means (n=8) and errors are $\pm$ SEM.

Table 3-3. Effect of temperature on the K_m values for substrates of glutathione reductase from euthermic prairie dog and bovine liver.

Temperature	K_m (μM) 37°C	K_m (μM) 5°C
Prairie dog		
GSSG	38 ± 1.80	4.03 ± 0.19^a
NADPH	81.1 ± 1.40	105.7 ± 3.80^a
Bovine		
GSSG	170 ± 14.0^b	$76.7 \pm 6.30^{a,b}$
NADPH	75 ± 0.2	$231 \pm 0.9^{a,b}$

Data are means $\pm$ SEM, $n=6$. ^a = $P < 0.05$ difference between 37 and 5 and ^b = $P < 0.05$ difference between bovine and prairie dog.

Figure 3-2. Effect of assay pH on the activity of purified prairie dog and bovine glutathione reductase at 5 and 37°C using bis-tris propane. Maximal activity for each enzyme was set to 100. Data are means $\pm$ SEM, n=3. The pH values were measured in cuvettes both before and after the reaction was run; values were measured at room temperature and corresponding pH values at either 5 or 37°C were calculated using the known change in pH of the buffer with temperature.

- Prairie dog GR 5°C
- Prairie dog GR 37°C
- ▲ Bovine 5°C
- ▼ Bovine 37°C

Figure 3- 3. Effect of assay temperature on the relative activities of glutathione reductase from bovine and prairie dog liver, assayed under optimal substrate conditions. Data are means $\pm$ SEM, n=8.

Figure 3- 4. Arrhenius plots for glutathione reductase from prairie dog and bovine liver. (A) shows data for the entire temperature range; (B) shows the linear portion between 2.5 and 34°C; (C) shows the linear portion between 34 and 52°C. Lines show the linear regressions. Data are means $\pm$ SEM, n=8.

different for either K_m GSSG or K_m NADPH. The reverse reaction was not examined because it lacked physiological relevance.

Table 3-4 shows the effects of inhibitors on GR at two assay temperatures. GSH is a product inhibitor and the two enzymes showed relatively low and similar I_{50} values at 37°C. However, the effect of temperature on the I_{50} GSH differed between the enzymes; at 5°C, I_{50} GSH of the prairie dog enzyme decreased by 50 % whereas that of the bovine enzyme increased by 50 %. Bovine GR was only weakly inhibited by inorganic salts. I_{50} values at 37°C being, in all cases, well above the normal physiological range of the compounds *in vivo*. I_{50} values for all four salts also increased further when assessed at 5°C. The prairie dog enzyme responded differently, however. I_{50} values for salts were overall lower than those for the bovine enzyme and all I_{50} values decreased significantly at 5°C by amounts ranging from 15 % (NH_4Cl) to 63% (NaCl).

Figure 3-5A shows fluorescence spectra of prairie dog and bovine liver glutathione reductase at 2.5°C; the spectra were normalized and overlaid to show differences and similarities between the two enzymes. Each protein was scanned three times to determine consistency and the spectra shown were chosen by random selection. For the most part, the spectra were very similar with only minor observable differences in the emission profiles of the two enzymes between 325 to 310 nm; these are not easily interpreted. The difference in the λ_{max} for the two proteins was the most substantial difference between the two enzymes being 340 nm for the PD and 344.2 nm for the bovine enzyme. Another notable point was that for equal protein concentrations, the PD had a greater maximum I value than the bovine enzyme (Figure 3-5B and C). This was

Table 3-4. I_{50} values for product and salt inhibitors of glutathione reductase from prairie dog and bovine liver at two assay temperatures.

Temperature	37°C	5°C
	I_{50} (mM)	I_{50} (mM)
Prairie dog		
I_{50} NaCl	661 ± 1.8	243 ± 4.3^a
I_{50} KCl	576 ± 2.9	293 ± 8.5^a
I_{50} $(\text{NH}_4)_2\text{SO}_4$	678 ± 3.3	411 ± 10^a
I_{50} NH_4Cl	371 ± 6	315 ± 8.6^b
I_{50} GSH	1.45 ± 0.11	0.72 ± 0.036^a
Bovine		
I_{50} NaCl	889 ± 5.6	1090 ± 9^a
I_{50} KCl	1062 ± 3.1	1407 ± 10.3^a
I_{50} $(\text{NH}_4)_2\text{SO}_4$	821 ± 5.2	1020 ± 6.5^a
I_{50} NH_4Cl	459 ± 4.1	502 ± 5.1^b
I_{50} GSH	3.2 ± 0.24	4.63 ± 0.38^b

Data are means $\pm$ SEM, n=6. ^a -Significantly different from the corresponding value at 37°C, $P < 0.005$; ^b - $P < 0.05$.

Figure 3- 5. Fluorescence scans of glutathione reductase from bovine and prairie dog liver. The protein content of the cuvette was 7 μ g in 3 ml of 20 mM phosphate-Tris pH 7.5 and 1 mM EDTA. (A) normalized fluorescence scan of bovine and prairie dog GR at 2.5°C. (B) scans of prairie dog GR at three temperatures showing the changes in the spectra with increasing temperature. (C) scans of bovine GR at three temperatures showing the changes in the spectra with increasing temperature.

not a large difference but it was consistent and observable at all temperatures tested. Over the temperatures examined the spectral changes for both the PD and bovine were very similar (Figure 3-5).

Chemical denaturation with GuHCl was more detrimental to the structure of GR than was thermal denaturation (Figure 3-6 A and B). The change in protein structure caused by guanidine hydrochloride tends to be linearization rather than the coagulation of protein, which can be caused by heating a protein in solution (Creighton, 1989). The effects of GuHCl denaturation on GR showed a further drop in intensity from the thermally denatured enzyme for both animals (Figure 3-6). The hibernating GR also displayed a further red shift in the λ_{max} between the chemically and thermally denatured enzyme. To further examine GuHCl effects on the enzyme, size exclusion chromatography on the HPLC was used in the presence versus absence of 6 M GuHCl to determine if subunit disassociation might occur. There was a 10 % reduction in the apparent molecular weight from 106 ± 3 kDa to 95 ± 3 kDa when the enzyme was run on the column in the presence of 6 M GuHCl. This reduction was not enough to suggest separation of the subunits. Glutathione reductase activity was sensitive to any concentration of GuHCl. Enzymatic activity of prairie dog GR was completely lost in the presence of 0.1 M GuHCl added to the cuvette, whereas the bovine enzyme was completely inactive by 0.25 M. The bovine and euthermic prairie dog enzymes displayed a constant change in the fluorescence intensity with changing temperature (Figure 3-7A). The plot of I_{340} versus temperature (Figure 3-7B and C) shows the amount of physical movement in the protein structure with small changes in temperature. From the plot of I

vs. temperature, it is clear that there was a substantial amount of similarity between the PD and the bovine enzyme (Figure 3-7A). When the curves for the two enzymes are viewed separately some differences in the thermal denaturation profile are found. The decrease in fluorescent intensity with temperature normally occurs at -1% in intensity for each 1°C increase in temperature (Creighton, 1989). The temperature profile for GR has a slope of 0.00858 (2 to 42°C) and 0.0127 (2 to 52°C) for the PDL and bovine enzyme respectively (Figure 3-7B and C). An abrupt change in the slope was then observed starting at 46 to 48°C for the PD and above 52°C in the case of the bovine enzyme. The slope of the PD GR then quadruples to 0.0331 and rises by nearly double to 0.02 for the bovine enzyme. Above 56°C and 60°C the slope decreases, indicating that very little change occurs in protein conformation at higher temperatures. The thermal denaturation of bovine GR occurred between 56°C and 60°C. The increase in the slope of the intensity vs. temperature curve is an indication of the loss of 4° and 3° structure and resulting changes to the environment near the Trp residues. From 60°C to 64°C there was almost no change in fluorescence indicating that very little heat labile structure was left. The prairie dog enzyme showed a change in slope starting at 48 or 52°C and leveled out by 56°C (Figure 3-7B). The starting point for the denaturation curve seems to cover the temperature range from 48 to 52°C. The change in slope at 48°C was substantial, resulting in a reduction in intensity of 26% for the PDL and 16% for the RL GR; both of these changes occurred over 8°C (Figure 3-7A and B). Furthermore, the decrease in fluorescence intensity at high temperature also corresponded to a reduction in enzyme activity (Figure 3-3). At 50°C or higher, there was a rapid loss of activity and the bovine

Figure 3-6. Comparison of native (N), thermally-denatured (T), and chemically-denatured (C) glutathione reductase. Chemical denaturation was done using 6 M guanidine hydrochloride whereas thermal denaturation is from a 60°C scan of the protein sample; the native state is a scan at 2.5°C. (A) bovine; (B) prairie dog GR. The protein content of the cuvette was 7 µg in 3 ml of 20 mM phosphate-Tris pH 7.5 and 1 mM EDTA. Each spectrum shown was a random selection of the three spectra run at each temperature.

Figure 3-7. A plot of the fluorescence intensity at 340 nm with changing temperature for both the PD and the bovine enzyme overlaid (A) prairie dog (B) and bovine (C) glutathione reductase. Spectra were run in 20 mM phosphate pH 7.5 with 7 μ g of protein in a total volume of 3 ml.

enzyme was inactive at 60°C. The decline in activity of the prairie dog enzyme started at 46°C and resulted in complete enzyme inactivation by 56°C. The reduction in I_{340} coupled with the loss of enzyme activity suggests that thermal denaturation of the protein is occurring. It is also apparent that the thermal denaturation of prairie dog GR starts and finishes before the bovine enzyme and the enzyme from the hibernating species is less stable thermally.

Measurement of the λ_{max} showed no relevant trends with changing temperature (Figure 3-8). For prairie dog GR, there were significant changes to the λ_{max} from 346 to 340.5 nm over the temperature range tested with a λ_{max} of 346 occurring at 10°C and 340.5 nm at 24°C (Fig. 8A). The prairie dog enzyme also showed a constant increase in the λ_{max} from 24°C to 40°C indicating that water is being removed from the core of the protein. The bovine enzyme showed a much more constant environment for the Trp residues from 16°C to 32°C and to either side of this temperature range there was substantially more variability in the protein structure (Figure 3-8B). This variability of λ_{max} for GR indicates that the environment of the Trp residues in GR varies with temperature. The occurrence of a λ_{max} at 346 suggests that these residues were mostly exposed to water (Lakowicz, 1983). These data also suggested that there might be substantial movement of Trp in and out of an aqueous environment within the enzyme.

Figure 3- 8. A plot showing the effects of temperature on λ_{max} of (A) prairie dog liver and (B) bovine GR. Data shown are for the same protein sample equilibrated to different temperatures. Conditions are the same as Figure 3-7.

Discussion

A comparison of GR purified from liver of hibernating and euthermic prairie dogs indicated that there was no change in this enzyme with hibernation. The enzyme from both sources was identical in a variety of physical parameters such as a pI 6.1, specific activity of 206 (euthermic) and 177 (hibernating) U/mg, native size 105 ± 2 kDa, identical subunit molecular weight by SDS-PAGE (Figure 3-1), and retention time on reverse phase HPLC. Furthermore, prairie dog GR is similar to the mouse enzyme in both its isoelectric point (pI 6.46) and the specific activity of the purified protein (158 U/mg) (Lopez-Barea and Lee, 1979). Both the native and subunit molecular weights for prairie dog GR are also consistent with the enzyme from other sources in the Swiss Protein data base. A homology search of the Swiss Protein data base PC gene software confirmed that the sequence homology of the enzyme is highly conserved over a wide variety of animals and, overall, the enzymes from most sources share over 85% sequence homology. Structural information from studies on other GR enzymes suggest an intramolecular disulfide bond and the fact that GuHCl treatment was unable to separate the subunits of prairie dog GR also indicates the presence of this bond in the enzyme from this hibernating mammal. Glutathione reductase activity from the prairie dog was less temperature stable than the bovine or mouse enzyme (Lopez-Barea and Lee, 1979). It was also more sensitive to salt inhibition and chemical denaturation by GuHCl. The fluorescent spectra of prairie dog and bovine GR showed a dramatic decrease in the I_{340} from the native state but there was only a small shift in the $\lambda_{\max}$. Hence, there are only

minor changes in the environment near the trp and there is almost certainly no opening (linearization) of the protein structure. Furthermore, the lack of a substantial red shift in the max during thermal denaturation suggests that there is a structural collapse and aggregation with thermal denaturation.

There are differences in the GR from the prairie dog and bovine sources. The K_m for GSSG from the prairie dog enzyme was significantly lower than that observed for the bovine enzyme at 37°C; in fact, the two enzymes are near the extremes of the values reported from other sources which are typically near 59 μM (horse liver) (Garcia-Alfonso et al., 1993). The bovine enzyme is in the upper range of reported K_m values at 170 μM , whereas the prairie dog is at the other extreme with a K_m of 38 μM at 37°C. By contrast, the K_m values for NADPH were typical of other GRs. The enzyme from other mammalian sources like mouse, rat and horse liver show K_m values in the range 50-120 μM for NADPH (Icen, 1967; Nakashima et al., 1976; Lopez-Barea and Lee, 1979; Carlsberg and Mannervik, 1981; Garcia-Alfonso et al., 1993).

The bovine and prairie dog GR showed differences in the I_{50} values for several salts (Table 3-4); in all cases I_{50} values of the salts tested being significantly larger for the bovine enzyme at both 5 and 37°C. Hence the prairie dog GR is more sensitive to salt than the bovine enzyme and more sensitive at low temperature than at high. However, overall, the I_{50} values for any of the salts were high and this suggests that the importance of salt effects in enzyme control in vivo maybe rather limited. GR from both prairie dog and bovine sources was sensitive to product inhibition by GSH and the I_{50} for GSH (1.5-3.2 mM at 37°C) was within the range of physiological concentrations for this peptide.

This suggests that product inhibition maybe very important to enzyme control *in vivo* because product concentration varies inversely with substrate levels (in most cases the total glutathione pool in a tissue remains constant). This means that enzyme activity would be rapidly responsive to any cellular conditions that increased the rate of GSH consumption. The I_{50} value for GSH changed at low temperature, decreasing for the prairie dog enzyme and increasing for the bovine enzyme. From a physiological point of view this effect could be adaptive in making the enzyme more sensitive to the GSH pools at low temperatures such as occur during hibernation.

The temperature versus activity profile (Figure 3-3) clearly shows identical temperature optima and similar temperature profiles for the prairie dog and bovine enzymes. The Arrhenius plots for both enzymes are very similar with the break occurring at the same temperature (Figure 3-4). The break in the Arrhenius plot occurred near the start of the temperature optima spike. In the case of both enzymes there was an increase in the E_a just as the enzyme was approaching the physiologically important temperature of 37°C.

The initial fluorescence spectra of the two proteins taken at low temperature (2.5°C) showed that the prairie dog and bovine GR were very similar (Figure 3-5). The emission spectrum for this protein is primarily composed of Trp, because Trp is present in the sequence and when it is present it comprises most of the spectra. To further ensure that Trp fluorescence was being observed excitation was at 285 nm (Creighton, 1989). Prairie dog GR showed a greater intensity value and a lower $\lambda_{\max}$ than the bovine enzyme. With increasing temperature, there was a great deal of variation in the $\lambda_{\max}$ for bovine GR

(Figure 3-8). The shift of the λ_{max} of Trp toward the red end of the spectrum is suggestive of exposure to water and thus the opening or relaxing of the protein structure. The fluctuations in the λ_{max} of GR from both prairie dog and bovine sources suggest that a lot of movement was occurring in the protein structure with temperature changes. The bovine enzyme demonstrates almost random motion with temperature changes with a decrease in the λ_{max} near 37°C. Figure 3-8 shows relatively consistent fluctuation of bovine λ_{max} around about 343 nm, whereas the PD enzyme has some large and probably significant variations.

CHAPTER 4:
Purification and Characterization of Aspartate
Aminotransferase

Introduction

Aspartate aminotransferase (AspAT), also known as glutamate-oxaloacetate transaminase (EC 2.6.1.1), catalyses the reaction:

The enzyme is pyridoxal phosphate dependent and is comprised of two identical subunits.

The subunit interface forms the active site and the pyridoxal phosphate-binding site.

AspAT occurs both in the cytosol and compartmentalized in the mitochondria, and may be present as a mixture of isozymes in the cytosol. In most cells there is normally a cytosolic and a mitochondrial form of the enzyme present (Obaru et al., 1986), but at least 3 resolvable isozymes of AspAT have been found in some mammalian tissues (Kuramitsu et al., 1985; Horio et al., 1988). The function of AspAT in the cell is to interconvert aspartic acid and glutamate as part of protein catabolic and anabolic pathways.

Blood glucose is regulated in all hibernators (Tashima et al., 1970). The glucose used during hibernation is formed largely as the result of gluconeogenesis (Tashima et al., 1970; Wang, 1989). Hibernators do not store large amounts of glycogen before hibernation and thus, glucose in the blood of hibernating animals comes primarily from new synthesis using a significant amount of amino acids as starting materials (Tashima et al., 1970; Wang, 1989). The use of proteins for fuel is common in hibernation and it has been determined that the contribution of amino acids to overall gluconeogenesis varies with the animal, ranging from 20 % in the golden-mantled ground squirrel (Tashima, S.J.

et al., 1970) and 47 % in the big brown bat (Yacoe, 1983). Proteins from a number of tissues are used for energy during hibernation. For example, the ground squirrel shows a loss of 12 % of total protein in heart, 22 % in liver, and 39 % in muscle over the course of the winter (Tashima, S.J. et al., 1970; Wang, 1989). Due to the important structural and functional roles of protein in cells, it is important for the hibernator to carefully regulate the use of protein. The hibernator must fast for months but resume normal activity upon waking and this suggests that protein catabolism must be carefully controlled to prevent wasting of tissues (Wang, 1989). The use of amino acids for gluconeogenesis is further complicated by the need to dispose of the resulting ammonia.

During torpor all hibernators get most of their energy from lipids. The exception to this is during waking, when glucose use returns to near normal during periods of arousal (Tashima et al., 1970; Wang, 1989). The transition to lipid use has been implicated in the regulation of the glycolytic pathways in hibernators. Fatty acid metabolism seems to increase gluconeogenic rate (Wang, 1989) and down regulate glycolysis (Brooks and Storey, 1992; De la Roche, 1995). Evidence shows that the most significant controls are exerted on the enzymes of glycolysis (Brooks and Storey, 1992; Storey, 1989; Storey and Storey, 1990; Storey and Storey, 1990).

AspAT is involved in protein and amino acid metabolism and very high activities of the enzyme are found in tissues of the golden-mantled ground squirrel: 1.7 U/mg in skeletal muscle, 0.83 U/mg liver, and 1.3 U/mg heart (De la Roche, 1995). Furthermore, the maximal activity of this enzyme has also been observed to change as a result of hibernation in other hibernating species (Lin et al., 1997). The activity of this enzyme did not change in the muscle of ground squirrels during hibernation, but AspAT activity

increased in the heart which correlates with an increase in protein catabolism (De la Roche, 1995). Indeed, heart AspAT activity increased to the levels found in skeletal muscle during hibernation. In both of these tissues, there were also increases in the activities of enzymes of gluconeogenesis during hibernation. The activity of AspAT also changed in some other tissues of the golden-mantled ground squirrel during hibernation. Activity increased in heart and kidney but decreased in white adipose and liver (De la Roche, 1995). Furthermore, high activities of AspAT in all tissues (> 700 mU/mg protein) indicates that this is a well-used enzyme system. Recent work on the liver of the Richardson's ground squirrel also showed changes in the activity of AspAT during the seasonal cycle (Lin et al., 1997).

The present study was designed to examine potential structural and kinetic changes in ground squirrel AspAT between hibernating and euthermic states as well as between a hibernating species (ground squirrel) and a nonhibernating species (pig). Fluorescence analysis of the proteins in solution was used to analyze differences in the temperature stability of the enzymes and in their resistance to chemical denaturation.

Methods and Materials

Enzymes (porcine heart aspartate aminotransferase, malate dehydrogenase), α -ketoglutarate, and high purity guanidine hydrochloride (GuHCl) were purchased from Boehringer Mannheim (Montreal Que.). Buffers, aspartic acid, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Resource S and Q columns

were from Pharmacia. All water was purified by distillation followed by a Millipore deionization and charcoal filtration system.

Richardson's ground squirrels, *Spermophilus richardsonii*, were captured near Calgary, Alberta in the summer of 1994. They were held in the animal care facility at the University of Calgary on a natural (fall) photoperiod and fed *ad libitum* on rat chow supplemented by peanut butter. After two months, half of the animals were induced to hibernate by placing their cages in a cold room at 5°C with water but without food. Animals were determined to be truly hibernating if they were torpid for at least five days between periodic arousals. After one month, both euthermic and hibernating animals were sacrificed; in their last cycle, hibernating individuals were sampled after they had been torpid for at least 2 days. All animals were killed by decapitation and excised tissues were immediately immersed in liquid nitrogen and subsequently stored at -70°C.

Samples of frozen skeletal muscle were homogenized in 10 volumes of 20 mM Tris-HCl buffer, pH 6.5, containing 5 mM EDTA, 5 mM EGTA, and 1 mM PMSF. The homogenate was then centrifuged at 26,000 x g for 25 min at 4°C. The supernatant was removed and brought to 50 % saturation with ammonium sulfate. After stirring at 5°C for 30 min, the slurry was centrifuged at 26,000 g for 30 min. The supernatant containing AspAT activity was removed and readjusted to 70 % saturation which precipitated the enzyme. After further stirring and centrifugation as above, the pellet from the 70 % saturated fraction was resuspended in 20 mM Tris pH 7.5 containing 1 M salt (0.5 M KCl + 0.5 M (NH₄)₂SO₄). This sample was loaded onto a phenyl-Sepharose column (15 ml) equilibrated with the same buffer. The column was then washed with 20 column volumes

of 20 mM Tris until the absorbance of the eluant at 280 nm fell to zero. AspAT activity was then eluted with a linear gradient of 20 mM Tris to ddi-water. The active fractions were pooled and buffered with 1 M Tris pH 7.5 to a final concentration of 10 mM Tris pH 7.5. The sample was then passed through a column (3 ml) of Cibacron blue agarose equilibrated with 10 mM Tris pH 7.5 and the eluant was collected and adjusted to pH 8.5. The sample (peak activity fractions) was then filtered through a 0.45 μ m syringe filter and injected onto a 1 ml resource Q column on an HPLC (see HPLC section) equilibrated with 10 mM Tris pH 8.5. The flow through was collected which contained the AspAT activity, pH was adjusted to 7.5, and then the sample was concentrated by dialysis against solid polyethylene glycol 8000. Prior to storage, 2 mM pyridoxal phosphate and 20 % v/v glycerol were added to stabilize enzyme activity. Prior to fluorescence work, the sample was run on a SEC HPLC column for final purification and buffer exchange.

HPLC separations were performed on a Beckman HPLC system with a UV/VIS detector at either 225 or 280 nm. Reverse-phase separations were done on a Zorbax RP-300 C-8 (2.1 x 150 mm) column with a flow rate of 0.2 ml/min. Samples were loaded at 15% acetonitrile and 65% water containing 0.12% trifluoroacetic acid (TFA) and a linear gradient was run to 75% acetonitrile and 35% water 0.12% TFA over 65 min. Prepacked Resource Q and S (1.0 and 5.0 ml) were purchased from Pharmacia. These columns were run at 3 ml/min with (A) buffers which were 10 mM Tris pH 8.5 (Resource Q) or 10 mM acetate pH 5.5 (Resource S) and (B) buffers which were the corresponding, buffer, A with 1.0 M KCl added. Gradients were started 10 min after sample injection and went from 0 to 100% B in 10 min. Fractions (3 ml) were collected starting at the sample injection.

SEC chromatography was performed with an Altex TSK SW 2000 (7.5 x 300 mm) in series with a TSK SW 3000 (7.5 x 600 mm); both columns were used for all native molecular weight determinations. These columns were run isocratically in 50 mM potassium phosphate pH 7.0 containing 50 mM KCl (buffer A) at a flow rate of 0.715 ml/min. For SEC chromatography with GuHCl, buffer A was mixed with an appropriate portion of 6 M GuHCl in 20 mM phosphate pH 7.5 (buffer B) to produce a specific final concentration of GuHCl concentration. SEC columns were calibrated using blue dextran (2000 kDa), thyroglobulin (670 kDa), ferritin (443 kDa), phosphofructokinase (360 kDa), pyruvate kinase (230 kDa), catalase (232 kDa), aldolase (160 kDa), glutathione reductase (102 kDa), hexokinase (102 kDa), adenylate kinase (68 kDa), and triosephosphate isomerase (53 kDa) as molecular weight standards.

Enzyme kinetic analysis was conducted using a Dynatech MR 5000 microplate reader. The conversion of NADH to NAD⁺ was observed as a decrease and the reverse reaction as an increase in absorbance at 340 nm. The standard assay contained L-aspartate 250 mM, pyridoxal phosphate 0.11 mM, NADH 0.15 mM, MDH 1 U, α -KG 15 mM, Tris buffer 100 mM pH 7.5. All substrates and solutions were made up in the Tris assay buffer and the pH adjusted to 7.5 with HCl or KOH. The concentrations reflect the final concentration of the compound in solution to a total volume of 250 μ l for a microplate well and 1.0 ml for cuvette. Data was acquired and stored using Bio-Links software, 55 with A₃₄₀ readings taken 11 seconds apart after an initial shake of 5 seconds. To control reaction temperature, the entire microplate reader was placed in a controlled-temperature incubator; temperature in a microplate well was measured using a

microthermister before and after assay. Data was analyzed using the microplate analysis program (MPA) (Brooks, 1994) and a kinetics program (Brooks, 1992).

Gel electrophoresis by SDS-PAGE was performed according to Laemmli (970) using a Bio-Rad mini gel system (Protean 2). Samples were desalted by benchtop centrifugation through small columns of Sephadex G-10 followed by concentration in a Centricon 10 concentrator (Amicon). Samples were then dissolved in reducing buffer containing 50 mM dithiothreitol (DTT), 20% v/v glycerol, 1% w/v SDS, 50 mM Tris pH 8.8 and 0.04% w/v bromophenol blue. After boiling for 5 min, samples were loaded and run on 10% acrylamide gels with broad range molecular weight standards (Bio-Rad). Blotting to PVDF (Immobilon-N, Millipore) was done in an Owl semi-dry blotter at 500 mA for 45 min in 25 mM Tris, 192 mM glycine and 10% v/v methanol.

Protein sequencing was done on either a Beckman 6000 Prosequencer or an Applied Biosystems 470 protein sequencer with an online 163 PTH analyzer. Samples were sequenced from both PVDF (blotted) and liquid according to manufacturer's specifications and instructions.

Fluorescence studies were conducted in quartz cuvettes using purified AspAT (20 μ g in all cases) in 20 mM Tris buffer pH 7.5 + 1 mM EDTA; total volume was 3 ml. A temperature-controlled cell holder with stirring was used to mix the sample; stirring was stopped during fluorescence measurements. For denaturation studies, guanidine hydrochloride (GuHCl) was added to the cuvette with stirring and then spectra were recorded after 30 minutes of equilibration. For temperature studies, temperature was increased slowly (0.5°C/ min) and then samples were equilibrated for 15 min prior to measuring spectra. Emission spectra were collected using a Perkin Elmer LS50B fluorometer. Excitation was at 280 nm and spectra were scanned between 300 and 400 nm with a slit width of 2.5 nm and a scan rate of 50 nm min⁻¹. All spectra were corrected for buffer and water emissions by subtraction of the appropriate blank before

determination of relative intensity (I) and emission maximum (λ_{max}). Renaturation studies were done by dissolving the enzyme in a small volume (100 μl) of 3 or 6 M GuHCl for 35 minutes and then rapidly diluting sample with buffer to a final volume of 3 ml. Samples were equilibrated for 1 hour prior to fluorometry or enzyme assay.

Results

The purification protocol showed some interesting features of ground squirrel skeletal muscle AspAT. The enzyme did not bind to any of several ion exchange columns that were tried. For example, the enzyme would not bind to the cation exchanger (Resource S) at pH values as low as 5 or to the anion exchanger (Resource Q) at pH values as high as 9.5 in dilute buffers (10 mM). However, although AspAT would not bind, various contaminating proteins would, and hence the Resource Q column proved useful in the overall purification scheme. The protein bound well to phenyl-Sepharose at 1.0 M salt, but did not bind to propyl-, butyl-, or ethyl-Sepharose. The AspAT did not elute until late in the reverse gradient (almost 0 mM salt) which provided a good way of removing salt from the enzyme preparation and eliminating substantial contamination (Table 4-1). The phenyl-Sepharose column resolved two peaks of AspAT activity. The first peak (isozyme 1) to elute contained about 5 % of the total activity and the second peak (isozyme 2) comprised 95 % (Table 4-1). This distribution was noted for extracts from both hibernating and euthermic animals. Separation of the phenyl-Sepharose peak 2 by reverse phase HPLC yielded 3 peaks. Sequence analysis of the peaks indicated that the most abundant protein was creatine kinase, the second was

AspAT by N-Terminal sequence homology, and peak 3 was N-terminally blocked.

Chromatography with blue Agarose and Resource Q removed creatine kinase and the other protein (peak 3), respectively. The final purified preparation of isozyme 2 resulting after size exclusion HPLC was essentially the same for the enzyme isolated from muscle of hibernating versus euthermic ground squirrels; purification was about 150-fold with a 25-30 % yield and a final specific activity of 502-515 units/mg protein (Table 4-1). The specific activity of the purified isozyme 1 after the same purification procedure was 465 U/mg protein. Analysis on SDS-PAGE of purified isozymes 1 and 2 showed the presence of a single band with a subunit molecular weight of 54 ± 2 kDa (Figure 4-1A). Figure 4-1A shows an equal protein mixture of the hibernating and the euthermic isozyme 2 (the major component) from phenyl-Sepharose chromatography. N-terminal sequence analysis of the purified isozyme 2 from both euthermic and hibernating ground squirrels showed a sequence: _APPSVFAEVPQA(E or Q) which matches well with the sequences of cytosolic AspAT from other mammals as provided on the Swiss protein data base. Attempts to sequence isozyme 1 proved unsuccessful. It appeared to be N-terminally blocked but the protein had a molecular weight of 58.3 kDa by SDS-PAGE, which is very similar in size to the mitochondrial form of AspAT (which tends to be slightly larger than the cytosolic form).

SEC removed two low molecular weight contaminants from the commercial porcine heart AspAT preparation prior to use and hence, the ground squirrel enzyme was also run on this column prior to use to place both enzymes in the same final buffer.

Native molecular weights for the enzymes were determined by SEC to be 105 ± 3 kDa,

108 $\pm$ 3 kDa and 103 $\pm$ 3 kDa for ground squirrel isozymes 2 and 1 and the porcine enzyme, respectively. A small amount of isozyme 1 was purified to homogeneity for evaluation of specific activity, SDS-PAGE, reverse phase HPLC and N-terminal sequencing and results reported from this enzyme are from this purified sample. The two isozymes eluted on reverse phase HPLC at 49.6 minutes for isozyme 2 and at 50.8 minutes for isozyme 1 (Figure 4-1B). The relative peak areas for the two isozymes approximated the proportions of their activity in the two peaks eluted from the phenyl-Sepharose column. The long retention (elution at 63-68 % acetonitrile) of these peaks supports AspAT being a hydrophobic protein. The porcine enzyme had a similar elution profile as the ground squirrel on reverse phase HPLC but a slightly lower molecular weight than the ground squirrel enzyme by SEC and SDS-PAGE. This indicates that there similarities between the porcine AspAT and Isozyme 2 from ground squirrels. The low yields of isozyme 1 made structural studies impractical and thus, the dominant form of AspAT, isozyme 2, was used for the bulk of the studies (Table 4-1). Isozyme 2 appeared to be the same enzyme in both euthermic and hibernating tissues. This was supported by reverse phase HPLC (49.6 $\pm$ 0.1 min retention), SDS-PAGE (single band with a mw 55.9 $\pm$ 2 kDa for mixed samples), and SEC HPLC (103 $\pm$ 3 kDa for the native protein) which were all unable to resolve mixtures purified AspAT from hibernating versus euthermic animals.

The pH profiles for the euthermic ground squirrel as well as pig AspAT both showed wide optima when assays were conducted at 37°C (Figure 4-2A). The ground squirrel enzyme showed a wide pH range (pH 6.6-8.3) over which activity was above

50% of the maximum, which occurred at pH 7.9. A reduction in assay temperature to 5°C caused the pH optimum to shift slightly to 8.2, but the general shape of the curve remained similar (Figure 4-2B). At both temperatures activity dropped off rapidly below pH 7. The porcine enzyme showed an optimal pH near 7.1 at both temperatures although at low temperature the profile was sharper (Figure 4-2).

The comparison of the substrate affinities for aspartate and α -ketoglutarate of AspAT from muscle of hibernating and euthermic ground squirrels did not reveal any significant differences between the enzyme from the two sources at either high or low assay temperatures (Table 4-2). However, temperature change did elevate the K_m for α -ketoglutarate of both enzymes, K_m increasing by 3.4- and 4.0-fold at 5°C for the euthermic and hibernating enzymes, respectively. The porcine enzyme showed a similar K_m for α -ketoglutarate to the ground squirrel enzymes at 37°C but its K_m did not change when temperature was lowered (Table 4-2). However, the K_m for aspartate differed significantly from the ground squirrel enzymes at both temperatures and rose by 3.1-fold with the decrease in assay temperature to 5°C.

Figure 4-3 shows the effect of assay temperature on the K_m for α -ketoglutarate (α -KG). At temperatures between 12 and 37°C, the K_m α -KG of both ground squirrel enzymes remained relatively constant between 0.15 and 0.23 mM with no difference noted between the enzyme from hibernating or euthermic animals. Below 12°C, however, K_m values increased sharply reaching about 0.9 mM at 5°C and rising to 2.3 mM at 0°C.

Figure 4-1. (A) SDS-PAGE gel of purified ground squirrel skeletal muscle and commercial porcine heart AspAT stained with Coomassie blue. Lanes 1 and 4 contain broad range molecular weight standards. Lane 2 shows ground squirrel muscle AspAT after the Resource Q ion exchange purification step; the sample shown was a mixture of equal amounts of protein of hibernating and euthermic isozyme 2. Lane 3 shows porcine AspAT after desalting but with no other purification. (B) The reverse phase chromatogram of the final preparations of ground squirrel and porcine AspAT after SEC chromatography. The ground squirrel sample was a equal volume mixture of AspAT isozymes 2 and 1 which eluted at 49.6 and 50.8 min, respectively

Figure 4-2. pH optima for purified euthermic ground squirrel and porcine AspAT at 37°C (A) and 5°C (B) in the forward direction. Data are means $\pm$ SEM, n=3

Table 4-1. A typical purification for AspAT from the hibernating and the euthermic ground squirrel.

Tissue	Total Protein (mg)	Total Activity U	Specific Activity U/mg	Fold purification	Yield (%)
Hibernating					
Muscle crude (cytosol)	2560	8475	3.3		
Ammonium sulphate 50-70% saturation	496	7543	15.2	4.6	89
Phenyl Sepharose*	57	6102	106	32	72
Cibacron Blue agarose	17	5169	305	92	61
Resource Q	7.8	3813	492	149	45
SEC-HPLC	4.2	2119	515	156	25
Euthermic					
Muscle crude (cytosol)	2631	8930	3.4		
Ammonium sulphate 50-70% saturation	461	6786	14.7	4	76
Phenyl Sepharose*	37	5625	150	44	63
Cibacron Blue agarose	15	4286	282	83	48
Resource Q	7.7	3661	478	141	41
SEC-HPLC	5.3	2679	502	147	30

*Phenyl-Sepharose separated two isozymes; isozyme 1 represented about 5% of the total activity and isozyme 2 comprised 95%. For the remainder of the purification scheme, the data shown is for isozyme 2 only.

Table 4-2. Km values for the forward reaction of AsP_{AT} from skeletal muscle of euthermic and hibernating ground squirrels and porcine heart at two temperatures, 37 and 5°C.

Tissue	Temperature (°C)	Aspartate Km (mM)	α -ketoglutarate Km (mM)
Ground squirrel Euthermic	37	10 $\pm$ 0.72	0.257 $\pm$ 0.01
	5	9.63 $\pm$ 0.28	0.883 $\pm$ 0.0053 ^b
Ground squirrel Hibernating	37	11.3 $\pm$ 0.36	0.236 $\pm$ 0.0041
	5	8.75 $\pm$ 0.74	0.936 $\pm$ 0.0085 ^b
Porcine	37	5.5 $\pm$ 0.32 ^a	0.397 $\pm$ 0.023
	5	17.3 $\pm$ 1.4 ^{ab}	0.289 $\pm$ 0.041

Values are means $\pm$ SEM, n=4. a – Significantly different from the AsP_{AT} of the other species, P > 0.05; b – significantly different from the corresponding value at 37°C for the same enzyme, P > 0.05.

Porcine AspAT behaved differently, showing increased K_m values over the range 18-37°C but rising sharply to about 0.9 mM at 15-17°C. The K_m value returned to about 0.2 mM at 3-10°C but increased again at 0°C. K_m values for α -KG differed between the species at all temperatures between 0 and 17°C ($P < 0.05$), except at 12°C.

The effect of salts and reaction products on the activity of AspAT is detailed in Table 4-3.

The three salts examined, NaCl, KCl, and $(\text{NH}_4)_2\text{SO}_4$, all activated AspAT. In all cases the K_a was high, greater than 100 mM, and the fold activation at optimal salt concentrations was between 1.5 and 2.8 fold. The K_a values for NaCl and KCl of the ground squirrel enzymes increased significantly when assayed at 5°C, rising by 31-49 %, compared with corresponding values at 37°C. K_a KCl of the porcine enzyme also rose by 35 % at the lower assay temperature, but K_a NaCl fell at low temperature. The effect of temperature on K_a $(\text{NH}_4)_2\text{SO}_4$ was opposite, the K_a decreasing significantly at 5°C for all three enzymes and falling to 55-60% of the value at 37°C. The fold activation values for NaCl, KCl, $(\text{NH}_4)_2\text{SO}_4$ were 1.52, 2.03, 2.46, respectively, and were unaffected by temperature for the ground squirrel enzymes. The reaction products, glutamate and oxaloacetate inhibited all three enzymes although at very high concentrations which, especially for oxaloacetate, are much higher than concentrations in vivo (that are typically < 0.1 mM). The I_{50} values for glutamate were also high relative to in vivo concentrations of the amino acid, which are usually in the 1-5 mM region. I_{50} values for both products were the same for AspAT from both hibernating and euthermic animals.

Table 4-3. The effects of salt and enzyme products on the activity of purified AspAT from the ground squirrel and pig heart muscle at 5 and 37°C.

Enzyme source	Hibernating			Euthermic			Porcine		
Compound	Temperature e (°C)	Effect I ₅₀ /Ka	Value mM	Effect I ₅₀ /Ka	Value mM	Effect I ₅₀ /Ka	Value mM		
NaCl	37	Ka	250 ± 15	Ka	223± 9.8	Ka	398 ± 23a		
	5	Ka	328 ± 22b	Ka	332 ± 15b	Ka	223 ± 18ab		
KCl	37	Ka	178 ± 11	Ka	200 ± 6.3	Ka	140 ± 3.5a		
	5	Ka	258 ±, 19b	Ka	272 ± 10.2b	Ka	189 ± 16a		
Ammonium sulphate	37	Ka	321 ± 21	Ka	298 ± 5.8	Ka	351 ± 9.5		
glutamate	5	Ka	180 ± 6b	Ka	164 ± 1.2b	Ka	214 ± 15b		
	37	I ₅₀	23 ± 0.96	I ₅₀	25 ±0.36	I ₅₀	53 ± 2.3a		
oxaloacetate	5	I ₅₀	51 ± 1.2b	I ₅₀	48 ± 0.29b	I ₅₀	28 ± 1.6ab		
	37	I ₅₀	103± 5.2	I ₅₀	126 ± 10	I ₅₀	76 ± 1.2a		
	5	I ₅₀	120 ± 9.4	I ₅₀	98 ± 6.3	I ₅₀	48 ± 0.27ab		

Values are means ± SEM, n=4. Significance denoted by, a, is different from the AspAT from the other species at $P>0.05$ and b, indicates a significant difference occurring with assay temperature, $P>0.05$.

Figure 4-3. The change in K_m for α -ketoglutarate with assay temperature for muscle AspAT from hibernating and euthermic ground squirrels and pig heart. Data are means $\pm$ SEM, $n=4$.

Arrhenius plots ($\log V$ versus $1/^\circ\text{K}$) for ground squirrel and pig AspAT were constructed from measurements of $V_{\max}$ activities at 15 temperatures between 2 and 45°C. All plots were linear over the full range tested, showing no breaks in slope. Activation energy, calculated from the slope of the line, was 8.34 ± 0.098 kcal/mole for the euthermic enzyme and 7.85 ± 0.12 kcal/mole for the hibernating enzyme (each $n = 6$), values that were not significantly different. The porcine enzyme had an E_a of 14.5 ± 0.53 kcal/mole ($n=6$), significantly different from the ground squirrel enzyme ($P<0.005$).

Spectral comparisons by fluorescence of purified AspAT from muscle of euthermic and hibernating animals showed no differences in shape or $\lambda_{\max}$ under a variety of conditions. Spectra were compared at three temperatures (2°C, 37°C, 60°C), and in the presence of varying concentrations of guanidine hydrochloride (GuHCl = 0.0, 1.5, or 6 M). Normalization of the spectra for each condition, to compensate for variations in protein concentration, resulted in spectra, which were in all cases indistinguishable.

However, the fluorescence spectra of the ground squirrel (euthermic muscle) and porcine enzymes were similar in shape but had different $\lambda_{\max}$ values when assessed at 2°C (Figure 4-4). The spectra of ground squirrel and porcine AspAT could not be superimposed at 2°C because of the differences in the 1st and 2nd derivatives of the spectra both above 344.2 nm and below 320 nm. The $\lambda_{\max}$ of 333.8 nm and 338.0 nm for the ground squirrel and porcine enzymes, respectively, was the major difference between the two spectra at 2°C (Figure 4-4).

The effects of temperature on the spectra of AspAT showed indications of structural and stability differences between the porcine and ground squirrel enzymes (Figure 4-5). The ground squirrel enzyme showed considerably larger changes in the λ_{max} and intensity values with temperature than did the porcine enzyme. These indicated a higher thermal stability of the conformation of porcine AspAT. The ground squirrel and porcine enzymes showed similar temperature profiles at low and intermediate temperatures (2 and 37°C). Above 40°C, however, the ground squirrel showed larger and more rapid spectral shifts with increasing temperature.

Figure 4-6 compares the effects of temperature on the normalized fluorescence intensity and the activity of ground squirrel (A) and porcine (B) AspAT. Both enzymes showed a decrease in fluorescence intensity with increasing temperature. This relationship was linear for the porcine enzyme (slope = 0.00832), whereas the ground squirrel enzyme showed a two-phase response, with a slope of 0.0087 over the range 2 to 46°C which doubled to 0.019 between 50 and 60°C. The reduction in intensity that started at 50°C resulted in a total decrease in I of 38% over 11°C (Figure 4-6A). Corresponding changes in the activity profile showed an optimal temperature range from 27 to 36°C for the ground squirrel enzyme and a distinct optimum at 34°C for the porcine enzyme. Furthermore, the ground squirrel enzyme retained significantly higher relative activities ($P < 0.05$) than the porcine enzyme at temperatures ranging from 18 to 35°C (Figure 4-6). The temperature maxima for both enzymes occurred at 34°C which was followed by a sharp decline in activity which became undetectable by 42°C. Maximal enzyme activity did not correspond to a significant change in either the I or λ_{max} for

either enzyme (Figure 4-6 and 7). Figure 4-7 shows the effect of temperature on the λ_{max} of the enzymes. The ground squirrel enzyme showed a consistent red shift trend starting near 31°C, which increased dramatically in slope at 47°C (corresponding to the complete loss of enzymatic activity; Figure 4-6). The porcine enzyme did not show a significant or abrupt change in λ_{max} over the 2°C to 61°C temperature range examined (Figure 4-6B). Both enzymes showed very dramatic red shifts at high temperatures starting at 47°C for the ground squirrel and 52°C for the porcine enzyme (Figure 4-7). This change in the λ_{max} indicates a substantial change in the protein conformation occurring at these temperatures which in the case of the ground squirrel was within a few degrees of the loss of activity. The red shift in λ_{max} represented an increase of 5.5 nm from 336.0 to 341.5 for the porcine enzyme, whereas the shift for the ground squirrel enzyme was 8.7 nm from 332.8 to 341.5 (Figure 4-7). The dramatic change in the λ_{max} for both enzymes occurred at a higher temperature than the loss of activity. This rapid change in λ_{max} indicates a substantial change in the structure of AspAT from both animals.

Chemical denaturation with GuHCl produced several changes in the emission spectra of AspAT (Figure 4-8). The presence of 5 mM DTT in the 6 M GuHCl did not alter the λ_{max} or intensity (spectrum not shown), indicating that there are no disulphide bonds that affect the spectrum of either the ground squirrel or the porcine enzyme at 6 M GuHCl.

Figure 4-4. Normalized fluorescence emission spectra of porcine and ground squirrel AspAT at 2°C.

Figure 4-5. The fluorescence emission spectra of ground squirrel (I) and porcine (II) AspAT at three temperatures 0, 37, 61°C.

Figure 4-6. The effect of temperature on fluorescence intensity and maximal activity of ground squirrel (A) and porcine (B) AspAT. Activities are shown relative to the maximal activity. Activities were measured from an aliquot (25 μ l) of the enzyme after recording of the spectra from the fluorometric cell. Temperature of the assay cuvette was maintained within $\pm 2^{\circ}\text{C}$ of the temperature in the fluorometric cell.

Figure 4-7. Effect of temperature on the λ_{max} of ground squirrel and porcine AspAT. This plot illustrates the changes in maximal emission wavelength during thermal denaturation of AspAT.

Figure 4-8. The fluorescence emission spectra of AspAT at several concentrations of GuHCl. The spectra were run at 20°C for ground squirrel (I) and porcine (II) AspAT. The concentrations of GuHCl shown are: A, 0 M; B, 1.0 M; C, 2.5 M; D, 6.0 M

The emission spectrum for ground squirrel AspAT showed an increase in the intensity and a red shift of the λ_{max} with increasing concentrations of GuHCl. The same relative pattern was seen for the porcine enzyme. Figure 4-9 compares the shift in λ_{max} with the decrease in maximal activity of the enzyme with increasing GuHCl. The ground squirrel enzyme was completely inactivated in the presence of 1.16 M GuHCl and the porcine by 1.0 M GuHCl. Activity losses in both cases corresponded with the red shift of λ_{max} which rose from about 339 nm in the absence of GuHCl to 350 nm for ground squirrel and 347 nm for porcine AspAT at the GuHCl concentration at which all enzymatic activity was lost. Higher concentrations of GuHCl resulted in a continuing steep increase in λ_{max} for both enzymes up to about 360 nm at 3 M after which only a small increase in λ_{max} of about 2 nm occurred up to 6 M. The effects of GuHCl on fluorescence intensity are shown in Figure 4-10. For the ground squirrel enzyme the fluorescence intensity reached a minimum of 8.27 at 0.16 M and then climbed to 12.65 at 1.5 M GuHCl, with a particularly dramatic increase in the intensity between 0.61 and 1.5 M GuHCl which corresponded with loss of activity seen in Figure 4-9. Further increases in GuHCl concentration led to a continuing rise in intensity up to 6 M GuHCl. The porcine enzyme showed the major increase in fluorescence intensity over a lower GuHCl concentration range (0-0.32 M), followed by a general plateau up to 2 M and then a further increase at higher GuHCl (Figure 4-10).

Analysis of the effects of GuHCl on the apparent molecular weight of the enzymes as determined by SEC indicated that a reduction in the molecular weight of the ground squirrel enzyme occurred close to 1.48 M GuHCl. The molecular weight was

stable at 87 ± 3 kDa up to 1.36 M after which a decrease to 79 ± 3 kDa was observed. At 1.48 M GuHCl the molecular weight had dropped to 56.4 ± 4 kDa suggesting that separation of the dimeric AspAT into monomers had occurred. SEC chromatography of the porcine enzymes showed a stable molecular weight of 85 ± 3 kDa at low GuHCl concentrations, but a drop to 72 ± 3 kDa at 1.16 M. At 1.32 M GuHCl, molecular weight was further reduced to 50.5 ± 3 kDa with no further changes up to 6 M denaturant.

Figure 4-9. Effect of chemical denaturation with GuHCl on the λ_{max} and maximal activity of AspAT for the ground squirrel (A) and porcine (B) enzymes. Spectra were run at 20°C after allowing 30 min for sample equilibration with the denaturant. Samples of protein from the fluorometric cuvette (150 μl) were then taken and assayed at the same final GuHCl concentration at 20°C within 10 min of the spectra being run.

Figure 4-10. The effect of GuHCl concentration on the emission intensity of ground squirrel and porcine AspAT

Discussion

AspAT purified from the muscle of hibernating versus euthermic ground squirrels had identical molecular weights as judged both by SEC (native) and SDS-PAGE (denatured). Furthermore, neither phenyl-Sepharose chromatography nor reverse phase HPLC were able to separate mixtures of the hibernating and euthermic forms of either isozyme 1 or 2. Finally the N-terminal sequence of isozyme 2 was the same for the euthermic and hibernating enzymes and both isozyme 1 proteins were N-terminally blocked. Hence, the evidence leads to the conclusion that neither isozyme 1 nor 2 was modified in the hibernating state. However, ground squirrel skeletal muscle clearly contained two isoforms of AspAT and these were separable by phenyl-Sepharose chromatography and reverse phase HPLC (Figure 4-1). There was a slight difference in the native size of the two isozymes and a slight difference in subunit molecular weight as judged by SDS-PAGE. Many other studies have noted multiple forms of AspAT in mammalian tissues, and both cytosolic and mitochondrial forms occur in most tissues (Sonderregger et al., 1982; Kuramitsu et al., 1985; Obaru et al., 1986, 1988; Horio et al., 1988; Nagashima et al., 1989; Reyes et al., 1993). It is reasonable to suggest that isozyme 1 is the mitochondrial form of the enzyme, although the N-terminal block on isozyme 1 did not allow comparison of the sequence homology for identification. There was also no apparent change in the relative amounts of the isozymes 1 and 2 during hibernation. The lack of hibernation-induced modification of the enzyme was further supported by the fact that the enzymes (isozyme 2) purified from muscle of hibernating

versus euthermic ground squirrels showed no significant differences in K_m values for the substrates of the forward reaction (aspartate and α -ketoglutarate) when assayed at either euthermic (37°C) or hibernating (5°C) body temperatures (Table 4-2). However, both enzyme forms differed in K_m values from the porcine enzyme and whereas the K_m for aspartate of the porcine enzyme was affected by temperature, it was the K_m for α -ketoglutarate of the ground squirrel that was temperature-sensitive. The K_m values for the porcine heart enzyme determined here are similar to the results reported in other studies of the porcine heart enzyme which reported K_m values of 2.0 to 4.0 mM for aspartate and 0.15 to 0.2 mM for α -ketoglutarate at 20°C (Martinez-Carrion et al., 1967; Banks et al., 1968).

The effect of temperature on the K_m values for α -KG show further differences between the ground squirrel and porcine enzymes (Figure 4-3). The K_m of the porcine enzyme was largely stable from 0 to 37°C except for a transient increase at 15-17°C. However, the ground squirrel enzyme showed a sharp increase in K_m α -KG at temperatures below 12°C that lead to a nearly 10-fold increase in K_m at 0°C, compared with the enzyme assayed at higher temperatures. The effects of temperature change on the pig heart enzyme would have little or no physiological relevance for a core organ in an endothermic animal. However, changes in body temperature over at least a 30°C range during the hibernation cycle of ground squirrels is common. Hence, temperature effects on the properties of enzymes could be very important in modifying enzyme function and metabolic flux through different pathways in the hibernating state. The increase in K_m α -KG of the ground squirrel enzyme at 5°C would reduce the affinity of the enzyme for

this substrate at the low temperature. This could reduce enzyme function in the hibernating state, although other factors including substrate availability, modulator effects and enzyme amount would also influence the role of the enzyme in the hibernating animal. Indeed, analysis of the maximal activities of multiple enzymes in several tissues of the golden-mantled ground squirrel (*Spermophilus lateralis*) showed increased maximal activities of AspAT in tissues of hibernating animals that also showed elevated protein catabolism during hibernation (Lyman et al., 1982; Wang, 1989). At temperatures below 5°C the K_m for α -KG was very large, suggesting a way of regulating AspAT activity based on cellular α -KG concentrations by shifting the reaction equilibrium. The use of amino acids for fuel (after conversion to glucose via gluconeogenesis) occurs mostly when the animal is rewarming and undergoing shivering thermogenesis and in the euthermic state to maintain blood glucose levels during periods of arousal (Tashima et al., 1970; De la Roche, 1995). Hence, the most important adaptation of ground squirrel AspAT is probably the increased temperature optima, which allows near maximal activity starting at 26°C (Figure 4-6).

Product inhibition and activation by salts at both high and low temperature affected AspAT activity (Table 4-3). The changes to K_a values for the salts with temperature are physiologically relevant with K_a values between 180 to 398 mM. This data could also indicate that AspAT may be stabilized by the presence of salts such as NaCl, KCl, and $(NH_4)_2SO_4$; this effect is well known for other proteins. Of these salts the most probable salt to have a stabilizing effect in vitro was $(NH_4)_2SO_4$ since this salt is a well known stabilizer of enzyme conformation. Stabilization by NaCl and KCl are of

physiological significance since AspAT is exposed to these salts *in vivo*. The concentration of K^+ is high in the cell and Na^+ is high in other fluids. Finally, AspAT is a relatively hydrophobic protein and it is possible that high ion levels promote subunit assembly and/or makes subunit separation less favorable. Inhibition by glutamate and oxaloacetate occurred at extremely high concentrations (Table 4-3). AspAT showed the lowest I_{50} values for glutamate with an increase in this I_{50} occurring at 5°C for the ground squirrel enzyme and a decrease for the porcine enzyme. There was little change in the I_{50} oxaloacetate value of the ground squirrel enzyme with temperature although the porcine enzyme showed a decrease in I_{50} at low temperature. However, there is little physiological relevance of inhibition by either oxaloacetate or glutamate, since the levels of neither of these compounds *in vivo* approach levels that would be inhibitory to the enzyme.

The spectra of ground squirrel AspAT showed indications that the Trp residues are more shielded in this enzyme than in the porcine form (Creighton, 1989). The λ_{max} was significantly lower for the ground squirrel enzyme at 2°C than the porcine (Figure 4-4) and this suggests that the structure of the ground squirrel enzyme is more compact or tighter at low temperature (Creighton, 1989). This would help to counter the effects of cold, which normally cause proteins to relax and open their structure slightly (Somero, 1995, 1996). The loosening of structure with cold is the result of a weakening of hydrophobic bonds at low temperature (Douzou, 1977; Comstock, 1979). As the temperature increased there was little change in the λ_{max} up to the temperature at which thermal denaturation of the protein began, but a constant decrease in intensity was

observed with increasing temperature (Figure 4-7). The rapid change in the λ_{max} and the fluorescence intensity of ground squirrel AspAT at 50°C suggests some radical changes to protein conformation at high temperature (Creighton, 1989). The increase in the λ_{max} indicates an opening of the protein structure and increasing the exposure of Trp residues to water. The decrease in intensity could be a sign of quenching of the Trp fluorescence resulting from a refolding of the protein (Reyes et al., 1993). This is a typical pattern of thermal denaturation of a protein where heat can cause precipitation, aggregation, and random refolding (Creighton, 1989; Somero, 1995, 1996). By this temperature (50°C) a total loss of enzyme activity had already occurred indicating that enzyme activity is more temperature sensitive than the structure. Overall the proteins from both animals were relatively similar with respect to increasing temperature (Figure 4-6 and 7). The lower temperature stability and broad temperature (favoring low temperature) range of activity of the ground squirrel enzyme could provide an advantage in helping to promote protein catabolism at the height of shivering thermogenesis. Coupled with enhanced gluconeogenesis, this would have a clear advantage in providing energy (glucose) to the muscles. The lower thermostability of this protein would not be a detriment to metabolism. The enzyme might have a higher turnover rate than other AspATs with a higher thermostability. The increased low temperature activity would be an advantage in the waking and rewarming process for ground squirrels when the activity of AspAT is required for gluconeogenesis.

Guanidine hydrochloride affected the structure and activity of the porcine enzyme at lower concentrations than it affected the ground squirrel enzyme. An increase in

fluorescent intensity was seen at low GuHCl concentrations for both enzymes but the porcine enzyme was more dramatically affected than the ground squirrel one (Figure 4-7). Activity of the ground squirrel enzyme was also slightly more resistant to the denaturant than the porcine enzyme (Figure 4-6). The increase in fluorescence intensity during chemical denaturation is an indication of fluorescent quenching occurring in the native state of AspAT. Quenching has been attributed to the presence of pyridoxal phosphate (PP) in the protein, which is thought to be incorporated in the AspAT subunit during folding. It is located in the core of the protein near Trp 140 in the *E. coli* enzyme (Nagashima et al., 1989). The PP is also in close proximity to the active site of the enzyme. Trp 140a is located very close to the PP which could cause quenching (Horio et al., 1988; Nagashima et al., 1989; Reyes et al., 1993). Other fluorescent studies of AspAT have suggested that the Trp fluorescence was quenched with PP being the primary suspect (Reyes et al., 1993). The increase in intensity observed with chemical denaturation of AspAT might be an indication of movement of the protein near PP, resulting in a larger contribution from the active site Trp to the emission spectra. Furthermore, since the active site is at the subunit interface, perturbations of the subunits could increase the exposure (contribution) of any of the other 9 Trp in each subunit (Horio et al., 1988). The fully denatured AspAT (in 6 M GuHCl) showed a dramatic increase in intensity and a characteristic red shift of the λ_{max} suggesting a highly denatured state. This is supported by the final λ_{max} value (362 nm), which is very close to that of Trp dissolved in water (360 nm). Separation of the subunits of AspAT occurs near 1.46 M GuHCl as determined by SEC, which is after the total loss of enzymatic

activity. The λ_{max} and I both show stabilization near and after (respectively) the subunit separation. The stabilization of the λ_{max} was more pronounced for the ground squirrel enzyme (Figure 4-9), indicating a higher resistance to chemical denaturation for this enzyme compared with the porcine enzyme. Furthermore, it suggests that the denaturation of ground squirrel AspAT occurs as a multi-step process, each of which has a different resistance to GuHCl. Both enzymes then showed significant changes in the λ_{max} and I, probably due to the unfolding of strong 3° and 2° structures in the presence of GuHCl (Figure 4-9 and 10). Potential intermolecular disulfides that were present in the native enzyme did not alter the emission spectrum in the presence of 6M GuHCl since the addition of 5.0 mM DTT did not significantly alter the Trp emission spectrum.

Furthermore, the expected molecular weight shift with subunit separation suggested that there were no intermolecular disulphides in either enzyme. Neither of the AspATs examined showed a radical departure from previous studies with the mitochondrial precursor or mature enzyme, although slightly higher tolerances of the activities to GuHCl were observed with the ground squirrel and porcine enzyme as compared to previous studies with the mitochondrial enzyme (Reyes et al., 1993). The increase in intensity with increasing GuHCl was also observed with the mitochondrial AspAT (Reyes et al., 1993). This suggests that the enzymes share conformational similarity, as far as the shielding of some Trp in the native conformation. The increase in I during chemical denaturation is thought to occur because of shielding of a Trp in the native conformation by pyridoxal phosphate (Reyes et al., 1993). This is also likely to occur in

the cytosolic AspATs since there is a large degree of catalytic and active site homology between the two enzymes.

It is clear that the AspAT proteins isolated from muscle of hibernating and euthermic ground squirrels are the same enzyme. All of the evidence from physical and activity studies confirms that there was no change to AspAT during hibernation. Overall, the ground squirrel AspAT showed a greater stability to GuHCl denaturation and less thermal stability than the porcine enzyme. The ground squirrel enzyme also showed a broader temperature optimum with near maximal activity occurring over a 4°C temperature range, and this showed that as the need for amino acids increases during warming the enzyme activity increases (Figure 4-6). This did not occur with the porcine AspAT, which had a narrow temperature optimum. Both temperature activity profiles were similar although were more spread out with the ground squirrel enzyme to favor activity at lower temperatures. The increased low temperature activity would be an asset to the rewarming of the ground squirrel muscle and may represent an adaptive change in this enzyme. The E_a for ground squirrel AspAT was significantly lower than the corresponding value for the porcine enzyme. This is potentially an adaptive change in the enzyme; with a lower activation energy, catalysis could occur with less energy input into the system and at a lower temperature which can be seen in Figure 4-6. Since AspAT is an equilibrium enzyme and not a regulatory point in metabolism, there are not a lot of physiological effectors of its activity. Substrate inhibition could occur since glutamate and oxaloacetate showed inhibitory effects but these effects would be minimal because the I_{50} values for these compounds are so large (Table 4-3).

Overall, the differences between the ground squirrel and porcine enzyme are probably a mixture of tissue and species differences. There are structural and kinetic differences between the two enzymes but these changes are not striking examples of cold adaptation. The broadening of the temperature optima and lower E_a are the only potential evidence for temperature adaptation in AspAT. There are a variety of kinetic and structural differences between the porcine and ground squirrel AspAT. Furthermore, there is no difference between the hibernating and euthermic forms of AspAT indicating that there is no need to modify AspAT structure or activity to compensate for a different metabolic role. Varying the amount of this enzyme in tissues (De la Roche, 1995; Lin et al., 1997) and well defined factors like Q_{10} effect, pH changes, are probably enough to control this enzyme activity *in vivo*.

CHAPTER 5: Purification and Kinetics of Glutamate Dehydrogenase

Introduction

Glutamate Dehydrogenase (GDH, EC 1.4.1.3) catalyzes the following reaction:

The mammalian enzyme uses both forms of nicotinamide nucleotide with comparable facility (Frieden, 1965; Goldin and Frieden, 1971; Smith et al., 1975). GDH is an important enzyme in several pathways. It provides an essential link between carbohydrate and amino acid metabolism (Male and Storey, 1982) and has been implicated in the urea cycle (Williamson et al., 1976; Wanders et al., 1983). In mammalian tissues there is only one isozymic form of GDH whereas invertebrates may have two forms of the enzyme, a catabolic and an anabolic GDH (Storey et al., 1978; Male and Storey, 1983). Bovine liver GDH is regulated by the $\text{NAD(P)}^+/\text{NAD(P)H}$ ratio and more specifically by preferring NADP^+ for the oxidative deamination reaction (Male and Storey, 1982).

Glutamate dehydrogenase is involved in at least three pathways: the urea cycle (Wanders et al., 1983; Williamson et al., 1976), amino acid catabolism and amino acid anabolism, glutamate being a key intermediary in the transfer of amino groups to and from other amino acids (Storey et al., 1978; McGivan and Chappell, 1975). GDH is also involved in the transport of carbon across the mitochondrial membrane by the glutamate transport systems (McGivan and Chappell, 1975; Wanders et al., 1983) by providing the glutamate and releasing the α -ketoglutarate at the ends of the transport system. With the

input of the aminotransferases, GDH can be seen as a distribution point for α -amino functionalities (Britton et al., 1992). GDH can feed carbohydrate metabolism from the amino acid pool and *vice versa*. GDH is such a key central locus in anabolism that it requires stringent control to respond to a variety of metabolic conditions. The regulation of GDH has been examined by a number of investigators (LeJohn, 1967; Storey et al., 1978). Glutamate dehydrogenase is regulated by several allosteric effectors *in vivo* including activation by ADP and inhibition by ATP, GDP, and GTP (LeJohn, 1967, 1968; Storey et al., 1978). GDH activity is also controlled by changing substrate concentrations, substrate/product effects and competitive inhibition by selected amino acids (Goldin and Frieden, 1971; Smith et al., 1975; Wanders et al., 1983).

The regulation of enzymatic activity occurs on several levels. First, there is the physical environment of the enzyme: pH, salinity, protein concentration, thermodynamics of the system, and temperature. Secondly, limits are set by the concentrations of substrates, co-factors, and products present *in vivo*. Thirdly, there are the influences of inhibitors and activators, which act either at the active site or at allosteric sites to alter enzyme activity. The combination of all of the above listed factors controls the function of an enzyme *in vivo* to meet cellular needs. The first set (class 1) of factors are normally rigidly maintained in mammalian cells, although local changes in some factors can occur in an organelle or tissue (e.g. pH changes during burst work in skeletal muscle). Widespread changes in any of these factors causes a systemic response, and a large stress on the organism. A change in one of these environmental variables will affect all of the proteins in the system to some degree unless the protein has been adapted

to the altered conditions. The second type (class 2) of effectors function at a distance from GDH. For example, changes in other pathways that alter pH, substrate concentration, will tend to change the flux that the enzyme is maintaining. In the case of GDH this could involve input from glutamate transport, the TCA cycle, and urea production. GDH has three substrates: glutamate, NH_4^+ , and α -ketoglutarate. Glutamate dehydrogenase is a crossover point between amino acid and carbohydrate metabolism and therefore must influence and be influenced directly by the levels of all these substrates. The third type (class 3) of regulation allows metabolism that is not directly connected to GDH to affect the activity of the enzyme, providing input regulating GDH activity from the rest of the cell. These effectors allow the enzyme to be fine-tuned by changing the capabilities of the enzyme through the modulation of the physical characteristics of GDH like protein conformation, K_m , and $V_{\max}$.

The above mentioned classification lists factors that influence metabolic activity and affect GDH activity *in vivo*. In most mammals, metabolism is maintained in a very well controlled internal environment; in fact, mammals have effectively isolated most of their tissues from class one changes. Thus, most proteins need function within only a very narrow set of conditions of temperature, pH, ionic strength, etc. Mammalian hibernation presents an exception to this rule since during hibernation there are dramatic changes in body temperature as well as changes in other class one conditions that are the result of temperature decrease.

In mammals, hibernation produces substantial physiological changes both on a whole animal and a cellular level. The changes in temperature and pH are well known;

core body temperature can drop as low as 5°C and cellular pH changes may or may not follow alaphastat predictions with changing temperature, depending on the tissue (McArthur et al., 1990; Wang, 1989). Metabolic rate may be suppressed by up to 95%, furthermore, during hibernation the animal has no intake of food or water, and does not eliminate wastes (Willis, 1982, 1989). For most of the hibernation cycle the animal is in a state of torpor (Loahr and Risser, 1977; Wang, 1989), but short waking bouts are interspersed during which normal metabolism and body temperature are restored (Nedergaard and Cannon, 1990; Wang, 1989). The hibernating system provides a unique environment for analyzing enzyme function over a wide range of metabolic conditions (McArthur et al., 1990). A key part of arousal from torpor is a major rise in fuel catabolism for the purpose of heat generation. This starts in the brown adipose tissue, which burns lipid to produce heat. As the body warms shivering thermogenesis begins to take over the warming. This results in an incresed level of glycolysis in the muscles to provide the energy for shivering. There is a dramatic rise in metabolism during warming with increases in amino acid catabolism, predominantly Ala and Asp, to feed the TCA cycle or glucogenesis.

Temperature has a significant effect on protein structure by changing bond strengths, causing conformational changes and denaturation. Changes to the structure of a protein will affect the properties, shape and interactions at the catalytic and allosteric sites. Protein conformation is vital for activity and binding of both subunits and small molecules. The cellular conditions of hibernation change the environment of enzymes, but enzymes must continue to perform their metabolic functions properly. The question

is what changes, if any, have been made to GDH from hibernating mammals, as compared with other mammals, or whether changes in enzyme structure/function are even necessary to support enzyme function during hibernation. To answer this question, I undertook a kinetic study of GDH isolated from tissues of euthermic and hibernating ground squirrels and compared the enzyme with its homologue from a nonhibernating mammal in order to highlight any adaptive changes in enzyme structure/function that optimize GDH for function in the hibernating animal.

Methods and Materials

Enzymes (bovine liver glutamate dehydrogenase, and lactate dehydrogenase), α -ketoglutarate and GTP were purchased from Boehringer Mannheim (Montreal, PQ). Buffers and all other substrates were from Sigma Chemical Co. (St. Louis, MO). All water was purified by distillation followed by a Millipore filtration system. Cibacron Blue and GTP agarose chromatographic resins were purchased from Sigma Chemical Co.

Richardson's ground squirrels, *Spermophilus richardsonii*, were captured near Calgary, Alberta in the summer of 1994. They were held in the animal care facility at the University of Calgary on a natural (fall) photoperiod and fed *ad libitum* on a diet of rodent chow supplemented with peanut butter. After two months, half of the animals were induced to hibernate by placing their cages in a cold room at 5°C with access to water but without food. Animals were determined to be truly hibernating if they were torpid for at least five days between periodic arousals. After one month, both euthermic

and hibernating animals were sacrificed. In their last cycle, hibernating individuals were sampled after they had been in torpor for at least 2 days. All animals were killed by decapitation and excised tissues were immediately immersed in liquid nitrogen and subsequently stored at -70°C .

Samples of frozen liver were homogenized in 10 volumes of 20 mM Tris pH 7.5, 5 mM EDTA, 5 mM EGTA, 1 mM PMSF and then centrifuged at $26,000 \times g$ for 25 min at 4°C . The supernatant was removed and applied to a 10 ml Cibacron blue affinity column equilibrated in 20 mM Tris, pH 7.5. After washing until absorbance at 280 nm fell to zero, enzyme was eluted off the column with a linear gradient of 0 to 1 M KCl in 20 mM Tris pH 7.5. The enzyme was then dialyzed against 20 mM Tris pH 7.5 and concentrated by dialysis against polyethylene glycol 8000. After concentration, the enzyme was loaded onto a 5 ml GTP agarose column equilibrated in 20 mM Tris, pH 7.5; after washing until A_{280} fell to zero, GDH was eluted with a 0 to 1 M linear gradient of KCl in the same buffer. Final purification was done by SEC-HPLC in 50 mM phosphate buffer containing 50 mM KCl. The active fraction was then concentrated in a Centricon 100 and stored in 50% v/v glycerol at 5°C .

HPLC separations were performed on a Beckman HPLC system with a UV/VIS detector at either 225 or 280 nm. Reverse-phase separations were done on a Zorbax RP-300 C-8 (2.1 x 150 mm) column with a flow rate of 0.2 ml/min. Samples were loaded at 35% acetonitrile and 65% water containing 0.12% trifluoroacetic acid (TFA) and a linear gradient was run to 65% acetonitrile and 35% water 0.12% TFA over 55 min. An Altex TSK SW 2000 (7.5 x 300 mm) or a TSK SW 3000 (7.5 x 300 mm) column was used for

native molecular weight determination. These columns were run isocratically in 10 mM phosphate pH 7.5 with 100 mM KCl at a flow rate of 0.65 ml/min. The size exclusion columns were calibrated using blue dextran (2000 kDa), thyroglobulin (670 kDa), ferritin (443 kDa), phosphofructokinase (360 kDa), pyruvate kinase (230 kDa), catalase (232 kDa), aldolase (160 kDa), glutathione reductase (124 kDa), hexokinase (102 kDa), adenylate kinase (68 kDa), and triosephosphate isomerase (53 kDa) as molecular weight standards.

Gel electrophoresis: SDS-PAGE was conducted according to Laemmli (1970) using a Bio-Rad mini gel system (protein 2). Samples were desalted through Sephadex G-10 spun columns followed by concentration in a Centricon 100 concentrator (Amicon). Samples were then dissolved in reducing buffer containing 50 mM dithiothreitol (DTT), 20% v/v glycerol, 1% w/v SDS, 50 mM Tris pH 8.8 and 0.04% w/v bromophenol blue. After boiling for 5 min, samples were loaded and run on 10% acrylamide gels with broad range molecular weight standards (Bio-Rad): myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97.4 kDa), serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21.5 kDa), lysozyme (14.4 kDa), and aprotinin (6.5 kDa). Gels were run at 42 mA per gel for 30 min at room temperature. Blotting to PVDF (Imobilon-N Millipore) was done in an Owl semi dry blotter at 500 mA for 45 min in 25 mM Tris, 192 mM glycine and 20% v/v methanol. Gels were stained with 0.1% w/v Coomassie blue R-250 in 50% v/v methanol and 5% v/v acetic acid. Destaining was done in 50% methanol + 5% acetic acid. Blots were stained in the same way although there was no acetic acid in the stain/destain.

Amino acid composition: Samples were hydrolyzed in the gas phase (Picotag workstation Waters) with constant boiling HCl (Pierce) at 110°C for 20-22 h under vacuum. Samples were dissolved in 150 µl of sample buffer (Beckman) of which 50 µl was analyzed on a Beckman 6300 amino acid analyzer according to manufacturer's specifications.

Protein sequencing: Sequencing was conducted on either a Beckman 6000 Prosequencer or an Applied Biosystems 470 protein sequencer with an online 163 PTH analyzer. Samples were sequenced from both PVDF (blotted) and applied to sequencing membranes according to manufacturer's specifications.

Kinetic properties: Kinetic studies used a Dinattech MR 5000 microplate reader to monitor the conversion of NADH to NAD⁺ (or vice versa) at 340 nm; readings were taken 11 seconds apart after an initial shake of 5 seconds. Data was acquired and stored using Bio-links software 55. Assay temperature was regulated by placing the microplate reader in a controlled temperature incubator and well temperature was measured with a microthermister before and after the assays. Data were analyzed using microplate analysis and kinetics programs (Brooks and Storey, 1992; Brooks, 1994). Optimal assay conditions for the forward reaction were: 50 mM glutamate, 1.5 mM NAD⁺ in 20 mM bis-tris propane pH 7.5. In the reverse direction, optimal conditions were 100 mM NH₄⁺, 0.15 mM NADH (in 20 mM bis-tris propane pH 8.0), 7.5 mM α-ketoglutarate, 1 mM ADP, 1 U of LDH and 50 mM Tris, pH 7.5, in a final well volume of 230 µl. Reactions were started by the addition of 20 µl of enzyme solution. When activators and inhibitors were tested, these were dissolved in assay buffer and replaced in an appropriate volume

of buffer in order to keep the total well volume constant. For the pH curves, the buffer used was bis-tris propane because of its wide pH range.

Results

The purification of GDH from *S. richardsonii* used two affinity columns, Cibacron blue and GTP agarose as well as SEC chromatography and resulted in a final specific activity of 190 U/mg for the enzyme from liver of euthermic animals and 201 U/mg for the hibernating enzyme (Table 5-1). These preparations, when run on SDS-PAGE, each produced a single band after staining with Coomassie blue (Figure 5-1). The molecular weight for the two GS enzymes was similar at 59.5 ± 2 kDa and the bovine enzyme was 57.3 ± 2 kDa (Figure 5-1). Native molecular weights for EGDH, HGDH, and BGDH determined by HPLC SEC showed values of 331 ± 3 kDa, 335 ± 3 kDa, and 320 ± 3 kDa, respectively. The bovine and GS enzymes were resolvable in mixed samples (BGDH & EGDH, BGDH & HGDH) by SEC chromatography, but the hibernating and euthermic GS GDHs were not resolvable; in mixed samples of HGDH and EGDH the peak only broadened.

Figure 5-1. SDS-PAGE of GDH from the hibernating and euthermic ground squirrel and the bovine liver. The gel was 10% acrylamide and stained with Coomassie blue R-250. Lane 1 and 5, contained broad range molecular weight standards; lane 2, hibernating GDH; Lane 3, euthermic GDH; lane 4, bovine GDH.

Table 5-1. A typical purification of glutamate dehydrogenase from the liver of the hibernating and the euthermic ground squirrel. The assay for activity was run in the reverse direction as described in the methods and materials.

Tissue	Total Protein (mg)	Total Activity $\mu\text{moles/min}$	Specific Activity $\mu\text{moles/min/mg}$	Fold purification	Yield (%)
Euthermic					
Liver crude (cytosol)	5521	324	0.059		
Blue Agarose	1.66	206	124	2101	24
GTP Agarose	1.06	179	168	2847	55
SEC	0.75	142	190	3220	44
Hibernating					
Liver crude (cytosol)	4125	297	0.72		
Blue Agarose	1.62	187	115	159	63
GTP Agarose	0.7	127	181	251	43
SEC	0.57	115	201	279	39

However, analysis of the purified proteins by reverse phase HPLC showed some slight contamination which amounted to only 1 or 2 % of the total protein (as assessed by relative peak areas from $A_{225\text{ nm}}$ measurements) (Figure 5-2). Because of the minor nature of the contamination, these enzyme preparations were used for kinetic study without any further treatment. Throughout this study, comparisons were made with purified commercial GDH from bovine liver which was diluted 20-fold for use in kinetic studies. Before use, its purity was confirmed on SDS-PAGE (Figure 5-1). The samples of GDH from the GS blotted to PVDF did not give any sequence and thus were N-terminally blocked.

Figure 5-3 shows pH optima for the forward and reverse directions of GDH at two assay temperatures, 37°C and 5°C, and comparing the enzymes from euthermic and hibernating ground squirrels with the bovine liver enzyme. For my purposes, the forward reaction of the enzyme is defined as the one that uses glutamate + NAD^+ whereas the reverse reaction is defined as the one that uses α -ketoglutarate + ammonia + NADH as substrates. In the forward direction at 37°C all three enzymes showed broad pH optima (Figure 5-3A). GDH from euthermic ground squirrels (EGDH) showed near optimal activity over the range pH 7-9.5 and lost activity at acidic pH values, activity falling to 52% on the maximum at pH 6. The enzyme from hibernating ground squirrels (HGDH) or the bovine enzyme (BGDH) showed a different pattern; optimum around pH 6-7 and gradually falling at higher pH values although still retaining 70-80 % of activity at pH 9.5. At low temperature (5°C) the three enzymes all showed virtually the same pH profile with a broad optimal range from about 6 to 9.5 (Figure 5- 3B). However, greater

loss of activity occurred at high pH values, EGDH activity being reduced to zero at pH 9.0 whereas HGDH and BGDH activities dropped to 40–45 % of optimal by pH 9.5. At 37°C in the reverse reaction, the pH profiles showed quite sharp optima at pH 8.0 for EGDH, BGDH, and 8.0–8.5 for HGDH (Figure 5- 3C). At higher or lower pH values, activity dropped rapidly to less than 30 % of optimum at pH 6 or pH 9.5. At 5°C in this direction, the sharp pH optimum was lost and a broad optimum range was seen from pH 7–7.5 up to 9.5 for all three enzymes (Figure 5- 3D). Activity was again very sensitive to acid and fell to less than 20% of the optimum at pH 6.0.

GDH purified from the liver of euthermic versus hibernating ground squirrels showed significant differences in kinetic properties that indicate that the enzyme is modified for function in the hibernating animal (Table 5-2). When assessed at 37°C, all K_m values were significantly higher than the corresponding values for EGDH. The K_m values at 37°C K_m values for HGDH were 2.25-, 9.5-, and 2.56-fold greater for ammonium ion, α -ketoglutarate, and L-glutamate, respectively. However, when assayed at 5°C, this relationship was reversed and HGDH showed significantly lower K_m values for all three substrates than did EGDH; in this case, K_m values for HGDH were just 49, 12, and 49 % of the K_m values of EGDH for ammonium ion, α -ketoglutarate, and L-glutamate, respectively. Note that in both cases, low K_m values occurred at the favored body temperature associated with each state (ie. at 37°C for the euthermic enzyme and 5°C for the hibernating enzyme). Thus, when EGDH was assayed at 5°C, the enzyme showed reduced affinity for both ammonium ion and α -ketoglutarate, K_m values being 3.5 and 35.5 higher at 5°C than at 37°C.

Figure 5-2. Reverse phase HPLC chromatogram showing each of the GDHs used for this study: EGDH, HGDH, and BGDH. Samples were run after final purification on SEC. Protein elution was monitored at 225 nm and retention time is in minutes.

Figure 5-3. Effect of pH on the activity of ground squirrel and bovine glutamate dehydrogenase at two assay temperatures. (A) forward, glutamate-utilizing, reaction at 37°C; (B) forward reaction at 5°C; (C) reverse, α -ketoglutarate-utilizing, reaction at 37°C; (D) reverse reaction at 5°C. Symbols show GDH from: squares, euthermic ground squirrel; triangles, hibernating ground squirrel; circles, bovine. Data are means $\pm$ SEM, n = 4 and show activities relative to the maximal activity of each enzyme which is set to 100.

Hence, the reverse reaction of EGDH is clearly impaired by low temperature. By contrast, low assay temperature had the opposite effect on K_m values for ammonium ion and α -ketoglutarate of HGDH, and both decreased (by 25 and 56 %, respectively) at 5°C compared with 37°C, giving the enzyme greater substrate affinity at low temperature. K_m glutamate values showed a different pattern. Both enzymes showed lower K_m values for glutamate at 5°C, compared with 37°C, but the effect of low temperature was much stronger on HGDH. Thus, K_m glutamate of EGDH at 5°C was 25 % of the 37°C value whereas for HGDH at 5°C, K_m was reduced to 5% of the 37°C value.

Table 5-2 also shows K_m values for bovine GDH at the two assay temperatures. K_m values for glutamate of BGDH were virtually identical with those of EGDH at both assay temperatures. K_m α -ketoglutarate also followed a pattern similar to that seen for EGDH with K_m increasing greatly (by 7.5-fold) at the lower assay temperature. However, K_m for ammonium ion behaved oppositely and at 5°C was reduced to 23% of the value at 37°C, a pattern that more closely resembled that of HGDH.

Although ammonium ion is the substrate for GDH, Table 5-3 shows that the type of ammonium salt used influences the maximal activity of the enzyme indicating that the anion has some effect on enzyme activity. At 37°C, the ratio of relative activities in the presence of 500 mM ammonium salt was 100:81:44:15:27 for EGDH, 100:81:43, 19:32 for HGDH, and 100:70:34:40:46 for BGDH. The ratios and rankings were identical for HGDH and EGDH, but BGDH response to different ammonium salts was somewhat different, particularly in the relatively higher activity seen in the presence of the chloride and acetate salts. For all of the enzymes, the preferred ammonium source at 37°C was

NH_4HCO_3 followed by ammonium sulphate. The ratios changed substantially at low assay temperature and relative V_{max} activities for the five ammonium salts were 100:39:13:23:64 for EGDH, 100:25:8:11:31 for HGDH, and 100:11:6:6:20 for BGDH at 5°C. NH_4HCO_3 was still the best source of ammonium ion but ammonium acetate replaced ammonium sulfate in second place. All three enzymes showed the same rank order in their use of the different ammonium salts at 5°C.

ADP is a well-known activator of GDH from most sources, and the ground squirrel liver enzyme is not an exception. Activation constants for ADP for EGDH and HGDH differed significantly at both assay temperatures with HGDH being less sensitive to the nucleotide at 37°C and more sensitive at 5°C than EGDH (Table 5-4). However, values for the fold activation by optimal ADP were similar for both enzymes at either temperature. Low temperature increased the sensitivity of both enzymes to ADP, lowering K_a values to only 18 and 2 % of the corresponding values at 37°C. ADP also activated bovine GDH with a K_a value of 13 μM at 37°C. However, BGDH lost its sensitivity to ADP when assayed at 5°C.

All three enzymes were also activated by AMP at 37°C with K_a values that were similar to (HGDH) or higher than (EGDH, BGDH) the corresponding values for ADP. Sensitivity to AMP activation was lost at 5°C for both HGDH and BGDH although EGDH showed increased sensitivity to AMP at low temperature, following the pattern seen for ADP. When AMP effects were tested in the presence of ADP (2 mM), no additional activation was found indicating that the effects of the two nucleotides are not additive.

Table 5-2. A table of K_m values of the three substrates of GDH NH_4^+ , glutamate, and α -KG for HGDH, EGDH, BGDH, at 37 and 5°C.

Substrate	Ammonium ion	α -ketoglutarate	Glutamate
Enzyme source	K_m (mM)	K_m (mM)	K_m (mM)
Euthermic GS 37°C	7 ± 0.69^b	0.103 ± 0.0082^b	2.03 ± 0.17
Euthermic GS 5°C	24.7 ± 0.0094^{ab}	3.66 ± 0.34^{ab}	0.5 ± 0.05^a
Hibernating GS 37°C	15.8 ± 1.44	0.982 ± 0.075	5.2 ± 0.47^b
Hibernating GS 5°C	12.1 ± 0.95	0.431 ± 0.018^{ab}	0.246 ± 0.016^a
Bovine 37°C	17.8 ± 0.15	1.4 ± 0.10	2.6 ± 0.22
Bovine 5°C	4.1 ± 0.25^{ab}	$10.5 \pm .32^{ab}$	0.47 ± 0.035^b

K_m are a mean ($n=4$) and errors are $\pm$ SEM. Significant differences at the $P > 0.05$ level for: differences between 37 and 5°C for the same enzyme (a), differences between GDH from differing sources at the same temperature (b).

Table 5-3. The table of NH_4^{4+} preference for hibernating and euthermic GS and bovine GDHs at 37 and 5°C.

Substrate Enzyme source	NH_4HCO_3 Vmax (mU)	$(\text{NH}_4)_2\text{SO}_4$ Vmax (mU)	NH_4NO_2 Vmax (mU)	NH_4Cl Vmax (mU)	$\text{NH}_4\text{Acetate}$ Vmax (mU)
Euthermic GS 37°C	2.5 ± 0.091	2.03 ± 0.11	1.1 ± 0.06	0.389 ± 0.026	0.677 ± 0.12
Euthermic GS 5°C	0.406 ± 0.018	0.16 ± 0.032	0.054 ± 0.054	0.092 ± 0.021	0.26 ± 0.025
Hibernating GS 37°C	2.1 ± 0.15	1.7 ± 0.21	0.897 ± 0.084	0.389 ± 0.031	0.677 ± 0.012
Hibernating GS 5°C	0.828 ± 0.047	0.21 ± 0.033	0.064 ± 0.0099	0.092 ± 0.0021	0.26 ± 0.0025
Bovine 37°C	2 ± 0.088	1.4 ± 0.14	0.67 ± 0.031	0.796 ± 0.078	0.927 ± 0.48
Bovine 5°C	0.89 ± 0.057	0.097 ± 0.0091	0.049 ± 0.0031	0.054 ± 0.012	0.178 ± 0.014

Vmax values are a mean (n=4) and errors are ± SEM. The assays were run at 100 mM NH_4^{4+} . Other than the source of ammonia the assay was run as described in the methods and materials.

L-Leucine is a commonly reported activator of GDH, but in the present study leucine had no effect on any of the three enzymes when assayed at 37°C (Table 5-4). At 5°C, however, both EGDH and HGDH were activated by the amino acid with the enzyme from the hibernator having a significantly lower K_a value (40 % of the value for EGDH).

Sodium chloride activated both EGDH and HGDH at 37°C at high concentrations with significantly different K_a values of 260 mM and 331 mM, respectively, but similar fold-activation values, about 7 fold (Table 5-4). However, the salt had no effect on any of the three enzymes at 5°C. At 37°C, however, NaCl had a different effect on BGDH and inhibited the enzyme (Table 5-5). Potassium chloride activated all three enzymes at 37°C with K_a values of 42 mM, 344 mM, and 10.1 mM for EGDH, HGDH, and BGDH respectively. At 5°C the response to KCl was distinctly different for each enzyme. K_a KCl for EGDH showed a small but significant drop at 5°C, compared with 37°C. However, K_a KCl of HGDH at 5°C fell dramatically to only 0.8% of the value at 37°C. KCl became an inhibitor of BGDH at 5°C (Table 5-5). A decrease in the fold activation by KCl was also observed at the lower assay temperature for both ground squirrel enzymes (Table 5-4).

Sodium acetate also showed mixed effects on the three enzymes depending upon assay temperature. The salt was an activator of HGDH and BGDH at 5°C with low K_a values (1.74 and 2.32 mM, respectively) but a limited fold activation (1.53 and 1.72 fold) in the forward direction (Table 5-4). In other instances, however, sodium acetate was an inhibitor of the enzyme, inhibiting all three enzymes at 5°C (Table 5-5). In addition, the

salt was an inhibitor of EGDH at 37°C, with an I_{50} value on only 320 mM, very different from the I_{50} value of 902 mM for the same enzyme at 5°C.

Other salt inhibitors of GDH are shown in Table 5-5. With the salts NaCl and KCl inhibition is only seen with the bovine enzyme. Effects of sodium iodide and potassium nitrate were limited to 37°C (Table 5-5), neither salt affecting the enzymes at 5°C. BGDH was the most sensitive to NaI with an I_{50} value of 41 mM compared to 149 and 124 mM for EGDH and HGDH, respectively. KNO_3 affected the EGDH and HGDH equally with I_{50} values of 10-11 mM at 37°C whereas the I_{50} for BGDH was significantly lower at 6 mM.

Nucleotide inhibitors are also shown in Table 5-5, ATP, and GTP being well known inhibitors of the enzyme from many sources. ATP inhibited all three enzymes at 37°C but had no effect at 5°C. The I_{50} values at 37°C were distinct for each of the three enzymes and ranged from 0.74 to 1.76 mM. When ATP effects were tested in the presence of the activator ADP (2.0 mM) inhibition by ATP became stronger, with the I_{50} values falling to 30%, 34%, and 17% of the values measured in the absence of ADP. GTP was a powerful inhibitor of all three enzymes at 37°C with sensitivity ranging from an I_{50} GTP value of, 19 μ M for EGDH to 163 μ M for BGDH. At 5°C, however, both ground squirrel enzymes showed reduced sensitivity to the inhibitor with I_{50} values increasing by 3.3 and 2.4 fold for EGDH and HGDH, respectively, whereas BGDH responded oppositely with increased sensitivity to GTP at the lower temperature (I_{50} fell to 23 % of the value at 37°C). The presence of ADP in the assay mixture at 37°C increased the effectiveness of GTP as an inhibitor in all cases by reducing the I_{50} value to

6, 27, and 6 % for EGDH, HGDH, and BGDH, respectively, of the corresponding values in the absence of ADP.

Table 5-6 shows metabolite and salt activators of the reverse reaction. ADP was a powerful activator of all three enzymes at 37°C with K_a values ranging from 29-41 μ M. Fold activation values differed distinctly, however, from 14-fold activation of EGDH to only 1.7-fold activation of BGDH. At 5°C the responses of the three enzymes to ADP differed. EGDH showed no significant change in K_a value whereas HGDH showed increased sensitivity to ADP, the K_a value at 5°C dropping to 29 % of the value at 37°C. BGDH, by contrast, lost all sensitivity to ADP activation at 5°C. A similar result was observed for AMP effects on the reverse reaction. All three enzymes showed similar K_a values at 37°C. When assayed at 5°C, EGDH showed no change in the K_a value but both BGDH and HGDH were unaffected by AMP at 5°C. The amino acid leucine had no effect on the activity of GDH in the reverse direction at 37°C, but did affect the HGDH and BGDH at 5°C, BGDH showing a much lower K_a Leu than the enzyme from the hibernator (Table 5-6). Fold activation by Leu at 5°C was about 2-fold in both cases.

GDH was also activated by salts in the reverse direction (Table 5-6). NaCl activated all three forms of GDH at 37°C. EGDH showed the highest K_a value (25 mM) and HGDH the lowest (10.6 mM) although fold activation was similar (about 3-fold) for both. At 5°C, however, NaCl had no effect on the enzymes as was also observed in the forward direction. Activating effects of KCl were restricted to the ground squirrel enzymes and this salt did not affect bovine GDH (Table 5-6). The K_a values at 37°C differed dramatically between EGDH (126 mM) and HGDH (3 mM) and fold activation.

Table 5-4. The table of Ka values (mM) and fold activation for activators of GDH in the forward reaction at both 37 and 5°C for all three GDH enzymes. Except where indicated the reaction were run in the absence of the activator ADP.

Enzyme source	Euthermic	Fold	Hibernating	Fold	Bovine	Fold
ADP 37 C	0.181 ± 0.0038 ^b	10.67 ± 0.59	0.576 ± 0.052 ^b	7.8 ± 0.74	0.013 ± 0.0089 ^b	14.8 ± 0.82
ADP 5 C	0.032 ± 0.0023 ^a	3.84 ± 0.4	0.011 ± 0.0013 ^a	3.84 ± 0.49	No effect	
AMP 37 C	0.724 ± 0.042	6.42 ± 0.45	0.358 ± 0.023	12 ± 1.06	0.052 ± 0.44 ^b	2.02 ± 0.049
AMP 5 C	0.11 ± 0.0085 ^a	1.45 ± 0.089	No effect		No effect	
AMP + ADP 37 C	No effect		No effect		No effect	
Leucine 37 C	No effect		No effect		No effect	
Leucine 5 C	1.19 ± 0.11 ^b	1.46 ± 0.068	0.444 ± 0.02 ^b	1.33 ± 0.098	No effect	
Leucine + ADP 37 C	No effect		No effect		No effect	
NaCl 37 C	260 ± 22 ^b	6.82 ± 0.25	331 ± 29 ^b	7.18 ± 0.61	No effect	
NaCl 5 C	No effect		No effect		No effect	
KCl 37 C	42.6 ± 3.4 ^{ab}	5.64 ± 0.53	344 ± 30 ^{ab}	5.35 ± 0.41	10.1 ± 0.96 ^b	1.21 ± 0.045
KCl 5 C	33.4 ± 4.1 ^a	2.18 ± 0.16	2.7 ± 0.27 ^a	3.22 ± 0.24		
Sodium acetate 5 C			1.74 ± 0.097	1.53 ± 0.095	2.32 ± 0.16	1.72 ± 0.09

Where it is indicated (+ADP) the ADP concentration in the final reaction mixture was 2.0 mM. Reactions were run at optimal substrate concentrations as described in the methods and materials. Fold activations were calculated from the maximal effect of the activator of GDH activity at optimal substrate concentrations for the enzymatic reaction. Values reported are means, n=4 and errors are SEMs. Significant differences (P>0.005) between temperatures, 37 and 5°C, were denoted by, ^a and ^b, indicated a significant (P>0.005) difference between enzymes at the same temperature.

Table 5-5. The table of I_{50} values for euthermic and hibernating GS glutamate dehydrogenase at two temperatures 37 and 5°C in the forward direction. Except where indicated the reaction were run in the absence of the activator ADP.

Enzyme source	Euthermic		Hibernating		Bovine	
	I_{50} (mM)		I_{50} (mM)		I_{50} (mM)	
ATP 37 C	0.74 ± 0.047 ^b	1.3 ± 0.1 ^b	1.76 ± 0.15 ^b			
ATP 5C	No Effect	No Effect	No Effect			
ATP+ADP 37 C	0.219 ± 0.02 ^b	0.436 ± 0.019 ^b	0.301 ± 0.0014 ^b			
GTP 37 C	0.0185 ± 0.0009 ^b	0.0761 ± 0.0068 ^b	0.163 ± 0.0094 ^b			
GTP 5 C	0.0613 ± 0.0051 ^a	0.185 ± 0.013 ^b	0.037 ± 0.0038 ^a			
GTP+ADP 37 C	0.0012 ± 0.00007 ^b	0.0207 ± 0.0009	0.0101 ± 0.00041			
NaCl 37 C						
KCl 5 C					365 ± 35	
Sodium acetate 37 C	0.32 ± 0.027				25.4 ± 1.97	
Sodium acetate 5 C	902 ± 61 ^{ab}	174 ± 11 ^b			353 ± 28 ^b	
NaI 37 C	149 ± 9.6 ^b	124 ± 4.7 ^b			41.1 ± 3.2 ^b	
NaI 5 C	No Effect	No Effect			No Effect	
Potassium nitrate 37 C	10.2 ± 1	11.2 ± 1.1			6.29 ± 0.44 ^b	
Potassium nitrate 5 C	No Effect	No Effect			No Effect	

Where it is indicated (+ADP) the ADP concentration in the final reaction mixture was 2.0 mM. Reactions were run at optimal substrate concentrations as described in the methods and materials. Values reported are means, n=4 and errors are SEMs. Significant differences ($P>0.005$) between temperatures, 37 and 5°C, were denoted by, ^a, and ^b, indicated a significant ($P>0.005$) difference between enzymes at the same temperature.

Table 5-6. The table of activators for GDH in the reverse reaction at both 37 and 5°C for all three GDH enzymes. Reactions were run at optimal substrate concentrations as described in the methods and materials.

Enzyme source	Euthermic Ka (mM)	Fold	Hibernating Ka (mM)	Fold	Bovine Ka (mM)	Fold
ADP 37 C	0.029 ± 0.0023	14 ± 0.46	0.041 ± 0.00075	3.64 ± 0.13	0.035 ± 0.00082	1.68 ± 0.029
ADP 5 C	0.0320.0021 ^b	3.95 ± 0.35	0.012 ± 0.0011 ^a	3.61 ± 0.35	No effect	
AMP 37 C	0.101 ± 0.0099	2.56 ± 0.073	0.093 ± 0.0066	2.35 ± 0.047	0.114 ± 0.0036	1.61 ± 0.15
AMP 5 C	0.11 ± 0.0085	1.46 ± 0.089	No effect		No effect	
Leucine 37 C	No effect		No effect		No effect	
Leucine 5 C	No effect		2.32 ± 0.25 ^b	2.21 ± 0.21	0.153 ± 0.016 ^b	1.65 ± 0.12
NaCl 37 C	25.1 ± 1.4 ^b	3.21 ± 0.17	10.6 ± 0.91 ^b	3.09 ± 0.26	13.1 ± 0.15 ^b	1.98 ± 0.16
NaCl 5C	No effect		No effect		No effect	
KCl 37 C	126 ± 6.9 ^b	2.11 ± 0.2	2.99 ± 0.24 ^b	4.96 ± 0.23	No effect	
KCl 5 C	30.9 ± 2.3ab	2.19 ± 0.16	2.75 ± 0.27 ^b	3.22 ± 0.24	No effect	
Sodium acetate 5 C	3.98 ± 0.35	1.36 ± 0.058	4.21 ± 0.38	1.37 ± 0.79	4.37 ± 0.39	1.35 ± 0.055
NaI 37 C	31.1 ± 1.9 ^b	1.88 ± 0.1	106 ± 9.1 ^b	1.56 ± 0.1		

Fold activations were calculated from the maximal effect of the activator of GDH activity at optimal substrate concentrations for the enzymatic reaction. Values reported are means, n=4 and errors are SEMs. Significant differences ($P>0.005$) between temperatures, 37 and 5°C, were denoted by, ^a, and ^b, indicated a significant ($P>0.005$) difference between enzymes at the same temperature.

values were different, being 2.1- and 5-fold, respectively. With the drop in assay temperature to 5°C the K_a KCl for EGDH decreased by 75% compared with the 37°C value but the K_a KCl of HGDH did not change. By contrast, KCl inhibited BGDH at 5°C (Table 5-7). Sodium acetate activated all three enzymes at 5°C (Table 5-6) but was an enzyme inhibitor at 37°C (Table 5-7). Sodium iodide also had mixed effects on the enzymes, activating the ground squirrel enzymes at 37°C but inhibiting the bovine enzyme at the same temperature. At 5°C, NaI inhibited both EGDH and BGDH but had no effect on HGDH. Potassium nitrate was also an inhibitor of all three enzymes (Table 5-7) with stronger effects on the ground squirrel enzymes at 37°C, compared with 5°C.

Nucleotide inhibitors of the reverse reaction are also shown in Table 5-7. ATP inhibited both EGDH and HGDH at 37°C with similar K_a values (about 0.7 mM) whereas K_a ATP of BGDH was about 5-fold higher. ATP had no effect on any of the enzymes at 5°C. GTP also inhibited only at 37°C and again the ground squirrel enzymes showed greater sensitivity (K_a values 10-18 μ M) to the nucleotide than did the bovine enzyme (K_a 143 μ M).

Table 5-7. The table of I_{50} values for euthermic and hibernating GS glutamate dehydrogenase at two temperatures 37 and 5°C in the reverse direction.

Enzyme source	Euthermic I_{50} (mM)	Hibernating I_{50} (mM)	Bovine I_{50} (mM)
ATP 37 C	0.659 ± 0.063	0.7 ± 0.05	3.7 ± 0.15 ^b
ATP 5 C	No effect	No effect	No effect
GTP 37 C	0.0185 ± 0.001 ^b	0.01 ± 0.0005 ^b	0.143 ± 0.0088
GTP 5 C	No effect	No effect	No effect
KCl 5 C			29.4 ± 1.97
Sodium acetate 37 C	63.5 ± 3.1	47.4 ± 2.6 ^b	62.9 ± 5.4
NaI 37 C			705.3 ± 47
NaI 5 C	6.4 ± 0.53 ^b	No effect	31.1 ± 1.74 ^{ab}
Potassium nitrate 37 C	38.3 ± 1 ^b	19.4 ± 1.1 ^b	57.5 ± 0.44 ^b
Potassium nitrate 5 C	64.5 ± 4.1 ^{ab}	121 ± 7.9 ^{ab}	40.3 ± 2.9 ^{ab}

Reactions were run at optimal substrate concentrations as described in the methods and materials. Values reported are means, n=4 and errors are SEMs. Significant differences ($P>0.005$) between temperatures, 37 and 5°C, were denoted by, a; b, indicated a significant ($P>0.005$) difference between enzymes at the same temperature

Discussion

A variety of differences were found in kinetic constants for substrates, activators and inhibitors between the purified enzymes from hibernating and euthermic GS and the bovine liver enzyme. The three forms of GDH (euthermic and hibernating GS and bovine) shared only a few factors in common including responses to some activators and inhibitors. In both the forward and reverse reactions, ADP and AMP were activators at 37°C (Table 5-4 and 6). These nucleotides are well-characterized activators of GDH activity (Frieden, 1965; Smith et al., 1975; Fisher, 1985; McGivan, 1988). The other two nucleotides, GTP and ATP, are well-studied inhibitors of GDH activity (Frieden, 1965; Smith et al., 1975). These two compounds inhibit all of the GDHs examined in both the forward and reverse directions (Tables 5-5 and 7). There were differences in the effectiveness of these compounds on each individual enzyme, which in many cases were statistically significant (Tables 5-4 to 7). Furthermore, when the temperature was lowered to 5°C, there were substantial changes to the effects of these nucleotides on enzymatic activity. In both reaction directions, ATP no longer had an effect on any of the GDH activities (Table 5-5 and 7) whereas AMP no longer had an effect on the hibernating or bovine enzyme in either reaction direction at 5°C but still activated EGDH activity. There was also a loss of effectiveness of ADP on BGDH activity at 5°C (Table 5-4). KNO_3 was the only salt that has a consistent effect in both reaction directions. KNO_3 was inhibitory at 37°C for all three GDHs, and furthermore, it is a more effective

inhibitor in the forward direction (Table 5-5). The effect of low temperature increased the I_{50} for EGDH and HGDH but decreased the effect on BGDH I_{50} (Table 5-5). The regulatory importance of KNO_3 to the GS enzyme is probably very limited *in vivo* since nitrate is not found in high concentrations in cells. NaI at 37°C was an inhibitor of GDH activity in the forward direction (Table 5-5). Furthermore, there was no effect from KNO_3 and ATP at 5°C but this was the same for all of the enzymes. At low temperature in the forward reaction, consistent results were seen for sodium acetate but not at 37°C (Table 5-5). In the majority of cases, there was a division of effects for the same temperature as observed in the case of KCl at 5°C (Table 5-4 and 5). The change in I_{50} , K_a , and K_m values further supports the presence of a hibernating form of GDH, which is different from the euthermic enzyme. For the majority of tested compounds, there were differences between the three forms of GDH. Similar effects of temperature, effectiveness, and species differences were observed in the reverse reaction (Tables 6 and 7).

Other than KCl and NaCl, the GDHs would not likely be exposed to the other ions in high enough concentrations to cause an effect on activity. NaCl had an effect on the activity of GDH in both directions. In the forward direction at 37°C concentrations above 200 mM were obtained for K_a and I_{50} values (Tables 4 and 5) which the enzyme is not likely to experience *in vivo*. In the reverse direction NaCl is an activator of GDH activity with K_a values of less than 26 mM which may have some regulatory importance. On the other hand, KCl affects GDH activity at relatively low concentrations in the case of EGDH and BGDH with K_a values of less than 43 mM in the forward direction. HGDH

was also activated by KCl but with a much higher K_a (344 mM). At low temperature, HGDH became far more sensitive to KCl and the K_a dropped to 2.7 mM. KCl also activates EGDH and HGDH in the reverse direction and had no effect on the bovine enzyme activity.

The nucleotide activators and inhibitors have physiological relevance. ATP and GTP are indicators of cellular energy levels; when concentrations of triphosphate nucleotides are relatively high so is the available cellular energy. The inhibition of GDH by ATP and GTP would indicate that less GDH activity is required when it is not necessary for feeding the TCA cycle α -KG for energy production. ATP and GTP were better inhibitors of HGDH at 37°C in the forward reaction, which was not the case for the other GDHs (Table 5-5 and 7). ATP lost effectiveness with a reduction in temperature to 5°C in both directions. The activators ADP and AMP are indicators of low cellular energy and activate GDH to help fuel the TCA cycle for energy production. The K_a values of ADP for the GS GDHs were substantially lower in the reverse direction at 37°C (Table 5-4 and 6). AMP lost effectiveness on HGDH and BGDH at 5°C and has similar K_a values for all three GDHs at 37°C in the reverse direction (Table 5-6).

All of the GDHs studied were from the same tissue (liver) and the euthermic and bovine enzymes would experience similar physiological and environmental conditions normally, whereas HGDH operates under a distinctly different set of parameters *in vivo*. In the presence of ATP or GTP, the hibernator enzyme exhibits preferential inhibition of the reverse reaction (the I_{50} values for GTP and ATP are lower in this direction). I_{50} values for GTP with EGDH and BGDH are similar in both directions (forward and

reverse). The effect of ATP is not significant for EGDH, but showed a higher I_{50} in the reverse direction (Table 5-5 and 7). At high ATP and/or GTP concentrations, HGDH will run in the forward direction, the bovine enzyme will tend to work in reverse (reaction), and EGDH will not show a direction preference. The forward direction of the GDH reaction is catabolic, producing α -KG and ammonia, whereas the BGDH at a high-energy state would be running in anabolic mode for the production of glutamate. This is evidence of a catabolic GDH isozyme in a mammal (*S. richardsonii*). This is also a difference between the GDH from euthermic and hibernating GS liver, the EGDH has no directional preference at high ATP/GTP concentrations, whereas HGDH appeared to have a catabolic preference under these conditions.

Apart from the activators and inhibitors that had a common effect on GDH activity (GTP, ATP, ADP, KNO_3), the rest of the compounds tested (Tables 5-4, 5, 6, 7) showed some very different effects on the three forms of GDH. Between the three GDHs it was common to see a switch between the effects (inhibitor and activator) of a compound depending on temperature and/or reaction direction. The effects of temperature and reaction direction with the tested compounds clearly show differences between the GDHs. Such an effect was observed with NaCl in the forward reaction where it was an activator of the GS GDHs and an inhibitor of the bovine enzyme at 37°C, but had no effect on the activity at 5°C for any of the enzymes (Table 5-4 and 5). Most of the activators and inhibitors displayed this type of profile with a different effect for one of the GDHs compared to the others. Temperature is an important variable in the action of small molecules on the activity of GDH *in vitro* and is crucial only to the hibernating

animal *in vivo*. When assayed at 5°C there were dramatic changes in the effect of a number compounds on GDH activity ranging from a loss of effectiveness, to a switch between activator and inhibitor (or vice versa) (Tables 5-4 and 5). The effects of temperature on the hibernating enzyme may show adaptation to low temperature regulation of HGDH. It also helps to establish that two forms of GDH are found in the ground squirrel (euthermic and hibernating). Furthermore, the GS enzymes are functionally different from the bovine enzyme. Temperature effects are only physiologically relevant to the hibernating ground squirrel GDH since it must function over the range of 5 to 37°C. The effect of temperature may reflect the changing demands on hibernating GDH during torpor (5°C) and waking bouts (37°C) during hibernation. Both of the temperatures examined are relevant to HGDH activity and regulation *in vivo*, but the bovine and euthermic enzymes function only at 37°C.

The combination of temperature and allosteric regulation both have influence on enzymatic activity and add a level of complexity to GDH kinetics. To highlight some of the differences and similarities between the three forms of GDH, in both directions, and at two temperatures, ratios of effect in the forward and reverse directions provide some clarification of the relative directional effects on GDH activity. At low temperature the I_{50} values for ATP all increase (Table 5-8). This seems to show that the effect of ATP on the direction of GDH is substantially different for HGDH and BGDH with little preference being demonstrated by the euthermic enzyme.

Source of GDH	Change in I_{50} with decrease in temperature	Relative I_{50} ratios for ATP forward/ reverse directions
HGDH	Increase	1.86
EGDH	Increase	1.12
BGDH	Increase	0.45

Table 5-8. The effect of temperature on the effectiveness of ATP as an inhibitor of GDH activity. The relative ratios of ATP I_{50} values for three forms of GDH (forward / reverse reaction directions) at 37°C.

This suggests there are different directional preferences for GDH reaction direction at 37°C between the euthermic and hibernating GS and the bovine enzyme. It also implies that at euthermic temperatures and high cellular energy charge (Zubay, 1988) the HGDH showed a preference for the production of α -KG and this is opposite observed for BGDH.

The ratios for GTP I_{50} values show almost no directional preference on the reaction direction of the euthermic GS or bovine GDH (Table 5-9). The HGDH however, shows a strong preference for the forward reaction (conversion glutamate to α -KG) in the presence of high GTP levels and euthermic body temperature (37°C). This also suggests that GTP is potentially a directional control mechanism for HGDH but not for EGDH and BGDH.

Source of GDH	The ratio of I_{50} values, forward/reverse directions for GTP at 37°C
HGDH	7.61
EGDH	1.0
BGDH	1.14

Table 5-9. The relative ratios of I_{50} values for the forward over the reverse directions for GTP at 37°C.

The K_a ratios for ADP at low temperature show that at low temperature the two GS enzymes have no directional preference with ADP (Table 5-10). There is no effect on the BGDH activity at 5°C with ADP. At high temperature, there is a preference for the forward reaction from both of the GS enzymes and a opposite response from the bovine GDH (Table 5-10).

Source of GDH	K_a ratios for ADP at 5°C forward/ reverse directions	K_a ratios for ADP at 37°C forward/ reverse directions
HGDH	0.92	14.08
EGDH	1.0	6.96
BGDH	No effect	0.37

Table 5-10. The relative ratio of K_a values for ADP for the forward/reverse directions at 5 and 37°C.

The K_a ratios are more than two fold different, under the conditions tested and they show the same directional preference.

The ratios of K_m values show temperature and directional differences for GS and bovine GDHs. At low temperature a higher K_m for glutamate is observed while the opposite effect is observed for EGDH and BGDH (Table 5-11). The function of EGDH and BGDH at 5°C is not in the normal physiological range but it is for the hibernation enzyme, which spends much of the winter at this temperature.

Source of GDH	The ratios of K_m values for glutamate/ α -KG at 5°C	The ratios of K_m values for glutamate/ α -KG at 37°C
HGDH	1.8	5.3
EGDH	0.14	20
BGDH	0.05	1.9

Table 5-11. The relative ratios for the K_m values for the substrates glutamate and α -ketoglutarate at 5 and 37°C.

At 37°C, which is physiologically relevant for all three forms of GDH, the K_m for glutamate is always larger than for α -KG (Table 5-11). The most dramatic change with temperature occurred with EGDH with over a 100 fold change and the smallest change occurred with the hibernating enzyme. The K_m values for all three enzymes show a preference for α -KG as a substrate at 37°C.

The roles of each of these factors on GDH activity will vary between the conditions tested and the *in vivo* reality of enzyme function. These data do confirm that the HGDH, EGDH, and BGDH are three distinct forms of GDH. It is further suggested that changes in metabolism associated with hibernation result in a need for an isozyme that can perform under a different set of conditions during hibernation.

The GS enzymes showed the same order of preference for ammonium salt substrates, but the bovine enzyme differed with a preference for NH_4^+ acetate over NH_4NO_2 and NH_4Cl at 37°C (Table 5-3). The source of NH_4^+ can also be used to expand the examination of the effects of the counter ions on GDH activity. The best substrate for the reaction was ammonium carbonate, which is reasonable since carbonate has been known to activate GDH activity (Wanders et al., 1983). This was followed by ammonium sulphate, which is well known as an enzyme stabilizer at a variety of concentrations. Substrate preference (the effect of the counter ion) was also observed to vary with temperature for all three forms of GDH and thus substrate preference was different at 37 and 5°C (Table 5-3).

The bulk of the kinetic data suggests that HGDH, EGDH and BGDH are three distinct enzymes. The bovine and the euthermic GS enzyme function under similar

physiological conditions. Both of these animals are functioning within a narrow temperature range around 37°C. These animals are also awake and active. In contrast, the hibernator is functioning at two different body temperatures, 5°C during torpor and 37°C during waking bouts. Furthermore, the hibernator is in a fasting state and living entirely off stored fuel reserves that were accumulated prior to hibernation. During hibernation ground squirrels do not drink or excrete waste for the duration of hibernation.

The differences between HGDH and EGDH could be the result of several types of structural changes that effect enzyme activity. These include *de novo* protein synthesis, post-translational modification, refolding, and proteolysis to name a few possible modes of modifying a protein and changing enzymatic activity. The results of HPLC SEC gave native molecular weights of 331 ± 3 kDa euthermic, 335 ± 3 kDa hibernating, and 320 ± 3 kDa for the bovine enzyme. The bovine and GS enzymes were resolvable in mixed samples (BGDH & EGDH, BGDH & HGDH) by SEC chromatography, but the hibernating and euthermic GS GDHs were not resolvable; in mixed samples of HGDH and EGDH the peak only broadened. There was no difference in the molecular weight on SDS-PAGE of the hibernating and euthermic GDHs; thus subunit size is very similar and the two GS enzymes were slightly larger than the bovine enzyme (Figure 5- 1). Subunit weights for GDH by SDS-PAGE of 59.5 ± 2 kDa (EGDH and HGDH) and the bovine at 57.3 ± 2 kDa further indicated that the enzymes were hexamers under the conditions tested. The N-terminals of both the hibernating and euthermic GDHs were blocked, offering no information on sequence homology. The difference in reverse phase HPLC retention (Figure 5-2) suggests that there were small differences in the three forms of

GDH. The hibernating, euthermic and bovine glutamate dehydrogenases from the euthermic, hibernating and bovine sources are resolvable (from a mixture) by reverse phase HPLC, implying that they are different proteins. It appears that from the experimental data that the GS produces a new GDH during/for hibernation, since most of the other possibilities are improbable from the literature and experimental results. It has also been suggested that there may be a regulatory relationship between the tetrameric and hexameric forms of GDH *in vivo* (Britton et al., 1992). However, this could not be true of the studied GDHs since there was no significant difference in the native molecular weight between the enzymes to indicate changes in the number of subunits comprising each form of GDH under the conditions tested.

In summary, it is clear from the data that there are definite differences between the three forms of GDH studied (EGDH, HGDH, and BGDH). There are a number of similarities between the hibernating and euthermic forms of GS GDH. The physical data suggest that these differences are small and may only involve a few amino acids producing a functionally different enzyme to cope with the changes during hibernation.

GENERAL DISCUSSION

In the present studies, I have examined a variety of enzymes from diverse pathways to look at some of the biochemical adaptive strategies that hibernators may have developed to live at low temperature and in the absence of food and water (Hochachka and Somero, 1984; Storey, 1987, 1992; Storey and Storey, 1990; Marchand, 1991). The enzymes examined were GST, GR, AspAT, and GDH. A variety of different adaptive strategies were observed, specific to different enzymes. Thus, GST exhibited the production of a hibernation specific isozyme; GDH gave evidence of two enzyme forms with substantially different characteristics; and GR and AspAT, although without differences between hibernating and euthermic forms, were both different in the hibernating species from the homologous enzyme found in a nonhibernating mammalian species. These enzymes are from a variety of metabolic pathways and perform a variety of functions. GR and AspAT are equilibrium enzymes that are subject to very little regulatory control. GST is a member of a diverse group of enzymes which are involved in a variety of cellular functions connected with metabolic stresses. Finally, there is the complex metabolic role of GDH; this enzyme is the crossover point between three pathways, protein, carbohydrate, and NH_4^+ / urea metabolism.

The choice of two different experimental animals allowed for the examination of a larger population of hibernators; the total number of animals available of each species was small and the availability of tissue was limited. The resulting data allowed conclusions to be drawn that were also true of hibernator species in general. Both of these species share a number of characteristics that are important for this study. Both enter hibernation in the early fall, hibernate for the entire winter and emerge in the spring. The animals are of similar size, ground dwelling, and burrowing rodents. All of the

animals were wild capture followed by similar laboratory living environments and fed similar diets. Their hibernation characteristics are also similar in that they both have low core body temperature (minimum of 5°C), experience about 6 months of normal hibernation, and show similar patterns of torpor bouts. This could result in similar strategies for coping with the common stress of hibernation since very similar conditions exist for both species. The use of porcine, bovine, and rabbit enzymes as examples of “control” enzymes provided a diverse group of nonhibernating mammals. Overall the comparison of the enzymes between the euthermic (prehibernation) and the nonhibernating animal provides a benchmark for species differences between enzymes. The enzyme from the hibernating animal will show hibernation specific changes if they are present.

The lower thermal stability of enzymes (GR and AspAT) has a potential cost for the animal. A lower thermal stability could lower their half-lives in vivo and thus result in a higher turnover rate at warm temperature (Feller et al., 1994; Jaenicke, 1991). GR and AspAT seem to be designed to improve activity within 10°C of the euthermic normal ($\approx 37^\circ\text{C}$). There appear to be no specific adaptations to facilitate low temperature activity similar those observed in various ectothermic species (Leroi, et al., 1994; Marden, 1995; Somero and Yancey, 1978; Vetter, et al., 1996). Since the GR and AspAT do not show adaptation for increased function at low temperature, enzymatic activity is probably regulated by changing the amount of enzyme in the cell. This could also imply that the amount of enzyme activity in the cell and Q_{10} effects are enough to allow the enzymes to fill their metabolic roles at 37 and 5°C. The other shared property observed with AspAT

and GR was reduced thermal stability, which was observed with the enzymes from hibernating species, compared to the equivalent enzyme from a nonhibernating species. The lower thermal stability of AspAT and GR was accompanied by increased stability to denaturation with GuHCl. This could indicate a change in the type or strength of the inter- and intra-molecular interactions responsible for conformation and activity (Hand and Somero, 1982; Franks, 1987; Jaenicke, 1991).

The adaptation of the GST enzyme system of the muscle and liver of prairie dogs provides a typical example of GST response to a stress. The variations in subunit and isozyme composition of the GST family are well used as markers of a variety of stresses (Mannervik, 1985; McLellan et al., 1991; Tsuchida and Sato, 1992; Rushmore and Pickett, 1993; Lenartova et al., 1996). The isozyme pattern for both prairie dog liver and muscle changed during hibernation. The muscle produced a new GST subunit, PDM 18, and in the liver the amount of PDL 14 significantly increased during hibernation. First, the overall GST activity could indicate a variety of metabolic, environmental, and biochemical differences between animals (Rushmore and Pickett, 1993; Rozell, et al., 1993; Comstock, et al., 1994).

Secondly, the GST family of enzymes responds to stress by the production of an isozyme designed to cope with the problem (Singhal, Saxena et al., 1992; Tsuchida and Sato, 1992; Gulick and Fahl, 1995). The adaptability of the GST family of enzymes (α, μ, π -class) is well known and has been implicated in responses such as drug/pesticide resistance (Dwivedi et al., 1992; Susan and Rao, 1992; Dwivedi, et al., 1993), disease state (Tsuchida and Sato, 1992), and exposure to environmental stresses (Raza, et al.,

1995; Lenartova, et al., 1996). The response of GSTs to stress is a change in the isozyme pattern and/or production of an isozyme that will cope with the problem. Our results suggest that hibernation is another stress that is overcome by changes in GST isozyme pattern. The changes in the liver GST profile is good evidence that the tissue is under stress. Furthermore, this implies that there was a change in the role of GST in the muscle and liver tissue of the prairie dog during hibernation. Unfortunately, because of the variety of functions and natural substrates for GST it is not possible to discern the exact function of most isozymes *in vivo*. The identification and classification of the new isozymes PDM 18 and PDL 14 has given some information of the type of role the enzyme might have. The π -class GSTs are well known for their preferential use of epoxy substrates *in vitro*, but probably have other roles *in vivo*. It is possible that the increases in π -class GST is the result of hibernation related metabolic changes in the animal. The production of similar GST subunits in two very different tissues suggests a common causative agent, or a systemic change in metabolism or other stress that can effect several tissues.

The essential point from the GST studies (kinetics and spectroscopic) is the demonstration of protein adaptation resulting from hibernation in the prairie dog. The regulation of GST isozymes occurs at a genetic level and is inducible by exposure to certain stressors (Pickett and Lu, 1989; McLellan, et al., 1991). The production of a new class of GST indicates expression of a new gene product since all of the classes of GST (α , μ , π , θ) form different gene families. The euthermic PD muscle and liver tissue are dominated by μ -class GST isozymes suggesting that hibernation causes some major

cellular metabolic changes. The dominance of the μ -class GST also occurs in the rabbit, suggesting some metabolic similarities between the two species. The change in protein profile in the muscle and liver of the PD suggest that regulated changes in isozyme profiles are involved in the hibernation process. The general characteristics of the new GST isozyme PDM 18 did not show unusual kinetic parameters for π -class GSTs. The PDM 18 isozyme shared the same type of enhanced low temperature activity and a slightly higher resistance to GuHCl denaturation than the RL enzyme (as was also shown in the comparison of hibernator GR and AspAT with the corresponding enzymes from nonhibernators). The two most plausible reasons for a new/increased π -GST in the liver and muscle are (a) metabolic changes at low temperature (lipid byproducts) or (b) coping with physiological changes of hibernation (peroxidative damage, large temperature changes). The changes to GST subunit composition during hibernation also confirm that hibernation puts stress on the liver and muscle tissue of the PD.

GDH provides the most interesting finding of the study. The data are consistent with the presence of a second form of GDH induced in a mammalian tissue for hibernation poses some interesting possibilities and questions. The preponderance of evidence to date suggests that mammalian tissues typically contain only one isozyme of GDH (Frieden, 1965; Martinez-Carrion, Turano et al., 1967; McGivan and Chappell, 1975; Fisher, 1985; McGivan, 1988). Two forms of GDH have been found in other organisms that use circulating amino acids as an energy source, such as insect flight muscle (Male and Storey, 1983). However, in mammals the dominant role of GDH is anabolic rather than catabolic. For the most part mammals do not use amino acids for

energy production; the dominant fuels are glucose and lipids. The metabolic design of most mammals has very little need for a catabolic form of GDH since there are no large amino acid pools that can be used for quick energy. Fasting or starvation that causes protein catabolism is the exception to this rule and leads to the catabolism of amino acids to feed the TCA cycle. Since starvation is not a normal metabolic condition, the catabolic function of GDH is down regulated by high concentrations of ATP and GTP in the mitochondria. Fasting is a part of hibernation but in this case, fasting is the lifestyle during the entire hibernation bout (6 months). The primary use of amino acids during hibernation is for glucose production and hence, a primary function of GDH would be to fuel gluconeogenesis by channeling the carbon skeletons of amino acids into the reactions of gluconeogenesis. Furthermore, during the bouts of euthermia amino acids could also provide α -KG for gluconeogenesis and thus the role of GDH is primary catabolic during the hibernation cycle. GDH could be regulated *in vivo* by substrate availability or the effects of allosteric effectors (Schoolwerth, et al., 1978; McGivan, 1988).

The enzymes from hibernators that we examined share some substantial and interesting similarities, which seem to separate them from comparable enzymes of nonhibernating species. GST, GR, and AspAT from the ground squirrel and prairie dog show similar traits to cold adapted enzymes from cold adapted animals (Vetter and Buchholz, 1996; Vetter et al., 1996; Vetter and Buchhoiz, 1997). Cold adapted enzymes show some common traits including broad optima for pH and temperature (Vetter and Buchhoiz, 1997). There is also a great range of kinetic profiles that have been observed in cold adapted animals which are adapted for the environment and lifestyle of the

organism (Comstock, et al., 1979; Douzou, 1977; Hochachka and Somero, 1984; Vetter et al., 1996; Vetter and Buchhoiz, 1997). Hibernator enzymes were found to have higher activity at sub-optimal temperatures than the comparable enzymes from nonhibernating species. They also had broad pH optima and reduced thermal stability relative to the enzymes from nonhibernators. This provides evidence for cold adaptation of enzymes in hibernators. Furthermore, a reduced thermal stability is also observed in other cold adapted enzymes and could be evidence of a general change in inter and intra molecular bonds that benefits enzyme function at cold temperatures. An increased tolerance to chemical denaturation could further support changes in bonding (Comstock, 1979; Franks, 1987; Hatley and Franks, 1989).

GST shows the temperature related adaptations and further suggests adaptation to the metabolic changes associated with hibernation. The function of GST can be antioxidant and / or metabolic repair of lipid peroxidation and metabolism of electrophilic compounds (Pickett and Lu, 1989). The production of hibernation induced GST isozymes indicate the response to metabolic or cellular stress. This suggests that hibernation is putting further stress on this enzyme system beyond temperature. The factors that effect GST level and isozyme patterns are numerous; the most probable cause for the change in GST isozyme pattern during hibernation in the PD is a shift in metabolic (glycolytic to lipid) byproducts. Since the levels of GST activity do not change with hibernation this implies that the GST system is dealing with one or more new substrates under these conditions and this necessitates a change in isozyme rather than a need for more overall GST activity.

In the case of GDH, there was a replacement of the enzyme over the course of hibernation in the GS. The euthermic form of the enzyme (from nonhibernating GS) was replaced with a kinetically distinct hibernating isozyme. The reason for a change in GDH isozymes during hibernation could be adaptation of this enzyme to changes in regulation and function that occur during hibernation. During hibernation 20% of the glucose produced is from amino acids which is funneled into gluconeogenesis through GDH. In euthermic animals, the primary role of GDH is to support amino acid and protein production not catabolism since carbohydrate is available. With changes in metabolic function of a key regulatory enzyme like GDH, there is a need to adjust the enzyme activity and regulation to suite its new role. The production of a new isozyme of GDH for hibernation would allow the enzyme to fulfill its new metabolic role and respond properly to regulatory effectors under different metabolic conditions. This would be good evidence of protein adaptation for hibernation.

In summary the adaptation of enzymes for hibernation must take many forms to adjust for the stress of hibernation. Hibernation requires a number of substantial changes to occur to allow the cycle of cooling, torpor, warming and euthermia to occur with out food or water for months. We have examined a few proteins from two hibernators and have found evidence of adaptations to temperature changes during hibernation.

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IMAGE EVALUATION TEST TARGET (QA-3)

APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
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Fax: 716/288-5989

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