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**Glycogen degradation by α -glucosidase
from a hibernating ground squirrel, *Spermophilus lateralis*,
and a freeze tolerant frog, *Rana sylvatica***

by

Peggy Lee Schade, B.Sc. (Hons)

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

Master of Science

Ottawa-Carleton Chemistry Institute
Carleton University
Ottawa, Ontario
July, 1996

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the degree of Master of Science

Chair, Department of Chemistry

Thesis Supervisor

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1996

Abstract

Glycogen degradation in cells can proceed via two routes, the well known phosphorolytic pathway via glycogen phosphorylase (GP) and the glucosidic pathway involving α -glucosidase. The present study analyzes α -glucosidase and its potential for net contributions to organ glucose production in two species that exhibit unusual glucose requirements while overwintering at low body temperatures: the hibernating golden-mantled ground squirrel, *Spermophilus lateralis*, and the freeze-tolerant wood frog, *Rana sylvatica*. Mammalian liver (ground squirrel, rat) possessed two neutral isozymes of α -glucosidase and one acidic form (pH optima 7.0 and 4.0) whereas amphibian liver (wood frog, leopard frog) had one neutral and one acidic enzyme. Tissue distribution of acidic and neutral forms revealed highest activities in liver and kidney and significant changes in activity during hibernation or freezing in selected tissues. Compared with the amount of active GP, acid α -glucosidase could make a substantial contribution to net glucose production in several wood frog organs, notably heart. Liver isozymes were separated by isoelectrofocusing prior to characterization of substrate specificity and affinity, inhibitor effects, and thermal denaturation. Neutral isozymes of all species were similar showing limited use of glycogen, strong thermal denaturation at 45°C and above, and generally reduced substrate affinity at the low temperatures characteristic of the hibernating state. Acidic isozymes used glycogen, showed good thermal stability and substrate affinities were largely temperature-independent. Inhibition of acidic α -glucosidase by salts and carbohydrates was reduced for the wood frog enzyme, compared with the leopard frog, a factor that may improve enzyme function in the frozen state.

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Table of Contents

	Page
Title Page	i
Acceptance Sheet	ii
Abstract	iii
Acknowledgments	iv
Table of Contents	v
List of Abbreviations	vi
List of Tables	viii
List of Figures	x
Chapter 1: General Introduction	1
Chapter 2: Characterization of α -glucosidase from a hibernator. <i>Spermophilus lateralis</i>	7
Chapter 3: Characterization of α -glucosidase from a freeze tolerant frog, <i>Rana sylvatica</i>	64
Chapter 4: General Discussion	122
References	127

List of Abbreviations

- ATP, ADP, AMP - adenosine tri-, di-, and mono-phosphate
- % *a* - percentage of glycogen phosphorylase in the active form
- a* + *b* - total activity of glycogen phosphorylase, active + inactive forms
- BAT - brown adipose tissue
- E_a - activation energy
- EDTA - ethylenediamine tetraacetate
- EGTA - ethyleneglycol bis(β -aminoethyl ether) tetraacetate
- G1,6P₂ - glucose-1,6-bisphosphate
- G6P - glucose-6-phosphate
- G6PDH - glucose-6-phosphate dehydrogenase
- GP - glycogen phosphorylase
- GP_a, GP_b - active and inactive form of glycogen phosphorylase
- GS - glycogen synthase
- HK - hexokinase
- HIT - hibernation induction trigger
- I_{50} - the concentration of effector which reduces the initial enzyme velocity by 50%
- INP - ice nucleating protein
- IEF - isoelectric focusing
- K_m - Michaelis-Menten constant
- KP_i - mono- and di-potassium phosphate
- MU α G - methylumbelliferyl- α -glucoside
- NAD⁺, NADH - oxidized and reduced forms of nicotinamide adenine dinucleotide
- NADP⁺, NADPH - oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate
- PFK - phosphofructokinase

pI - isoelectric point

Q_{10} - the factor by which a 10°C change alters the rate of a chemical reaction

REM - rapid eye movement

T_b - body temperature

V_{max} - maximal enzyme velocity

List of Tables

		Page
2.1	KCl concentrations for mammalian α -glucosidases eluted from DE-52	25
2.2	pI values for mammalian α -glucosidases from IEF	26
2.3	$V_{\max}$ values for neutral α -glucosidases from mammalian tissue extracts	27
2.4	$V_{\max}$ activities for acidic α -glucosidase from mammalian tissue extracts	28
2.5	Activation energies for α -glucosidase isozymes from mammals	29
2.6	Activation energies for GP from mammalian liver	30
2.7	Maximal activities of α -glucosidase in crude liver extracts from euthermic <i>S. lateralis</i> and rat toward oyster glycogen and maltose	31
2.8	Maximal values for partially purified acidic α -glucosidase from mammalian liver toward maltose and glycogen	32
2.9	K_m for maltose and glycogen of partially purified euthermic <i>S. lateralis</i> and rat acidic α -glucosidase	33
3.1	KCl concentrations for frog α -glucosidases eluted from the DE-52	81
3.2	pI values for frog α -glucosidases from IEF	82
3.3	$V_{\max}$ values of the neutral isozyme from frog tissues	83
3.4	Acidic isozyme $V_{\max}$ values from frog tissues	84
3.5	GP total activity and % α in frog tissues	85
3.6	Activation energies for frog α -glucosidases	86
3.7	Activation energies for GP from frog	87
3.8	Maximal activities of crude α -glucosidases from frog with oyster glycogen and maltose	88

3.9	Maximal activities of partially purified acidic α -glucosidases from frog with oyster glycogen and maltose	89
3.10	K_m values from partially purified control <i>R. sylvatica</i> and <i>R. pipiens</i> acidic α -glucosidase	90
3.11	I_{50} values for frog liver partially purified α -glucosidases	91

List of Figures

	Page
2.1	pH profile for mammalian α -glucosidases 35
2.2	DE-52 elution profile of euthermic <i>S. lateralis</i> α -glucosidases 37
2.3	DE-52 elution profile of rat α -glucosidases 39
2.4	IEF profile for euthermic <i>S. lateralis</i> liver α -glucosidases 41
2.5	IEF profile for rat α -glucosidases 43
2.6	Effect of assay temperature on MU α G K_m values for mammalian neutral I α -glucosidase 45
2.7	Effect of assay temperature on MU α G K_m values for mammalian neutral II α -glucosidase 47
2.8	Effect of assay temperature on MU α G K_m values for mammalian acidic α -glucosidase 49
2.9	Effect of incubation temperature on mammalian neutral I activity after 2, 4 and 6 hours 51
2.10	Effect of incubation temperature on mammalian neutral II activity after 2, 4 and 6 hours 53
2.11	Effect of incubation temperature on mammalian acidic activity after 2, 4 and 6 hours 55
2.12	Inactivation of mammalian α -glucosidases at 45°C 57
3.1	pH profile of frog α -glucosidases 93
3.2	DE-52 elution profile of <i>R. sylvatica</i> α -glucosidases 95
3.3	DE-52 elution profile of <i>R. pipiens</i> α -glucosidases 97
3.4	IEF profile of control <i>R. sylvatica</i> α -glucosidases 99
3.5	IEF profile of <i>R. pipiens</i> α -glucosidases 101
3.6	Effect of assay temperature on MU α G K_m values for frog neutral α -glucosidase 103

3.7	Effect of assay temperature on MU α G K_m values for frog acidic α -glucosidase	105
3.8	Effect of incubation temperature on frog neutral α -glucosidase activity after 2, 4 and 6 hours	107
3.9	Effect of incubation temperature on frog acidic α -glucosidase activity after 2, 4 and 6 hours	109
3.10	Inactivation of frog α -glucosidases at 45°C	111

Chapter 1

General Introduction

Glycogen is a macromolecule of glucose units connected by $\alpha(1 \rightarrow 4)$ and $\alpha(1 \rightarrow 6)$ bonds. Endogenous stores of glycogen are concentrated in the liver and this glycogen is degraded by two pathways: phosphorolytic and hydrolytic. The former pathway is via glycogen phosphorylase (GP) which utilizes inorganic phosphate to cleave $\alpha(1 \rightarrow 4)$ bonds to within four units of the $\alpha(1 \rightarrow 6)$ branch point. The end three glucose units are transferred to the end of another chain of $\alpha(1 \rightarrow 4)$ linked units via $(\alpha 1,4 \rightarrow \alpha 1,4)$ glucantransferase and GP again cleaves these glucose units down to four units. The $\alpha(1 \rightarrow 6)$ branch point glucose is hydrolyzed by the $\alpha(1 \rightarrow 6)$ glucosidase activity of the debranching enzyme. The product of the phosphorolytic cleavage by GP is glucose-1-phosphate which is isomerized by phosphoglucomutase to glucose-6-phosphate and can be metabolized via glycolysis.

GP exists in two forms: an inactive (*b*) form; and an active (*a*) form. *GPb* can be activated to *GPa* by protein phosphorylation by a specific phosphorylase *b* kinase which transfers phosphate groups from ATP to two serine residues. *GPa* is deactivated by a phosphorylase phosphatase which removes these phosphate groups. The major stimulation of liver glycogen metabolism comes from the pancreatic hormone glucagon. Glucagon stimulates the production of cyclic AMP which activates cyclic AMP-dependent protein kinase which in turn activates phosphorylase *b* kinase by phosphorylation. This activated kinase then phosphorylates *GPb* to *GPa* and leads to enhanced glycogen breakdown.

Along with the hormonal control of GP activity is the nonhormonal control by an allosteric activator, 5'-AMP. Under normal conditions the ratio of ATP to AMP is high and the competitive binding of AMP does not occur. However, under extreme circumstances when AMP accumulates to high levels at the expense of ATP hydrolysis there is sufficient binding of AMP such that *GPb* can be allosterically activated and glycogen degradation proceeds.

In the hydrolytic pathway, $\alpha(1 \rightarrow 4)$ glucosidase (α -D-glucoside glucohydrolase, EC 3.2.1.20) cleaves the glucosidic bonds of the glycogen chains to produce glucose: the same debranching enzyme as in the phosphorolytic pathway is responsible for the hydrolysis of the $\alpha(1 \rightarrow 6)$ branch point. Interest in α -glucosidase has been the result of the discovery by Hers (1963) that humans suffering from Pompe's disease (a glycogen storage disease) lack this enzyme; this results in the accumulation of huge amounts of glycogen in the lysosomes that is unavailable to cytosolic GP and ends in their death within the first year of life. Lysosomes are acidic subcellular compartments which digest substances that pass through the cell wall by endocytosis as well as the catabolism and turnover of intracellular structures and organelles (cytoplasm, mitochondria, endoplasmic reticulum, glycogen). These substances are isolated within vacuoles which eventually become fused to lysosomes where the contents of the vacuoles are transferred and destroyed. The small molecules resulting from this digestion are passed through the lysosomal membrane and reused by the cell.

α -Glucosidase has been extensively studied in mammals (Burns, 1982; Calder and Geddes, 1989; Grinna and Robbins, 1979; Hers, 1963; Jeffrey *et al.*, 1970; Lejeune *et al.*, 1963; Matsui *et al.*, 1984; Onodera *et al.*, 1989 and 1994; Palmer, 1971; Radin *et al.*, 1989; Tashiro *et al.*, 1986), fish (Mehrani and Storey, 1993; Picukans and Umminger, 1979), amphibians (Fournier *et al.*, 1992; Fournier and Guderley, 1993) and invertebrates (Chuang *et al.*, 1992; Vega-Villasante *et al.*, 1993). Multiple forms of α -glucosidase have been grouped into acid α -glucosidases with pH optima of 4-5 and neutral α -glucosidases with pH optima of 6-7. From subcellular fractionation studies the acidic α -glucosidase appears to be associated with lysosomes and the neutral with endoplasmic reticulum.

Acid α -glucosidase is located in the lysosomes (Chuang *et al.*, 1992; Lejeune *et al.*, 1963; Murat, 1976) and its low pH optimum is in accordance with its

function as a catalyst in the degradation of oligosaccharides trapped in this environment. The enzyme shows activity towards naturally occurring substrates with its activity decreasing with increasing substrate size (Calder and Geddes, 1989). The enzyme is activated by cations (Na^+ , K^+ , Mg^{2+} and Ca^{2+}) (Calder and Geddes, 1989; Matsui *et al.*, 1984; Onodera *et al.*, 1989; Vega-Villasante *et al.*, 1993) and is competitive inhibited by turanose (a disaccharide) (Jeffrey *et al.*, 1970; Matsui *et al.*, 1984; Tashiro *et al.*, 1986) although this sugar is not hydrolyzed (Matsui *et al.*, 1984). Thermal lability at temperatures above 50°C was evident even after short periods of time (Chuang *et al.*, 1992; Matsui *et al.*, 1984; Onodera *et al.*, 1989). Analysis of purified α -glucosidase from the hepatopancreas of the shrimp *Penaeus japonicus* revealed that it is a monomer of 105,000 daltons (Chuang *et al.*, 1992).

Studies have indicated that neutral α -glucosidase is quite different in structure and function from the acid α -glucosidase. Its higher pH optimum implies that it is not associated with the lysosomes and through subcellular fractionation it has been found to be associated with microsomes in rat (Lejeune *et al.*, 1963) and trout liver (Mehrani and Storey, 1993). Burns and Touster (1982) found that the neutral isozyme from rat liver was a tetrameric glycoprotein containing 65,000 dalton subunits. Activity towards 4-methyl-umbelliferyl- α -D-glucoside, maltose and other small substrates has been demonstrated although there has been differing results with glycogen. The enzyme from carp, dog and rat liver, urine and plasma all exhibited activity with glycogen as a substrate (Murat *et al.*, 1978) but glycogen was not used by the neutral α -glucosidase activity found in trout liver (Mehrani and Storey, 1993). Although acid α -glucosidase is inhibited by turanose, the sugar does not inhibit the neutral isozyme (Lejeune *et al.*, 1963; Mehrani and Storey, 1993). The lability of this isozyme to freezing and thawing has been indicated (Castilla *et al.*, 1980; Murat *et al.*, 1978) in contrast to the more thermal tolerant acid α -

glucosidase. The roles of the neutral form have been proposed to be in oligosaccharide hydrolysis, the transfer of carbohydrate moieties, and the construction of glycoproteins (Castilla *et al.*, 1980; Murat *et al.*, 1978).

Initial studies considered that acid and neutral α -glucosidase had scavenger and biosynthetic roles, respectively, in animal cells but did not contribute to central energy metabolism which is regulated by GP. A study of glycogen metabolism in the carp, *Cyprinus carpio*, challenged this view when no GP activity was detected in the liver of this species. However, activity of α -glucosidase was high and it increased in response to hormonal stimulation (Murat *et al.*, 1978). Hence, it seemed that α -glucosidase had replaced the role of GP in this system. A subsequent reevaluation of the situation did detect GP activity in carp liver but the ratio of α -glucosidase to GP activity was very high and indicated the hydrolytic enzyme probably made a substantial contribution to total glycogenolysis in the liver (Picukans and Umminger, 1979). A study of α -glucosidase in trout liver also indicated that the enzyme could make a significant contribution to net cellular glycogenolysis in this species; furthermore, enzyme activity and substrate affinity increased in response to the same stimuli that activated GP (Mehrani and Storey, 1993). It appears, then, that α -glucosidase may play both qualitatively and quantitatively different roles in the glycogen and oligosaccharide metabolism of different vertebrate species.

This study is the exploration of the roles of α -glucosidase in animals which display unique glycogen metabolism that has been adapted to aid in winter survival. The hibernating ground squirrel, unlike most mammals, must sustain and regulate glycogen metabolism at very low body temperatures (as low as 3-5°C) during bouts of hibernation. The frogs uniquely accumulate extremely high concentrations of glucose as a cryoprotectant to preserve its cells during freezing and this cryoprotectant must be rapidly synthesized from liver glycogen within

minutes of the onset of ice formation. Thus, each system places novel demands on the metabolism of glycogen within its organs and each may make unique use of α -glucosidase as an important contributor to overall glucose production.

Chapter 2
Characterization of α -glucosidase from
a hibernator, *Spermophilus lateralis*

Introduction

Winter survival of mammals is dependent on food availability and/or body fuel reserves built up before its onset. Most species face a difficult dilemma. On the one hand, food is scarce or completely lacking during the winter and there is a physical limit to how much fat can be stored on the body. On the other hand, extra demands are placed on body fuel reserves for the cold ambient temperatures mean that mammals must commit a higher proportion of their total fuel consumption to the process of keeping warm. Hence, the demand for energy consumption is increased in a system that has fixed or declining fuel reserves. The smaller the mammal the more severe the dilemma for metabolic rate of mammals is inversely proportional to mass and heat loss from the body is proportional to the surface area-to-volume ratio which increases with decreasing body size. Thus, to maintain a constant body temperature (i.e. remain euthermic) a small mammal would have to increase its body lipid reserves by a much greater proportion than would a larger mammal. Furthermore, given that the animal is able to endure the winter, other obstacles must also be overcome, namely spring reproduction at a time when there is a scarcity of food and unpredictable winter storms are often lingering. Hence, stored body fuel reserves must also last well into the spring. For small mammals, these conflicting demands are impossible to accommodate and the only solution that permits winter survival is hibernation, a state of torpor where metabolic rate and body temperature are strongly suppressed to a level that can be sustained throughout the long, cold winter months using available body fuel reserves.

Hibernation

During the winter months, many small mammals abandon the attempt to maintain a constant high body temperature (T_b) and instead allow T_b to drop to a

few degrees above ambient temperature (Hudson, 1973). Net metabolic rate while hibernating can be as low as 1-10 % of the normal resting euthermic metabolic rate (Storey and Storey, 1990) and hence, the animals gain a 10-100 fold extension of the length of time that their body fuel reserves can support survival. Hibernation has been referred to as adaptive hypothermia where the body temperature approaches the ambient temperature; oxygen consumption is reduced; there are prolonged periods of apnea; dormancy occurs; heart rate is reduced; and the ability to arouse is retained.

Along with these processes, the decrease in T_b affects the activity of chemical processes and the establishment of equilibria. The rate of most chemical processes changes by 2-3 fold for every 10°C and this also applies to the overall metabolic rates of animals (Nedergaard and Cannon, 1990). Many hibernating mammals also show a Q_{10} for metabolic rate (generally measured by oxygen consumption) of 2-3. In some cases, however, the Q_{10} is substantially higher, many of the smallest hibernators showing Q_{10} values of about 4 (Gieser, 1988). This additional degree of metabolic rate suppression further extends the potential survival time of the animal and several mechanisms have been suggested for its occurrence, including the effect of the relative acidosis that develops due to intermittent breathing patterns while torpid, a 'hibernation trigger', and the specific down regulation of non-essential pathways in the hibernating state. Thus, an animal which enters into hibernation saves doubly by the reduction in body temperature and the active suppression of metabolism.

Onset of Hibernation

The cause for the onset of hibernation is unknown. It is thought to be a combination of any or all of these variables: scarcity of food; reduction in ambient

temperature: changes in photoperiod: and a neuropeptide called Hibernation Induction Trigger (HIT molecule: Galster 1978; Oeltgen *et al.* 1989)

The Hibernation State

Hibernation is characterized by the onset of a state of slow-wave (non-REM) sleep, and heat production is strongly reduced allowing T_b to decrease gradually (Nedergaard and Cannon, 1990). When closely monitored in the golden-mantled ground squirrel, *Spermophilus lateralis*, it was found that metabolic rate decreased first and then T_b fell. This indicates that hibernation is the result of a controlled suppression of metabolic rate which subsequently leads to the development of hypothermia (Heldmaier *et al.*, 1993; Song *et al.*, 1995). Metabolic rate can be depressed as a result of a number of factors including an alteration of the flow of reactants into metabolic pathways (e.g. via rate-limiting regulatory enzymes, changes in receptors and/or transporters), control of reaction velocities by changes in the concentrations of allosteric effectors or substrates, or by phosphorylation/dephosphorylation regulation of key proteins (Malan, 1993). In liver of the jumping mouse, *Zapus hudsonius*, for example, covalent modification inactivation of glycolytic regulatory enzymes served two functions. The first was the depression of glycolytic activity as part of the overall metabolic rate depression and the secondly was to aid in the reorganization of fuel use in the hibernating state to promote gluconeogenesis and restrict carbohydrate catabolism (Storey, 1987).

Arousal

Hibernating mammals typically sustain dormancy for several days to two weeks but then spontaneously arouse and rewarm. After about 12-24 hours they re-enter dormancy. Some species eat from food caches during this time but most do not. For those that do not feed (which includes ground squirrels), the purpose of an

arousal bout is largely unknown but it must serve an important purpose since it involves an enormous energy expenditure to rewarm the animal. Many theories about the purpose of periodic arousal have been put forward including the need to re-establish ionic balance, elimination of metabolic waste, and the replenishment of carbohydrate stores. The observation that when torpor was interrupted prematurely by environmental disturbances the animals spent less time at high T_b than during spontaneous arousal bouts supports this postulate (French, 1982).

To rewarm the body during arousal, hibernators rely first on the heat generated by non-shivering thermogenesis (uncoupled lipid oxidation by mitochondria) in brown adipose tissue (BAT) and, after the animal is partly rewarmed, shivering in skeletal muscles also contributes to heat output (Nedergaard and Cannon, 1990). BAT is mainly located in the thorax of the animal so that reheating of the core organs and of the central nervous system is favoured: thus, during arousal temperatures in these organs may be as much as 15°C higher than in the hind-quarters of the animal. During these arousal bouts T_b , respiration, heart rate and alertness are returned to euthermic levels.

The Hibernator

The hibernating species focused on in this study is the golden-mantled ground squirrel (*Spermophilus lateralis*). Squirrels are approximately 30 cm in length (Michener and Koepl, 1985), with a mass of about 200 g and they live in the mountains of northern California (Snapp and Heller, 1981). This species is an obligatory hibernator and animals can reduce their T_b to 5°C for up to 14 days at a time when kept at 3-5°C in the absence of food (Twente and Twente, 1965).

Fuel Stores and Usage

The usual response to a disruption in cellular energetics in animals is a rapid activation of carbohydrate catabolism. Because there is a limited store of carbohydrates, inhibition of metabolic responses for the catabolism of these resources must be employed until the cellular energetics have been re-equilibrated (Storey, 1989). Glycolytic enzymes have been shown to be inactivated during hibernation in *Jaculus orientalis* skeletal muscle (El Hachimi *et al.*, 1990), *Lepus hudsonius* and *S. lateralis* liver (Storey, 1989) and *Z. hudsonius* brain and muscle (Storey and Kelly, 1995). The net suppression of carbohydrate catabolism through the reversible inactivation of key enzymes (glycogen phosphorylase, phosphofructokinase, pyruvate kinase, and pyruvate dehydrogenase) helps to promote the utilization of lipids as the major fuel source used in the hibernating state (El Hachimi *et al.*, 1990; Nedergaard and Cannon, 1990; and Willis 1982).

Although a need to limit or inhibit carbohydrate catabolism exists during entry into hibernation, there must be a supply of glucose maintained throughout the period of reduced metabolism. For example, glucose is the primary fuel of brain and carbohydrate (glycogen) stores in liver in particular must continue to maintain blood glucose at appropriate levels as a fuel supply for those organs that need it. Because GPa is strongly suppressed, it is postulated that another mode of glycogenolysis may contribute to glucose output in hibernation. One possibility is the glucosidic pathway via α -glucosidase. Hence, the present study investigates α -glucosidase in hibernator organs and its possible role in the degradation of glycogen to equip the hibernator with a supply of glucose throughout the hibernation bout. As *S. lateralis* undergo dramatic T_b fluctuations during the winter, the effect of temperature on enzyme parameters including the energies of activation, structure/function relationships and substrate affinity and specificity could obviously be key to overall enzyme regulation and these are extensively explored

Materials and Methods

Chemicals and Animals

All chemicals were purchased from either Sigma Chemical Company or Boehringer Mannheim Biochemical. Twenty-one golden-mantled ground squirrels, *Spermophilus lateralis*, (both male and female) were captured in June 1992 in the White Mountains of California. They were housed individually at the University of California-Irvine in the animal care facility where the animals were kept for the next 7 weeks. After this time, 9 squirrels were sacrificed as controls and the remaining 12 animals were then induced to hibernate at 3.5°C. After 30 days of hibernation the remaining *S. lateralis* were sacrificed while torpid. All animals were decapitated and tissues were rapidly excised, immersed in liquid nitrogen and stored at -70°C until use. Both male and female rats, *Ratus ratus*, were obtained from the animal care facility at Carleton University. Rats were decapitated and tissues were rapidly removed, frozen in liquid nitrogen and stored at -70°C.

Tissue Preparation and Enzyme Extraction

Frozen tissue samples were quickly weighed and immediately homogenized 1:10 (w:v) in 50 mM imidazole-HCl (pH 7.0) buffer containing inhibitors of protein phosphatases, Ca²⁺-dependent proteases, and serine proteases: 100 mM NaF, 2.5 mM EDTA, 2.5 mM EGTA and 30 mM β -mercaptoethanol (reducing agent) (with a few crystals of phenylmethylsulfonyl fluoride added) for 10-20s in an Ultra-Turrax homogenizer at 70 % full speed. Homogenates were centrifuged at 14,000g for 20 min at 4°C and supernatants were saved for analysis.

α -Glucosidase and Protein Assays

Two methods were used to measure the activities of the acidic and neutral enzymes. The first method was a variation on the fluorometric assay of Salafsky

and Nadler (1973) which monitors the hydrolysis of the synthetic substrate 4-methylumbelliferyl α -glucoside (MU α G) to release the fluorescent product 4-methylumbelliferone. Prior to assay, aliquots of crude supernatant were diluted 1:100 v/v. For each assay, 0.02 mL of enzyme extract was added to 700 mM potassium phosphate buffer (pH 7.0) or 35 mM potassium acetate buffer (pH 4.0) with 0.5 mM MU α G and a final volume of 0.4 mL. Some assays also contained 40 mM turanose which inhibits acidic α -glucosidase with no effect on the neutral isozymes. Reaction tubes were incubated at 20°C for 20 min and then the reaction was stopped by addition of 2 mL of 1 M glycine-NaOH buffer, pH 10.5. Product formation was measured on an Aminco-Bowman spectrofluorometer at an excitation wavelength of 366 nm and an emission wavelength of 450 nm. A standard curve was constructed with 4-methylumbelliferone in amounts from 1 to 100 nmol in 2 mL of 1 M glycine-NaOH, pH 10.5. One unit of activity is defined as the amount of enzyme that hydrolyzes 1 nmol MU α G per minute at 20°C.

The second assay method utilized either oyster glycogen or maltose as the substrate. The glucose liberated by α -glucosidase hydrolysis of these carbohydrate substrates was measured using a coupled assay with hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6PDH) as shown in the following reaction scheme:

The formation of NADH was monitored fluorometrically using an excitation wavelength of 340 nm and an emission wavelength of 460 nm. Stock solutions of oyster glycogen were dialyzed at 5°C overnight against deionized, distilled water prior to use. $V_{\max}$ values were determined for crude α -glucosidase using 0.01 mL enzyme extract, 50 mg/mL glycogen or 15 mM maltose and either 1 M potassium

phosphate, pH 7.0 or 50 mM potassium acetate, pH 4.0 to a final volume of 0.2 mL. To some of the assays, 40 mM turanose was added to inhibit the acidic α -glucosidase. Reaction tubes were incubated for 60 minutes at 37°C. To stop the reaction, 0.5 mL Tris-HCl buffer (pH 9.0) was added and then 0.1 mL of this mixture was removed and added to the glucose assay reaction mixture containing 0.5 mM ATP, 0.5 mM NAD, 5 mM MgSO₄, 1 U G6PDH and 50 mM Tris buffer (pH 8.0) to a final volume of 1 mL. After taking an initial fluorescence reading, 1 U HK was added to each cuvette. The reaction was incubated for 30 minutes and then a final fluorescence reading was taken. Standard curves were prepared using 0-100 nM NADH in reaction buffer or 0-50 nM glucose added to the standard assay mixture. For the determination of throughput constant, K_m , values of partially purified enzyme, the assay mixture contained a range of substrate concentrations (0.1-50 mg/mL glycogen; 0.5-15 mM maltose) and was incubated at 5°C or 37°C for 4 hours.

Protein concentrations were determined using the Bio-Rad assay kit (Coomassie blue G-250 binding method) with bovine serum albumin as the standard.

DE-52 Column Chromatography

Aliquots of crude supernatant were desalted by centrifugation (IEC benchtop centrifuge) through small columns (1 x 5 cm) of Sephadex G-25 equilibrated in 50 mM imidazole-HCl (pH 7.0) buffer containing 100 mM NaF, 2.5 mM EDTA, 2.5 mM EGTA and 30 mM β -mercaptoethanol (Helmerhorst and Stokes, 1980). The enzyme extract was then applied to a column of DE-52 Sephadex (1 x 2.5 cm) equilibrated in 10 mM phosphate buffer, pH 7.0. The column was washed with 20 mL KP_i buffer and then α -glucosidase was eluted with a linear gradient of KCl (0-1 M, 10 mL); 1 mL fractions were collected and assayed using MU α G as substrate.

Isoelectric Focusing

Analysis of the various isozymes of α -glucosidase was completed using isoelectric focusing at 5°C. Column isoelectrofocusing was conducted in an 110 ml LKB column using a gradient of pH 3.5-10.0 ampholines in a 0-30% (w/v) linear sucrose gradient. One milliliter of a 1:10 (w:v) crude supernatant was placed in the center of the column and focusing was conducted overnight at 450 volts. Two milliliter fractions were drained and assayed for activity using MU α G as substrate. Peak fractions of one acidic and two neutral isozymes were pooled separately, dialyzed for 2 hours against 5 mM potassium phosphate (pH 7.0) and used as partially purified enzymes for some studies.

Arrhenius Plot

Arrhenius plots were constructed from $V_{\max}$ activity determinations conducted over the range 5°C to 65°C using crude extracts as the enzyme source and MU α G as substrate; incubation time at each temperature was 20 minutes.

pH Curve

The pH dependence of the α -glucosidase reaction was assessed over the range pH 3.0-8.5 using potassium acetate and KP_1 as buffers, the standard MU α G assay, and crude supernatant.

Thermal Inactivation

Partially purified enzymes from the IEF column were incubated at various temperatures (5°C to 55°C) in 5 mM KP_1 , pH 7.0. At intervals over a period of six hours, 50 μ L aliquots (10 μ g protein) were withdrawn and α -glucosidase activity was determined at 20°C using the standard MU α G assay.

K_m and Temperature Effect Analysis

K_m values for MU α G were determined using a substrate concentration range of 0.05-6 mM MU α G. To determine the effect of temperature on K_m , reactions were conducted using 0.03 mL partially purified enzyme incubated for 20 minutes at 5°C, 12°C, 20°C, 27°C, 37°C, 45°C or 55°C.

Glycogen Phosphorylase Assay

For the determination of glycogen phosphorylase (GP), frozen tissue samples were homogenized as described above and then the crude homogenate was allowed to settle on ice for a minimum of 30 minutes. Optimal assay conditions for the active *a* form of GP were: 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM MgCl₂, 0.2 mM EDTA, 200 mg/mL oyster glycogen, 10 μ M G1,6P₂, 0.4 mM NADP⁺, 0.7 U phosphoglucomutase and 0.2 U G6PDH. For the total (*a* and *b*) activity, 1.6 mM AMP was added to the assay. The activity of GP was followed by monitoring the consumption of NADPH at 340 nm using a Gilford 240 recording spectrophotometer and a Kipp and Zonen BD 111 chart recorder. Assays were initiated by addition of the enzyme preparation at 20 $\pm$ 1°C with a final cuvette volume of 1 mL plus added crude homogenate. Arrhenius plots were completed using V_{max} activity conditions over a temperature range of 5°C to 65°C.

Statistical Analysis

Student t-tests were conducted for the determination of statistical significance where a normal distribution and random errors were assumed.

Results

pH Curves

Local maxima were detected at pH 4.0 and pH 7.0 for both *S. lateralis* and rat liver α -glucosidase, assayed in crude extracts over the range pH 3.0-8.5 (2.1). The inclusion of turanose in the assay completely inhibited the acidic activity without affecting the neutral activity and 40 mM turanose was subsequently employed whenever needed to determine the true activities of the two forms.

DE-52 Column Chromatography

DE-52 ion exchange chromatography revealed the presence of two neutral isozymes and one acidic isozyme of the enzyme in both *S. lateralis* and rat liver. The elution profiles of the acidic and one of the neutral forms on DE-52 were similar (elution peaks at 224 and 245 mM, respectively) (Figure 2.2) for euthermic *S. lateralis* whereas the second neutral isozyme eluted at a much higher [KCl] concentration (499 mM) (Table 2.1). The elution profiles for liver α -glucosidases from hibernating *S. lateralis* were somewhat different (Table 2.1). The elution peak for the acidic enzyme was at a higher concentration (320 mM) whereas the second neutral peak eluted at a lower [KCl] (409 mM) than the corresponding euthermic enzymes. Figure 2.3 shows a representative plot of the DE-52 elution profile for rat liver α -glucosidase. Two neutral and one acidic form were again detected, eluting at KCl concentrations similar to those seen for the ground squirrel enzymes (Table 2.1).

Isoelectric Focusing

The three α -glucosidase isozymes from both *S. lateralis* and rat liver were readily separated using IEF. Each fraction from the IEF column was assayed at

both pH 7.0 and pH 4.0 to determine the number of isozymes. The three isozymes were well separated on IEF with only very minimal overlap (Figures 2.4 and 2.5). Isoelectric points for the three enzymes are shown in Table 2.2. Unlike the results for elution peaks on DE-52 chromatography, there were no significant differences in pI values of any of the enzyme forms between euthermic versus hibernating animals and, hence, the pI values were combined in Table 2.2. The two neutral enzymes were termed I and II based on their IEF elution profile and the mean pI values for ground squirrel neutral I, neutral II and acidic α -glucosidase were 6.39, 5.91 and 5.72, respectively. Corresponding values for the rat liver enzymes were very similar although the pI of neutral I was significantly higher (6.65) than that of the ground squirrel enzyme.

Tissue Survey

The tissue distribution of α -glucosidase activity was assessed in 5 ground squirrel tissues using crude extracts. Total neutral activity was highest in liver and kidney, intermediate in heart and skeletal muscle, and very low in white adipose tissue (Table 2.3). No significant difference in total neutral activity was found between euthermic and hibernating states in four tissues but neutral activity in liver decreased by 27% in hibernating ground squirrels. The maximal activity of neutral α -glucosidase in rat liver was significantly higher than in ground squirrel liver, exceeding the euthermic value by 2.5-fold and the hibernating value by 3.4-fold.

Contrary to this, activities of the acidic enzyme form in liver did not vary between species or between the euthermic and hibernating states (Table 2.4). Acidic activity in heart was also unaffected by the experimental state of the ground squirrels. However, acidic α -glucosidase activity in kidney decreased by 38% during hibernation whereas activity of the muscle enzyme increased by 2-fold.

Acidic α -glucosidase activity was highest in liver, intermediate in kidney, and low in heart and muscle.

Arrhenius Plots

Maximal activities of both neutral and acidic α -glucosidase were measured (using crude extracts) over a temperature range from 5°C to 65°C and data were plotted as an Arrhenius plot ($\log V_{\max}$ versus $1/^\circ\text{K}$). In all cases, the resulting plots were linear over the full temperature range tested. Activation energies were calculated from the slope of the line using the relationship:

$$\log_{10} V_{\max} = - (E_a/2.3R)(1/T) + \text{constant}$$

and are shown in Table 2.5. The activation energy of the acidic enzyme was the same in all cases (53.1-55.6 kJ/mol). However, the E_a of the combined neutral forms of α -glucosidase from hibernator liver was significantly higher (by 21%) than the value for the euthermic enzyme.

Glycogen can be degraded in animal cells by both the phosphorolytic (via glycogen phosphorylase) and glucosidic (via α -glucosidase) pathways. To determine whether the relative importance of these two modes of glycogen degradation could change at euthermic versus hibernating body temperatures, temperature effects on GP activities were also assessed via Arrhenius plots. All Arrhenius plots completed for GP were linear and Table 2.6 shows the activation energies for GP *a* and *b* from liver of euthermic and hibernating *S. lateralis* as well as rat liver. GP*b* activity was determined by subtracting the activity of GP*a* from the total GP activity. The activation energy for GP*a* was unaltered by hibernation in ground squirrels but the value for the rat enzyme was significantly higher than those for the ground squirrel enzyme. By contrast, the E_a values for the inactive *b* form from both rat liver and hibernating squirrels were not different but both were

significantly lower (by about one-third) than the value for the euthermic ground squirrel.

Glycogen and Maltose Activity of α -Glucosidase

The $V_{\max}$ activity of crude α -glucosidase from liver of euthermic *S. lateralis* and rat towards natural carbohydrate substrates, maltose and glycogen, was determined (Table 2.7). Crude extracts showed both neutral and acidic enzymatic activity using both the disaccharide and the polymeric substrates. Liver activities with either substrate were generally similar between species except that activity of the acidic isoform towards maltose was about 50 % less in rat, compared with squirrel, liver ($P < 0.05$). Table 2.8 lists the maximal activities as determined with maltose and glycogen as substrates using the partially purified acidic enzyme. With the partially pure enzyme, no neutral activity was detectable for either species or for either substrate. Similarly, no activity was observed with acidic rat α -glucosidase towards maltose. However, the acidic enzyme from both species used glycogen as a substrate although the activity of the rat enzyme was significantly lower than the activity of *S. lateralis* acidic α -glucosidase. *S. lateralis* acidic α -glucosidase also showed low activity with maltose as a substrate.

K_m values for these two substrates were assessed for the partially purified acidic enzyme at two temperatures (representing euthermic and hibernating body temperatures) (Table 2.9). *S. lateralis* acidic activity showed a K_m for maltose of 0.89 ± 0.03 mM at 37°C but enzyme activity with this substrate could not be detected at 5°C. Rat α -glucosidase showed similar behaviour with glycogen as the substrate; a K_m of 0.27 ± 0.03 mg/mL was determined at 37°C but enzyme activity was not detectable at 5°C. However, K_m values for glycogen for *S. lateralis* acidic α -glucosidase could be determined at both temperatures and a major effect of

temperature was found. K_m for glycogen increased by 8-fold with the decrease in assay temperature from 37 to 5°C.

Substrate Affinity of MU α G at Varying Temperatures

Figures 2.6, 2.7 and 2.8 illustrate the effect of temperature on the K_m values of the three α -glucosidase isozymes for the artificial substrate MU α G over the temperature range from 5°C to 55°C. Figure 2.6 shows that the substrate affinity constant of neutral I was constant and virtually identical (0.1-0.2 mM) in both species over a broad range from 12 to 45°C. This also remained true for the rat enzyme at both lower (5°C) and higher (55°C) temperatures but the K_m for MU α G of the squirrel enzyme rose at both these temperatures. The K_m was particularly sensitive to cold temperature, rising at 5°C by 9-fold over the value at 12°C and increasing by 4-fold between 45 and 55°C. A similar trend was seen for neutral II, the K_m of the rat enzyme remaining low and constant over the full temperature range tested (Figure 2.7). However, K_m for MU α G of ground squirrel neutral II was constant only between 27 and 45°C and rose at both higher and lower temperatures. K_m increased by 25-fold between 45 and 55°C and, like neutral I, was again much more strongly affected by low temperature, increasing by as much as 50-fold at 5°C, compared with 27°C. The K_m values for MU α G of acidic α -glucosidase are shown in Figure 2.8; these were much less affected by temperature than were the neutral enzymes. K_m values for the rat enzyme were again highly stable with changing assay temperature but unlike the neutral enzymes, substrate affinity increased at high temperature (K_m dropped by 2.5-fold between 45 and 55°C). K_m values for the ground squirrel enzyme were constant between 5°C and 37°C but decreased above this temperature.

Deactivation of α -Glucosidase by Temperature

Figure 2.9 shows the effects of incubation in 5 mM KP_i , pH 7.0 at different temperatures on the stability of neutral I α -glucosidase from *S. lateralis* and rat liver. Both enzymes were completely stable after 2, 4 or 6 hours when incubated at temperatures between 5°C and 37°C. However, both enzymes lost activity when incubated at 45°C or higher. Indeed, ground squirrel neutral I activity was undetectable after 4 h at 45°C.

Activity of neutral II showed a similar pattern, again fully stable over 2, 4 or 6 hours when incubated between 5 to 37°C (Figure 2.10). At 45°C, however, activity was strongly reduced to levels below the limits of detection of the spectrofluorometer after two hours, this effect being greater than that seen for neutral I.

Figure 2.11 shows the equivalent experiments conducted with acidic α -glucosidase. Activities of both the rat and golden-mantled ground squirrel enzymes again remained virtually constant over the temperature range from 5°C to 45°C, lacking the inactivation by 45°C seen for the neutral enzymes. The acidic enzyme from ground squirrel liver was also stable when incubated at 55°C whereas the enzyme activity from rat liver decayed rapidly at this temperature.

Differences in thermal stability can be one characteristic that differentiates isozymes and, in the case of hibernating mammals, changes in the thermal stability of enzymes may be one characteristic that is modified to create enzymes that can function effectively at both euthermic (37°C) and hibernating (about 5°C) body temperatures. To further characterize α -glucosidase from hibernating and non-hibernating mammals, thermal stability was monitored over time at 45°C (Figure 2.12). Enzymatic activity started to decay at this temperature (Figure 2.9-2.11) and hence differential thermal stability between isozymes and species might be readily detected at 45°C. The acidic activity of both species proved to be highly stable at

45°C with no significant change in activity detectable after 4 hours for the squirrel or 6 hours for the rat enzyme (Figure 2.12 A, B). However, the neutral activities from both species were unstable at 45°C. Activities of both *S. lateralis* isozymes decreased to zero after 4 hours whereas the activities of neutral I and II from rat liver decreased rapidly over the first 2 hours and then leveled off at one-fifth of the original value after 6 hours.

The high instability at 45°C of the neutral enzymes was further evidenced by the calculated half-times (time taken for initial activity to decrease by 50%) for activity loss. Half-times for activity decay by *S. lateralis* neutral I and II were 22 and 36 minutes, respectively. By contrast, values for rat neutral I and II were determined to be 85 and 26 minutes, respectively. In the ground squirrel there was a greater lability of the neutral I than neutral II whereas in the rat this was reversed. There was a distinct difference in the two neutral I isoforms as indicated by the rat form having a half-time for inactivation that was 4 times greater than the half-time for the *S. lateralis* enzyme. However, the half-time value for neutral II from both species was both around 30 minutes.

Table 2.1. Mean KCl concentrations (mM) at which neutral and acidic α -glucosidase isozymes eluted from DE-52 column chromatography.

Animal	Neutral Isozymes		Acidic Isozyme
Euthermic <i>S. lateralis</i>	245 $\pm$ 5	499 $\pm$ 75	224 $\pm$ 12
Hibernating <i>S. lateralis</i>	242 $\pm$ 8	409 $\pm$ 35	320 $\pm$ 77
Rat	234 $\pm$ 52	452 $\pm$ 84	285 $\pm$ 51

Elution profiles were analyzed using $V_{\max}$ assay conditions with 0.5 mM MU α G at either pH 7.0 or 4.0. Data are mean $\pm$ SEM, $n = 3$.

Table 2.2. Mean pI values for α -glucosidase from *S. lateralis* and rat liver from isoelectric focusing analysis.

Animal	Neutral Isozyme I	Neutral Isozyme II	Acidic Isozyme
<i>S. lateralis</i>	6.39 ± 0.06	5.91 ± 0.06	5.72 ± 0.06
Rat	$6.65 \pm 0.02^*$	6.16 ± 0.10	5.40 ± 0.10

Data are means $\pm$ SEM. $n = 3$ for rat, $n = 6$ *S. lateralis*. There were no significant differences between the pI values from liver of euthermic versus hibernating *S. lateralis* and hence data from both groups have been combined.

* - significantly different ($P < 0.025$) as compared to the *S. lateralis* neutral I.

Table 2.3. $V_{\max}$ values for the combined neutral isozymes I and II of α -glucosidase from various tissues of euthermic and hibernating ground squirrels as well as rat liver, measured at 20°C.

Tissue	<i>S. lateralis</i> , Euthermic	<i>S. lateralis</i> , Hibernating	Rat
Heart	21.8 $\pm$ 2.9	20.0 $\pm$ 2.1	
Kidney	153 $\pm$ 25	106 $\pm$ 22	
Muscle	24.7 $\pm$ 2.7	35.4 $\pm$ 3.2	
White Adipose	4.0 $\pm$ 0.4	3.2 $\pm$ 0.6	
Liver	114 $\pm$ 11	83.2 $\pm$ 8.7*	280 $\pm$ 19*§

Data are means $\pm$ SEM, ($n \geq 3$). Values are expressed in U/g wet weight (where a unit is defined as nmol MU α G hydrolyzed per minute).

* - Significantly different from the value for euthermic *S. lateralis* by the Student's t-test at a confidence level of $P < 0.05$; § - significantly different from the hibernating ground squirrel enzyme.

Table 2.4. $V_{\max}$ activities for the acidic isozyme of α -glucosidase from various tissues of euthermic and hibernating ground squirrels as well as rat liver, measured at 20°C.

Tissue	<i>S. lateralis</i> , Euthermic	<i>S. lateralis</i> , Hibernating	Rat
Heart	9.9 $\pm$ 1.1	10.2 $\pm$ 2.2	
Kidney	32.0 $\pm$ 1.2	23.1 $\pm$ 1.3*	
Muscle	2.9 $\pm$ 0.3	6.1 $\pm$ 0.4*	
Liver	57.2 $\pm$ 2.9	57.6 $\pm$ 6.8	60.4 $\pm$ 5.8

Mean values ($\pm$ SEM), $n \geq 3$, are expressed in U/gram wet weight (one U is equal to 1 nmol MU α G hydrolyzed per minute).

A significant difference between the activities in control and hibernating tissues is indicated by * with a confidence level of $P < 0.05$.

Table 2.5. Activation energies for α -glucosidase isozymes from ground squirrel and rat liver in kJ/mol.

Isozyme	Euthermic	Hibernating	Rat
	<i>S. lateralis</i>	<i>S. lateralis</i>	
Neutral (combined)	58.2 $\pm$ 2.9	70.7 $\pm$ 1.3*	56.1 $\pm$ 2.3
Acidic	53.1 $\pm$ 0.4	55.6 $\pm$ 0.7	55.6 $\pm$ 1.1

Data are means $\pm$ SEM (n = 4).

Assays were conducted at $V_{\max}$ MU α G concentration (0.5 mM) and activation energies were derived from the slope of Arrhenius plots.

* - Activation energy for the combined neutral isozymes from hibernating *S. lateralis* is significantly different ($P < 0.05$) from the corresponding values for the euthermic and rat enzymes by the Student-Newman-Keuls test.

Table 2.6. Activation energies for glycogen phosphorylase from ground squirrel and rat liver in kJ/mol.

	Euthermic <i>S. lateralis</i>	Hibernating <i>S. lateralis</i>	Rat
GP _a	74.9 ± 5.5	66.9 ± 4.4	95.0 ± 4.3 [§]
GP _b	93.3 ± 5.4	63.2 ± 6.1*	64.0 ± 5.3*

Data are means ± SEM (n = 4).

* - Significantly different (P < 0.05) as compared to the euthermic enzyme;

§ - significantly different from both the euthermic and hibernating enzymes.

GP_b E_a was determined by determining the GP_b activity (subtraction of GP_a activity from total GP activity) and using the Arrhenius equation for the activation energy.

Table 2.7. Maximal activities of α -glucosidase in crude liver extracts from euthermic *S. lateralis* and rats using oyster glycogen or maltose as the substrates.

Animal	Substrate	Combined	Acidic
		Neutral Isozymes	Isozyme
Euthermic	Maltose	30.8 $\pm$ 5.82	84.2 $\pm$ 13.1
<i>S. lateralis</i>	Glycogen	51.6 $\pm$ 12.0	118 $\pm$ 21.2
Rat	Maltose	24.1 $\pm$ 3.09	46.5 $\pm$ 7.52*
	Glycogen	31.3 $\pm$ 5.13	82.7 $\pm$ 15.6

Values are means $\pm$ SEM (n = 3-6) in units/mg protein where 1 unit is 1 nmol glucose produced per minute.

* - Significantly different ($P < 0.05$) from the corresponding value for *S. lateralis*.

Neutral activity was determined in the presence of 40 mM turanose and acidic activity was determined using the differential technique in the presence and absence of 40 mM turanose.

Table 2.8. V_{max} values for partially purified acidic α -glucosidase from *S. lateralis* and rat liver using maltose or glycogen as the substrate.

Substrate	Euthermic <i>S. lateralis</i>	Rat
Maltose	348 ± 101	n/d
Glycogen	$(2.3 \pm 0.2) \times 10^4$	$(1.3 \pm 0.2) \times 10^4^*$

Values are means $\pm$ SEM ($n = 3-4$) and are given in units (nmol glucose produced per minute) per mg protein.

* - Significantly different ($P < 0.05$) from the corresponding *S. lateralis* value.

There was no detectable activity for rat acidic α -glucosidase towards maltose.

No activity was detectable for either of the neutral isozymes towards either substrate.

Table 2.9. K_m values for maltose and glycogen for partially purified euthermic *S. lateralis* and rat acidic α -glucosidase at two temperatures.

Animal	Maltose		Glycogen	
	5°C	37°C	5°C	37°C
<i>S. lateralis</i>	n/d	0.89 ± 0.03	26.1 ± 0.97	$3.42 \pm 1.06^*$
Rat	n/d	n/d	n/d	0.27 ± 0.03

Values are means $\pm$ SEM ($n = 4$) and are given in units of mM for maltose and mg/mL for oyster glycogen.

*- denotes a significant difference ($P < 0.05$) from the corresponding K_m at 5°C; n/d indicates that no activity was detectable, hence K_m could not be determined.

Figure 2.1. Representative pH profile for *S. lateralis* and rat liver α -glucosidase. For extracts from both euthermic *S. lateralis* (solid symbols) and rat (open symbols), pH curves were determined for the neutral (●) in the presence and of 40 mM turanose and the acidic (■) activity was determined differentially by the difference between activities in the presence and absence of 40 mM turanose.

Figure 2.2. Elution profile of α -glucosidase activities from liver of euthermic *S. lateralis* on DE-52 ion exchange chromatography. Elution was with a linear 0-1 M KCl gradient in pH 7.0 phosphate buffer and fluorescence units were measured for assays of both the neutral pH 7.0 (●) and acidic pH 4.0 (■) isozymes using the substrate MU α G.

Figure 2.3. Elution profile of rat liver α -glucosidase neutral and acidic isozymes from DE-52 chromatography. Isozymes were eluted using a linear KCl gradient (0-1 M) in phosphate buffer, pH 7.0. Fluorescence units were determined using MU α G as substrate symbols are: neutral, pH 7.0 assay (●); acidic, pH 4.0 assay (■).

Figure 2.4. Representative isoelectric focusing elution profile for euthermic *S. lateralis* liver α -glucosidase. Activity was measured using optimal assay conditions with MU α G as the substrate at both pH 7.0 (●) and pH 4.0 (■). Broken line shows the pH gradient and the peaks for the two neutral (I and II) and one acidic (A) isozyme are labeled.

Figure 2.5. Representative isoelectric focusing for α -glucosidase from rat liver. Circles indicate neutral isozyme activity measured at pH 7.0 (peaks I and II) and squares indicate acidic isozyme activity (A) measured at pH 4.0; both used MU α G as the substrate. The pH gradient is plotted by the broken line

Figure 2.6. Effect of assay temperature on the K_m values for MU α G for partially purified α -glucosidase neutral isozyme I from euthermic *S. lateralis* and rat liver. *S. lateralis* K_m values are symbolized by circles and the K_m values for rat are symbolized by squares. All data are the mean of at least 3 preparations for both *S. lateralis* and rat. Error bars denote SEM and where not seen are within the dimensions of the symbol used.

Figure 2.7. Effect of assay temperature on the K_m values for MU α G for α -glucosidase neutral isozyme II from euthermic *S. lateralis* and rat liver. K_m values for euthermic *S. lateralis* (●) and rat (■). Other information as in Figure 2.6.

Figure 2.8. Effect of assay temperature on K_m values for MU α G of α -glucosidase acidic isozyme from *S. lateralis* and rat liver. Values for euthermic *S. lateralis* (●) and rat (■). Other information as in Figure 2.6.

Figure 2.9. Effect of incubation at different temperatures on neutral isozyme I activity after 2, 4 and 6 hours. The mean $V_{\max}$ activity for euthermic *S.lateralis* (●) and rat (■) enzymes are from a minimum of 3 preparations; error bars denote SEM and where not seen are within the dimensions of the symbol.

Figure 2.10. Effect of incubation at different temperatures on neutral isozyme II activity after 2, 4 and 6 hours. Other information as in Figure 2.9.

Figure 2.11. Effect of incubation temperature on acidic α -glucosidase activity after 2, 4 and 6 hours. Other information as in Figure 2.9.

Figure 2.12. Inactivation of the α -glucosidase isozymes by temperature over time at 45°C. The means of at least 3 preparations are symbolized by circles, squares, and triangles for neutral I, neutral II and acidic enzymes, respectively, for *S. lateralis* (A) and rat (B). Error bars denote SEM.

Discussion

Two different pH optima (pH 7.0 and pH 4.0) were found in both *S. lateralis* and rat liver for α -glucosidase activity, as seen in Figure 2.1, where the inflection point could be used for the determination of residues responsible for mechanistic action. Because there was a slight overlap of these activities, turanose was employed to obtain true activities at pH 7.0 and pH 4.0. Turanose proved to be a specific inhibitor of only the acidic form and showed no effect on the activity at pH 7.0. This finding was in agreement with previous studies conducted by Jeffrey *et al.* (1970), Matsui *et al.* (1984), and Tashiro *et al.* (1986) that also showed two different enzyme types, acidic and neutral, in mammalian liver.

Eluted fractions from the DE-52 ion exchange chromatography were assayed at the two pH values (with turanose present in the neutral assays) and a differential fluorescent reading was obtained for the acidic activity when assays were conducted with and without turanose. Two neutral isozymes were separated at differing ionic strengths whereas only one acidic form was evident in both euthermic and hibernating *S. lateralis* liver and also in rat liver. Although the neutral forms were separable using ion exchange, it can be seen from Figure 2.2 and 2.3 that the acidic form eluted at almost the same ionic strength as the first neutral isozyme. In liver from hibernating ground squirrels neutral α -glucosidase showed a somewhat different interaction with the ion exchange column such that the peaks from the elution profiles were shifted as seen from the mean KCl elution concentrations listed in Table 2.1. The second isoform interacted less with the DE-52 Sephadex as indicated by the lower [KCl] required to elute it from the column and inversely, the acidic form had stronger interactions with the column material and higher ionic strength was required. This suggested that charge state of the two isozymes was altered in hibernation.

To further investigate this and to find a means of clearly separating all three enzyme forms, the enzymes were analyzed by isoelectrofocusing. Multiple forms were also discerned from IEF profiles (Figures 2.4 and 2.5) but no difference in pI values in euthermic and hibernating *S. lateralis* was observed. From ion-exchange chromatography the difference in interactions indicated a change in the surface of the protein. However, because there were no observable differences in the pI values of the three isozymes in *S. lateralis*, it can be suggested that hibernation-induced changes in the enzyme behaviour on DE-52 may be due to a net charge difference on the protein. The relative activities of the three isozymes are also represented in these figures. The amounts of the three isozymes in euthermic ground squirrel liver increased in relative activity with decreasing pI values. By contrast, the rat IEF profile indicated that as the pI increased, the relative activity also increased. Due to the separation of all three isoforms, peak IEF fractions were isolated and used for further analysis of kinetic and characterization studies.

Although there was no difference in pI values for the enzymes from liver of euthermic and hibernating *S. lateralis*, there were significant differences in the $V_{\max}$ values as calculated for the combined neutral isozymes from crude extracts (Table 2.3). Neutral activity from liver of hibernating ground squirrel measured only 73% of the activity present in the euthermic animal. However, activities in all other tissues from *S. lateralis* remained constant following the entrance into torpor. Rat liver contained significantly higher levels of neutral activity in the liver than did ground squirrel. One role that has been proposed for neutral α -glucosidase is in the transfer of carbohydrate moieties and the structuration of glycoproteins (Castilla *et al.*, 1980; Murat *et al.*, 1978); hence, a decrease in activity in *S. lateralis* would be consistent with a reduced rate of biosynthesis of glycoproteins in the hibernating state.

Although the activity levels of the neutral isoforms changed from the euthermic to hibernating states and between species, there was no indication of a similar trend for the acidic activity from liver. Table 2.4 shows that there was a 2-fold increase in α -glucosidase acidic activity in the skeletal muscle during hibernation which could indicate the need for increased glycogen breakdown via α -glucosidase during hibernation as GP is inactivated during hibernation. However, the muscle still contained the lowest activity of the acidic isoform behind heart, kidney and liver.

Arrhenius plots were used to analyze the relative effects of temperature change on the glucosidic and phosphorolytic pathways of glycogen degradation and to look for possible temperature-induced conformational changes in the proteins that could affect their function at the hibernating T_b . All Arrhenius plots obtained were linear indicating that, within the range tested, temperature caused no major conformational changes to the protein that differentially affected enzyme activity. This implies that the enzymes will function normally over the range of natural body temperatures encountered by the ground squirrel.

Maximal activities of rat and *S. lateralis* liver α -glucosidase towards natural carbohydrates such as maltose and glycogen are important for the determination of the importance of α -glucosidase in the breakdown of carbohydrates *in vivo*. The acidic V_{max} values as determined for both species and both substrates were greater than the V_{max} activities from the combined neutral isozymes indicating the acidic form has a greater capacity for degrading carbohydrates than do the combined neutral forms. Similarly, the maximal velocity for α -glucosidase breakdown of glycogen was greater in both species as compared to the activity for the degradation of maltose. However, the V_{max} activities as determined on the partially purified isozymes indicated no neutral activity towards either maltose or glycogen but activity still remained for the acidic isozymes (Table 2.8). Studies have shown that

rabbit (Onodera *et al.*, 1994), pig (Tashiro *et al.*, 1986), rat (Jeffrey *et al.*, 1970; Calder and Geddes, 1989) and human (Hers, 1963) liver acidic α -glucosidase hydrolyzes a wide variety of substrates. As the acidic α -glucosidase is housed in the lysosome, this finding was expected as its function is to digest poly- and oligo-saccharides into glucose which are then exported out of the organelle and back into the cytoplasm to be recycled. No activity was observed for rat liver α -glucosidase towards maltose although activity towards glycogen was conserved. In the golden-mantled ground squirrel, there was degradation of both of these substrates although the activity towards maltose was only 1% of that towards glycogen. Since there was acidic maltose activity in extracts of crude rat liver but none detectable for the partially purified extract there may be another enzyme present in the crude preparation which degrades maltose but has a different pI from acidic α -glucosidase. However, it is obvious from the activity towards glycogen that this enzyme could be of importance for the regulation of glycogen metabolism and glucose production within the mammals studied.

As an animal enters torpor, its body temperature falls and the need to maintain the integrity of enzyme active sites becomes important over a range of temperatures. An increase in the K_m of *S. lateralis* neutral I and II α -glucosidase at low and high temperatures (Figures 2.6 and 2.7) indicates the reduction in affinity of the enzyme towards MU α G. Between 5°C and 37°C for ground squirrel there was no significant difference in K_m values for the acidic isozyme (Figure 2.8). Rat liver α -glucosidase was stable over the temperature range for both neutral isozymes and above 45°C for the acidic α -glucosidase. These findings are indicative of highly conserved conformational integrity at the active site of neutral I, II and acidic α -glucosidase from rat and from the acidic isozyme from *S. lateralis*.

Table 2.9 lists the K_m values for maltose and glycogen as determined for the acidic α -glucosidase from euthermic *S. lateralis* and rat liver at 5°C and 37°C.

K_m for maltose of the *S. lateralis* enzyme increased from 0.89 ± 0.03 mM at 37°C to a value greater than 50 mM at 5°C . This increase was also seen in the rat acidic form with glycogen as substrate but the affinity of the acidic α -glucosidase from *S. lateralis* towards glycogen dropped by almost 8 times. These observations indicate that although activity was still present, there was an alteration in the active site such that affinity for substrates was affected. These data, coupled with the previous discussion of affinity for MU α G indicate that temperature change has differential effects on the binding of different substrates, binding of the physiological substrates being apparently more sensitive to temperature change. The differential temperature effects on the K_m for different substrates probably indicates that some of the functional groups on the protein that are involved in substrate binding are different for the synthetic, disaccharide and polysaccharide substrates, and that these are differently affected by temperature change producing overall differences in the temperature effect on enzyme affinity.

Since hibernators are known to decrease their body temperatures from 37°C (euthermia) to 5 - 12°C (hibernation), the thermal stability of enzymes is one of the characteristics which could be altered to allow for effectively functioning proteins over a range of temperatures. As temperature increases, the strength of hydrophobic bonds increases at the same time as hydrophilic bonds become less stable. Hence, the stability of enzymes over a wide range of temperatures gives an inference into the relative importance of these types of bonds to the important protein sites. All three isoforms of *S. lateralis* α -glucosidase were stable up to 37°C and only the neutral isozymes showed substantial decreases in activity above this temperature. The differences in the temperature effects on the activities of these three forms is suggestive of structural anomalies and degree of hydrophobic versus hydrophilic interactions. Similarly, thermal stability has been determined for α -glucosidase from rabbit muscle (Matsui *et al.* 1984), rabbit liver (Onodera *et al.*

1989) and rat liver (Grinna and Robbins, 1979); these studies indicated stability up to and including 37°C but the enzymes denatured rapidly above this temperature.

Further analysis was completed at 45°C as there was a substantial change in V_{max} activities of the three isozymes studied at this temperature. The enzymes from hibernator liver (Figure 2.12A) showed no change in the activity of the acidic α -glucosidase over incubation time but a significant drop to undetectable activities for the neutral I enzyme after 4 hours and neutral II after 2 hours. Although rapid initial thermolability was observed in Figure 2.12B for rat neutral I and II, activity still remained at after extended exposure to this incubation temperature. A slower degradation of activity was evident for the acidic α -glucosidase although over the 6 hour incubation there was no observed slowing down of activity loss as in the neutral isozymes. For both of these species, there must be greater flexibility within the acidic α -glucosidase than either of the neutrals as indicated by the thermal stability.

The species difference between the hibernator and non-hibernator implies that there was a greater need of flexibility in the α -glucosidase from ground squirrel than rat. The rat neutral I and II did not denature completely implying a structural rigidity difference between the hibernator and non-hibernator which is substantiated by the active site conservation as indicated by the K_m values over the same temperature range.

Chapter 3
Characterization of α -glucosidase from
a freeze tolerant frog, *Rana sylvatica*

Introduction

In northern climates, winter is one of the obstacles faced by frogs in their fight for survival. There are two methods used by animals to survive subzero temperatures, avoidance and endurance. The former includes active migration to warmer climates or to locales where the ambient temperature will not drop to subzero levels (under water or underground) and is the major strategy utilized by most amphibians. The latter is to allow the freezing of extracellular water while utilizing various cryoprotective adaptations to preserve the liquid state of intracellular compartments. Recently, it has been discovered that four species of frogs (the wood frog, *Rana sylvatica*, the grey tree frog, *Hyla versicolor*, the spring peeper *Pseudacris crucifer* and the chorus frog, *Pseudacris triseriata*) use freeze tolerance for overwintering. These species are all terrestrial frogs which overwinter on the soil surface covered by forest debris (Storey, 1985a). As the winter progresses, snow covers the frogs insulating them from the most extreme subzero temperatures but even under the cover of snow the temperature in their hibernacula has been found to drop to -5°C and -7°C (MacArthur and Dandy, 1982; Schmid, 1982). Hence, these four species must have developed strategies for survival during and following the freezing of their tissues.

The focus of the present study is the wood frog, *R. sylvatica*. In the Ottawa area, males range in size from about 5-8 g and females from 8-13 g (Storey, 1985) and are between 3.5-8.3 cm in length. The species ranges throughout North America up to and beyond the Arctic circle: they live in moist woodlands in the east, open grasslands in the west and in tundra in the north (Behler and King, 1979). This species has a supercooling point of -1.87°C (Schmid, 1982) and extracellular ice forms between -2 and -3°C . Adults can survive several days of freezing at -2.5°

C with survival times improving when insulated in well protected and humid hibernation sites (Storey, 1984).

Freeze Tolerance

Freeze tolerance has been identified in a variety of invertebrates, including many species of insects, and recently in selected species of amphibians and reptiles. Natural freeze tolerance refers to the freezing of extracellular fluids only; the formation of intracellular ice is lethal. Adaptations for freeze tolerance have been associated with the distribution of ice and water as well as the preservation of subcellular organization as the cell dehydrates. The main adaptations studied in the freeze tolerant insects include: extracellular nucleating agents for the induction of controlled ice formation at relatively high subzero temperatures; limitation of intracellular dehydration and protection of subcellular components by the accumulation of low molecular weight cryoprotectants; bound versus free water content alterations; changes to intermediary metabolism and mitochondrial function, especially to pathways involved with cryoprotectant metabolism; and energy production from anaerobic pathways in the frozen state when oxygen is not available (Storey, 1986).

The investigation of these various adaptations as observed in insects has been ongoing in the freeze tolerant frogs. Recently an ice nucleating protein (INP) has been discovered in *R. sylvatica*. This INP has been found in plasma of the frog and was determined to not only induce the nucleation of ice crystals, but also to control their formation (Wolanczyk *et al.* 1990a; 1990b). All four species of freeze tolerant frogs also produce high concentrations of low molecular weight cryoprotectants with the onset of freezing, their role being to increase intracellular osmolality to limit the amount of cell water that is lost into extracellular ice crystals. *R. sylvatica*, *P. crucifer*, *P. triseriata* and immature *H. versicolor* all

accumulate glucose whereas the primary cryoprotectant of adult *H. versicolor* is glycerol (Storey and Storey, 1986). Unlike insects, there is no anticipatory accumulation of glucose during an autumn cold hardening period but rather cryoprotectant is only produced upon the onset of freezing (Storey and Storey, 1984; Storey, 1987b). This is advantageous for the frog as their overwintering locales are often so well insulated that they are not exposed to freezing temperatures and they save the metabolic expense associated with synthesizing a cryoprotectant unless it is needed.

Frogs are able to produce vast quantities of glucose as a cryoprotectant in a short period of time, but to ensure that the glucose pool is sustained throughout a freezing episode, inhibitory controls are also applied to the possible routes of cryoprotectant catabolism. In *R. sylvatica*, this includes a decrease in glycogen synthetase (GS) activity in liver in the frozen state (Russell and Storey, 1995). In addition, Storey (1987a) found that changes in intermediates of glycolysis indicated an inhibition of this pathway at the phosphofructokinase (PFK) locus during freezing that would help to channel carbon derived from glycogenolysis away from glycolysis and into the production of glucose. Inhibitory control of glycolysis appears to be centered on the control of phosphofructokinase and a strong depression of the levels of this enzyme by its key activator, fructose-2,6-bisphosphate, may be the primary factor responsible for blocking the catabolism of glucose in the freezing frog (Vazquez Illanes and Storey, 1993).

Onset of Freezing

Both freeze tolerant and intolerant frogs have supercooling capacities such that they have a whole body supercooling point within the range of -1.9°C to -3°C ; once the temperature falls below -3°C the onset of ice formation in freeze intolerant frogs is lethal. Freeze tolerant species have been known to survive in a frozen state

for days at a time at moderate subzero temperatures, however if the temperature drops too low or for extended periods of time the mortality rate increases (Storey, 1986).

As extracellular ice crystals grow they exclude solutes from their crystal lattice with the result that remaining extracellular fluid becomes more concentrated. This places a hyperosmotic stress on cells that leads to an efflux of cell water and a decrease in cell volume along with an increase in metabolite and ion concentrations in the cytoplasm. Cell shrinkage due to water loss to extracellular ice is tolerable to a point but if cell volume falls below a critical minimum, then irreversible damage is done to the bilayer structure of the plasma and organelle membranes. Thus, a key function of low molecular weight cryoprotectants, such as glucose, is to help retard cell shrinkage by osmotic means by increasing intracellular solute concentrations. Because frogs produce this cryoprotectant only as a direct response to the initiation of freezing in the body extremities, glucose production must be very rapid so that cryoprotectant is provided to all organs before blood circulation is cut off by plasma freezing. Freezing stimulates a rapid activation of liver glycogenolysis which can be detected within as little as 2 minutes after freezing begins and elevates liver and blood glucose levels by several fold within just a few minutes. Production continues over the next several hours with glucose continuously exported from the liver and taken up from the blood by other organs to elevate their cryoprotectant pools. Storey and Storey (1984) found that glucose levels in the liver rose from $5.53 \pm 1.02 \mu\text{mol/g}$ wet weight in 3°C -acclimated animals to $387.8 \pm 44.8 \mu\text{mol/g}$ wet weight in fully frozen animals; blood glucose in the same frogs rose from $2.43 \pm 0.35 \mu\text{mol/mL}$ to $214 \pm 15.6 \mu\text{mol/mL}$. Other tissues (heart, kidney and leg muscle) also showed major increases in glucose content, but to a lesser extent than in the liver. Facilitating the export of glucose from liver and its distribution and

uptake by other organs, is a seasonal increase in the numbers of glucose transporter proteins in the plasma membrane of wood frog organs (King *et al.*, 1994).

Cryoprotectant biosynthesis

The best known and most studied pathway of glycogen conversion to glucose involves three enzymes: glycogen phosphorylase (GP), phosphoglucomutase, and glucose-6-phosphatase. This pathway is an established pathway in the liver and can be stimulated readily through hormonal controls. As well as the stimulation of glucose production during freezing, there must be a concomitant removal of homeostatic control by pancreatic hormones over blood glucose concentration as well as the inhibition of mechanisms for the oxidation of glucose or for the restoration of glycogen stores (Storey, 1985).

Glycogen Stores and Usage

Although there is no anticipatory build-up of glucose as a cryoprotectant in the *R. sylvatica*, there is an increase of hepatic glycogen during the autumn so that the substrate for glucose synthesis is at hand whenever it is needed. As ice propagation commences, there is a tremendous increase (520%) in total GP activity with 37 to 80% of this GP being in the active form (Storey and Storey, 1984). This activation results in measurable increases of glucose in both the liver and the blood within the first 5 minutes of ice propagation (Storey, 1987a). Glucose continues to accumulate over the next few hours, reaching high concentrations. Previous studies of vertebrate liver GP, however, have shown that it is susceptible to inhibition by high glucose and, indeed, the isolated wood frog liver enzyme also showed such inhibition by glucose (Risman *et al.*, 1993). Thus, due to the need to accumulate glucose very rapidly over a short time during the early stages of freezing and recognizing that GP could be substantially inhibited by the accumulated glucose as

it reached higher levels, we wondered whether other enzymatic activities in liver might also contribute to net cryoprotectant synthesis during freezing.

One enzyme which may be of importance for the degradation of hepatic glycogen is α -glucosidase. This enzyme of the glucosidic pathway hydrolyzes glucose units off of the glycogen backbone directly without the necessity of others for the production of glucose. One report has linked the buildup of glucose following exercise in *Rana pipiens* muscle to the action of the glucosidic pathway and not the phosphorolytic pathway (Fournier *et al.*, 1992; Fournier and Guderley, 1993). Hence, a similar buildup of glucose in *R. sylvatica* during the formation and control of extracellular ice could be assisted by α -glucosidase.

The present study examined the possible role of α -glucosidase in the production of glucose from glycogen in the freeze tolerant wood frog. Throughout a freeze/thaw bout there are large alterations in the whole body temperature of the *R. sylvatica* and hence, the effect of temperature on activation energy, substrate affinity and structure/function relationships are also of importance in the control of enzymes of glycogen metabolism.

Materials and Methods

Chemicals and Animals.

All chemicals were purchased from either Sigma Chemical Company or Boehringer Mannheim Biochemica. Adult male and female *Rana sylvatica* and *Rana pipiens* were collected from breeding ponds in woodland areas in the Ottawa (Canada) area in spring 1993. Animals were placed on damp sphagnum moss in covered plastic containers and kept without feeding at a constant temperature of 5°C for 2 weeks. For freezing experiments with *R. sylvatica*, one container was transferred to an incubator at $-4 \pm 0.1^\circ\text{C}$ and frozen with the insulating moss; maximal ice formation required 24-36 h. The animals were held at -4°C for 24 hours and then half were removed individually, killed by double-pithing, and livers were rapidly excised and frozen in liquid nitrogen. The remaining frozen frogs were returned to 5°C and allowed to thaw and recover for 24 hours, then sacrificed as above. Control *R. sylvatica* and *R. pipiens* were held at a constant temperature of 5°C, and then sacrificed and livers sampled as above. Frozen livers were transferred to -80°C for long-term storage. Control refers to those animals sacrificed prior to freezing. Tissues taken from animals which were frozen for 24 hours are labeled frozen and those thawed for 24 hours following a 24 hour freezing bout are identified as recovered.

All assay conditions for *R. sylvatica* and *R. pipiens* α -glucosidase were consistent with those used for the *S. lateralis* enzymes. The methods stated below are in addition to the methods from Chapter 2.

Subcellular Fractionation.

Subcellular localization of enzyme activity was conducted using fresh liver samples from control animals following the procedure of De Duve *et al.* (1955). The liver was removed immediately upon sacrifice, weighed and then immediately homogenized in 2 mL 0.25 M sucrose, 10 mM Tris, pH 7.4 using a hand-held smooth-walled glass tube fitted with a Teflon pestle. The homogenate was centrifuged at 600g for 10 min and the supernatant was removed. The pellet (cell debris and nuclei) was resuspended in cold buffer and centrifuged again at 600g for 10 min. The second supernatant was combined with the first and then centrifuged at 8000g for 20 min. This supernatant was removed and saved and the pellet was resuspended in cold 10 mM Tris buffer, pH 7.4. Then, both the resuspended pellet and the supernatant were sonicated to ensure complete release of enzyme from any membrane association. Fractions were held on ice and then assayed for enzyme assay using glycogen as the substrate.

I₅₀ Values.

The concentration of effector which resulted a in 50 % reduction in initial enzyme activity towards MU α G was determined for NaCl, KCl, K₂SO₄, sucrose, glucose, turanose, fructose and glycerol.

Results

pH Curves

When aliquots of crude extract of liver were incubated with MU α G in buffer at pH values ranging from 3.0 to 8.5, two local maxima were evident at pH 4.0 and pH 7.0 (Figure 3.1). α -Glucosidase from liver of both frog species, *R. pipiens* and *R. sylvatica*, showed these maxima. The activity with a pH optimum at pH 4.0 is denoted as the acidic form and pH 7.0 as neutral. The acidic form showed minimal overlap in activity at pH 7.0 and vice versa for the neutral form at pH 4.0.

Turanose (40 mM) was utilized as a specific inhibitor of the acidic form for the differentiation of the different pH optima. It was determined that at 100 mM turanose there was no inhibition of the neutral forms from either *R. sylvatica* or *R. pipiens*. However, the acidic isozyme from *R. sylvatica* had an I_{50} value of 3.4 ± 0.2 mM and the *R. pipiens* enzyme showed an I_{50} of 2.1 ± 0.2 mM. As there was no acidic activity detectable in the presence of 40 mM turanose, this concentration was employed throughout the assays to differentiate between the two enzyme forms.

Subcellular Fractionation

The first pellet obtained following centrifugation separated the nuclei and fibrous tissue from the mitochondria and lysosome components. After the second centrifugation a precipitation of the mitochondria and lysosomes was achieved. Using citrate synthase as a marker protein for the mitochondria, it was determined for *R. pipiens* that 93 ± 4 % of the neutral and 79 ± 11 % of the acidic liver forms were contained in this fraction. A similar finding was determined for the *R. sylvatica* liver α -glucosidase isozymes (neutral: 64.3 ± 2 %; acidic: 91 ± 9 %)

DE-52 Column Chromatography

R. sylvatica liver α -glucosidase was assayed in fractions eluted from the ion exchange column at both pH 4.0 and pH 7.0 using MU α G as substrate. Figure 3.2 illustrates the peaks eluted using a linear gradient from 0-1 M KCl. From Table 3.1 there appears to be a separation in the isozymes as the peak fractions eluted at 316 mM and 281 mM for the acidic and neutral forms, respectively. However, it is obvious from Figure 3.2 that there is a large overlap in activity for the two isoforms and thus no separation exists. Figure 3.3 shows the comparable separation of the different forms in *R. pipiens* liver by DE-52 chromatography. Here, the two enzyme forms were largely separable; some neutral activity overlapped with the acidic form but the major peak of neutral activity was free of acidic activity.

Isoelectric Focusing

Isoelectric focusing of crude extracts also showed two forms of α -glucosidase in both species when column fractions were assayed at both pH 4 and 7 ($\pm$ 40 mM turanose). The isoelectric points of the enzymes are listed in Table 3.2 and a representative elution profile is shown in Figures 3.4 and 3.5 for *R. sylvatica* and *R. pipiens*, respectively. In both species the acidic isozyme showed a lower pI value than the neutral enzyme. The peak fractions from the IEF columns were used as a partially purified enzyme extract and subsequent studies were completed using these preparations.

Tissue Surveys

Maximal activities of neutral and acidic α -glucosidase in tissues *R. sylvatica* and *R. pipiens* are listed in Tables 3.3 and 3.4. Freezing exposure increased the activity of neutral α -glucosidase in heart, kidney, and skeletal muscle of *R. sylvatica*; values increased by 95, 54, and 46 %, respectively. In gut, however,

activity fell by 50 % in frozen animals. Activity was unchanged by freezing in brain and liver. After thawing, neutral α -glucosidase activity had returned to the control level in muscle whereas an apparent elevation of α -glucosidase in liver was not significantly different from the control value.

Activities of the neutral isozyme in three tissues of *R. pipiens* were significantly lower than the comparable values for control *R. sylvatica*; values were just 10, 34 and 36 % of the values in control *R. sylvatica* muscle, heart and liver, respectively. However, kidney of *R. pipiens* showed a 40 % higher enzyme activity than did *R. sylvatica*.

Acidic α -glucosidase activity was strongly decreased in kidney and gut of *R. sylvatica* during freezing exposure, falling to just 3.7 and 8.1 % of the corresponding control value. By contrast, activity rose by 3.6-fold during freezing in heart but was unchanged in other organs of the frozen frog. After thawing, activity was unchanged in muscle but was significantly reduced by 37 % in liver.

A comparison of acidic activities in control animals of the two species showed a higher activity in *R. pipiens* heart but in all other instances, lower activities of acidic α -glucosidase in *R. pipiens*, compared with *R. sylvatica* tissues. The largest difference was between kidney of the two species where *R. pipiens* exhibited only 3 % of the activity present in the *R. sylvatica* organ.

Table 3.5 shows the total activity of glycogen phosphorylase (sum of the active and inactive forms) in six tissues of the two species as well as the effect of freezing and thawing on the enzyme in *R. sylvatica* tissues. Freezing elevated total GP activity in three tissues of wood frogs, activity increasing by 10.9-, 5.3- and 2-fold in liver, kidney, and gut of frozen animals, respectively. After thawing, however, activity was suppressed again in all three tissues, activity in kidney and liver falling to only one-half of the original control value. Compared with *R. sylvatica*, total GP activity was significantly lower in all *R. pipiens* tissues except

for gut. The largest difference between the species was for liver where *R. pipiens* total GP activity was only 22 % that in wood frog liver.

Although the total activity may change, the active form (*a*) of GP is of interest to determine how much of the total GP is activated to breakdown the glycogen stores. As seen in Table 3.5, the percentage of the *a* form decreased in heart of frozen *R. sylvatica*. Except for brain, all other tissues, however, showed a major increase in the percentage of active GP during freezing. The largest increase in percent *a* was in the muscle which increased from 7.75 ± 1.93 % to 37.67 ± 3.48 %, a 5-fold increase. After 24 hours of thawing recovery, the freezing effect was reversed and values for % *a* in four tissues were not different from the corresponding controls. The % *a* was still somewhat elevated in muscle but in liver percent *a* dropped to only one-half of the control value.

A comparison of the % *a* values for control animals of the two species showed significantly higher values in *R. pipiens* muscle and gut. However, the percent *a* in control *R. sylvatica* liver was twice that in *R. pipiens* liver.

Arrhenius Plots

The effect of temperature on the activity of α -glucosidase from liver of control *R. sylvatica* and *R. pipiens* was assessed with $V_{\max}$ activity measurements over a temperature range of 5°C to 65°C (20 min incubation) and analysis of the data via an Arrhenius plot ($\log V_{\max}$ vs $1/^\circ\text{K}$). In all cases the relationship was linear over the full range of temperatures tested (correlation coefficient > 0.98). The activation energy E_a for the two isozymes was calculated from the slope of the plot (Table 3.7). Values ranged from 53.6 to 59.8 kJ/mol and there were no significant differences either between species or between acidic and neutral enzymes.

Comparable Arrhenius plots were completed for GP over the same temperature range and assessing both the *a* and *b* forms: activation energies are listed in Table 3.7. The activity for the inactive form was determined from the difference between the total activity (*a* + *b*) and the activity of the active form. All plots were linear over the full temperature range tested. No significant differences were obtained for the activation energies in *R. sylvatica* or in *R. pipiens* liver GP.

Activity of α -Glucosidase towards Maltose and Glycogen

The activity of α -glucosidase in crude liver extracts of either species towards maltose and glycogen was greater for the acidic than for the neutral isozyme (Table 3.8). In fact, there was no detectable activity of the *R. pipiens* neutral isozyme towards glycogen. Comparing the acidic enzymes, there was higher activity in *R. sylvatica* liver, compared with *R. pipiens*, using either substrate (1.8-fold greater for maltose and 2.8-fold for oyster glycogen). However, activity of the *R. pipiens* neutral enzyme using maltose was 3 times greater than the corresponding activity of the *R. sylvatica* enzyme.

Maltose to glycogen activity ratios for the crude enzyme preparation for the acidic isozyme from *R. sylvatica* and *R. pipiens* were 1:2 and 1:1.3, respectively. However, the same activity ratio of the partially purified acidic isoform obtained following IEF was 1:6.6 for the *R. sylvatica* and 1:3.9 for the *R. pipiens* (Table 3.9). Thus, for both species and sets of preparations greater glycogen degrading activity was observed than the activity for the hydrolysis of maltose. No activity was determined for the semi-purified neutral α -glucosidase from *R. sylvatica* with glycogen or maltose as the substrate. However, there was maltose activity for the neutral isozyme from *R. pipiens* (7.722 ± 1.469 U/mg protein) although no glucose production from glycogen was measured.

I₅₀ Values

An I₅₀ is defined as the concentration of effector that reduces enzyme activity by 50 %. As seen in Table 3.10, the neutral isozyme was largely unaffected by salts and carbohydrates other than glucose. None of the salts tested affected the *R. sylvatica* enzyme at levels within the expected physiological range however there was inhibition of the *R. pipiens* neutral isozyme with KCl (1.15 ± 0.10 M). The neutral enzyme from both species showed product inhibition by glucose, the enzyme from *R. sylvatica* being less affected by glucose (I₅₀ = 90 ± 10 mM) than the *R. pipiens* neutral isozyme (I₅₀ = 16 ± 2 mM).

Acidic α -glucosidase from *R. sylvatica* was affected by NaCl and KCl but not inhibited by K₂P₄ and K₂SO₄ over the concentration range tested. These salt concentrations are considerably above the physiological levels in frog cellular fluids. The acidic form was also susceptible to inhibition by all the carbohydrates tested (sucrose, glucose, fructose and glycerol). In particular, the acidic form showed strong product inhibition by glucose (I₅₀ = 17 ± 2 mM).

Similar trends were observed for the *R. pipiens* acidic isozyme which showed substantial inhibition by all the effectors tested except for K₂SO₄. Both NaCl and KCl resulted in a decrease in activity as well as K₂P₄. The carbohydrates were strong inhibitors of the *R. pipiens* enzyme, especially glucose (I₅₀ = 8 ± 1 mM). Notably, inhibition of the *R. pipiens* enzyme by ions or carbohydrates was in most instances stronger (I₅₀ lower) than the corresponding effects seen with the *R. sylvatica* enzyme.

Substrate Affinity at Varying Temperatures

The substrate affinity for MU α G can indicate a change in the active site as the temperature changes. Figure 3.6 shows the effect of assay temperature on the K_m for MU α G of the neutral enzyme from both species over the range 5-45°C. The

K_m of the *R. sylvatica* liver enzyme was largely unaffected by temperature change. However, the MU α G K_m of the *R. pipiens* enzyme increased significantly at 12°C and 20°C to twice that determined at higher and lower temperatures.

A comparable analysis of the effects of temperature on the K_m MU α G of acidic α -glucosidase is shown in Figure 3.7. Temperature change resulted in a significant drop in K_m at 27°C as compared to 5°C for the *R. sylvatica* enzyme. Otherwise K_m of the wood frog enzyme was unaffected by temperature change, ranging from 0.75-0.95 mM. Similarly, there was no effect of temperature change on the K_m of the *R. pipiens* acidic enzyme.

Table 3.11 shows K_m values determined at 5°C and 20°C for the *R. sylvatica* and *R. pipiens* acidic isozymes using maltose and shellfish glycogen as substrates. Temperature change did not alter the *R. sylvatica* α -glucosidase active site as indicated by the lack of effect of temperature on the affinity for either substrate. However, at 5°C there was a significant decrease in substrate affinity for the acidic α -glucosidase from *R. pipiens* as compared to the maltose K_m value at 20°C. A similar decrease in glycogen K_m with the *R. pipiens* acidic α -glucosidase was not observed.

Deactivation of α -glucosidase by Temperature

In the wood frog, enzymes are exposed to large temperature fluctuations and stability of an enzyme at varying temperatures becomes important. α -Glucosidase isozymes were incubated for varying times at different temperatures and then activity remaining was determined under standard assay conditions (MU α G as substrate, 20 minute incubation at 20°C in either pH 7.0 or 4.0 buffer). As shown in Figure 3.8, the activity of the neutral isozyme was stable after 2, 4 or 6 h incubation at temperatures between 5°C to 37°C. However at 45 or 55°C, activity was rapidly lost and was undetectable after only 2 hours incubation at 55°C or after 4 hours at

45°C. The enzymes from both *R. pipiens* and control *R. sylvatica* behaved similarly. Contrary to the results for the neutral form, the acidic enzyme was highly stable over the full temperature range (Figure 3.9). After both 2 and 4 hours incubation, full activity of both enzymes was retained at both 45 and 55°C and only the *R. pipiens* enzyme showed a decrease in activity of about 50 % after 6 hours at 55°C.

To further characterize the thermal lability of neutral α -glucosidase, enzyme activity loss at 45°C was monitored over a more detailed time course (Figure 3.10). Plots for both frog species showed similar results; a rapid decrease in activity was seen during the first 20 minutes and the activity continued to drop over the remainder of the 6 hours. It was determined that activity of the *R. sylvatica* neutral α -glucosidase decreased at a slower rate than its *R. pipiens* counterpart as the half-time (time where 50% of the initial activity remains) was 30.5 and 21.5 minutes, respectively. However, no activity of *R. sylvatica* neutral α -glucosidase remained after 6 hours whereas 13% of the initial activity of the *R. pipiens* enzyme could still be detected.

Table 3.1. KCl concentration (mmol/L) at which the peak fraction of α -glucosidase eluted from the DE-52 column for the *R. sylvatica* and *R. pipiens* liver enzyme.

Tissue	Neutral Isozyme	Acidic Isozyme
<i>R. sylvatica</i>	316 ± 10	281 ± 18
<i>R. pipiens</i>	273 ± 50	239 ± 39

The data presented are the result of the column run in triplicate.

Table 3.2. Isoelectric points (pI) for α -glucosidase from *R. sylvatica* and *R. pipiens* liver IEF analysis.

Tissue	Neutral Isozyme	Acidic Isozyme
<i>R. sylvatica</i>	6.00 ± 0.04	5.41 ± 0.10
<i>R. pipiens</i>	6.16 ± 0.13	6.06 ± 0.17

Values are from 3 separate analyses.

Table 3.3. $V_{\max}$ values of the neutral isozyme from various tissues of *R. pipiens* and *R. sylvatica*.

Tissue	<i>R. sylvatica</i> , Control	<i>R. sylvatica</i> , Frozen	<i>R. sylvatica</i> , Recovered	<i>R. pipiens</i>
Heart	34.9 $\pm$ 1.7	68.3 $\pm$ 5.0*		11.8 $\pm$ 1.3*
Kidney	241.5 $\pm$ 24.8	372.0 $\pm$ 32.7*		341.0 $\pm$ 11.8*
Muscle	26.3 $\pm$ 0.6	38.3 $\pm$ 5.4*	29.0 $\pm$ 8.3	2.53 $\pm$ 0.3*
Brain	26.8 $\pm$ 5.3	14.2 $\pm$ 1.9		27.4 $\pm$ 3.2
Gut	108.0 $\pm$ 13.9	50.8 $\pm$ 5.5*		121.0 $\pm$ 24.2
Liver	202.7 $\pm$ 36.3	257.0 $\pm$ 21.0	304.5 $\pm$ 23.5	72.5 $\pm$ 6.4*

Data were obtained in the presence of turanose and are given in mU per gram wet weight $\pm$ SEM. n = 4 where one unit is the number of nmol MU α G hydrolyzed per minute at 20°C.

A significant difference from control *R. sylvatica* of $P < 0.05$ is indicated by *.

Table 3.4. Acidic isozyme $V_{\max}$ values from various tissues of *R. pipiens* and *R. sylvatica*.

Tissue	<i>R. sylvatica</i> , Control	<i>R. sylvatica</i> , Frozen	<i>R. sylvatica</i> , Recovered	<i>R. pipiens</i>
Heart	11.7 $\pm$ 1.6	41.9 $\pm$ 14.6*		21.7 $\pm$ 1.9*
Kidney	460.5 $\pm$ 45.5	17.2 $\pm$ 5.8*		14.9 $\pm$ 3.5*
Muscle	16.0 $\pm$ 0.8	17.5 $\pm$ 0.9	14.6 $\pm$ 10.0	9.0 $\pm$ 0.4*
Brain	19.5 $\pm$ 3.1	17.1 $\pm$ 1.4		11.4 $\pm$ 0.8*
Gut	87.9 $\pm$ 34.3	7.1 $\pm$ 1.4*		9.0 $\pm$ 1.6*
Liver	904.0 $\pm$ 157.0	689.0 $\pm$ 73.5	577.0 $\pm$ 39.8*	319.0 $\pm$ 23.2*

Data are mU/gww, means $\pm$ SEM, n = 3-5 where units are the number of nmol MU α G hydrolyzed per minute at 20°C

* indicates a significant difference (P < 0.05) from control *R. sylvatica*

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Table 3.5. Glycogen phosphorylase total activity and percent *a* in various tissues of *R. sylvatica* and *R. pipiens*.

Tissue		<i>R. sylvatica</i> , Control	<i>R. sylvatica</i> , Frozen	<i>R. sylvatica</i> , Recovered	<i>R. pipiens</i>
Heart	Total	3.49 ± 0.39	3.61 ± 0.53	5.02 ± 0.79	1.28 ± 0.22*
	% <i>a</i>	2.50 ± 0.34	0.87 ± 0.13*	2.25 ± 0.48 [§]	2.67 ± 0.67
Kidney	Total	0.99 ± 0.13	5.22 ± 0.28*	0.46 ± 0.07* [§]	0.28 ± 0.03*
	% <i>a</i>	21.8 ± 2.27	44.7 ± 4.18*	17.0 ± 2.27 [§]	15.8 ± 2.75
Muscle	Total	30.5 ± 3.27	29.1 ± 3.12	40.9 ± 6.32	13.9 ± 0.90*
	% <i>a</i>	7.75 ± 1.93	37.7 ± 3.48*	16.0 ± 1.68* [§]	20.2 ± 2.96*
Brain	Total	3.07 ± 0.20	3.94 ± 0.44	3.69 ± 0.42	2.16 ± 0.15*
	% <i>a</i>	33.3 ± 2.25	26.7 ± 2.67	30.0 ± 1.15	33.7 ± 5.49
Gut	Total	0.36 ± 0.05	0.72 ± 0.09*	0.47 ± 0.06 [§]	0.54 ± 0.07
	% <i>a</i>	18.4 ± 3.23	54.3 ± 4.49*	13.8 ± 1.03 [§]	49.0 ± 3.14*
Liver	Total	4.08 ± 0.61	44.6 ± 5.61*	2.05 ± 0.15 [§]	0.88 ± 0.11*
	% <i>a</i>	35.4 ± 3.83	62.0 ± 5.28*	14.8 ± 2.06* [§]	16.3 ± 1.45*

GP is expressed as means ± SEM (n = 4) in units/gww at 20°C.

* - Significantly different (P < 0.05) from the corresponding control value for *R. sylvatica* whereas [§] is the comparison between the frozen and recovered values of *R. sylvatica*.

Table 3.6. Activation energies for *R. sylvatica* and *R. pipiens* liver α -glucosidase in kJ/mol.

Isozyme	Control <i>R. sylvatica</i>	<i>R. pipiens</i>
Neutral	54.0 $\pm$ 1.7	53.6 $\pm$ 0.3
Acidic	54.8 $\pm$ 2.8	59.8 $\pm$ 4.4

There was no significant difference between the means ($\pm$ SEM) for either isozyme from the two frogs (n = 4).

Table 3.7. Activation energies for liver GP from *R. sylvatica* and *R. pipiens* in kJ/mol.

Form	Control <i>R. sylvatica</i>	<i>R. pipiens</i>
<i>a</i>	62.8 ± 5.1	84.5 ± 8.3
<i>b</i>	63.2 ± 5.4	46.4 ± 5.4

Activation energies for the active (*a*) and inactive (*b*) forms of GP are listed as the means of four measurements ($\pm$ SEM). The *b* activity was calculated as the difference between the total (*a* + *b*) and *a* activity.

Table 3.8. Maximal activities of α -glucosidase in crude liver extracts from control *R. sylvatica* and *R. pipiens* using oyster glycogen or maltose as the substrate.

Animal	Substrate	Neutral Isozyme	Acidic Isozyme
<i>R. sylvatica</i>	Maltose	22.3 $\pm$ 4.56	198 $\pm$ 34.6
	Glycogen	178 $\pm$ 29.7	392 $\pm$ 75.4
<i>R. pipiens</i>	Maltose	66.9 $\pm$ 14.4*	108 $\pm$ 19.4*
	Glycogen	n/d	140 $\pm$ 23.5*

Values are means $\pm$ SEM (n = 3-6) and are given in units (nmol glucose produced per minute) per mg protein.

A significant difference ($P < 0.05$) from the $V_{\max}$ value for *R. sylvatica* with the corresponding substrate is denoted by *.

Table 3.9. $V_{\max}$ values for maltose and glycogen for partially purified acidic α -glucosidase from liver of control *R. sylvatica* and *R. pipiens*.

Substrate	<i>R. sylvatica</i>	<i>R. pipiens</i>
Maltose	$(6.1 \pm 0.5) \times 10^3$	$(2.2 \pm 0.2) \times 10^4^*$
Glycogen	$(4.0 \pm 0.7) \times 10^4$	$(8.7 \pm 1.7) \times 10^3$

Values are means $\pm$ SEM (n = 3) and are given in units (nmol glucose produced per minute) per mg protein.

A significant difference ($P < 0.05$) from *R. sylvatica* with maltose as substrate is denoted by *.

Table 3.10. I_{50} values for partially purified α -glucosidase isozymes from liver of control *R. sylvatica* and *R. pipiens*.

Effectors	<i>R. sylvatica</i>		<i>R. pipiens</i>	
	Neutral	Acidic	Neutral	Acidic
NaCl	> 3.0	1.49 ± 0.34	> 3.0	0.24 ± 0.04
KCl	> 1.5	1.62 ± 0.39	1.15 ± 0.10	0.78 ± 0.13
KP _i	> 0.75	> 0.75	> 0.75	0.19 ± 0.017
K ₂ SO ₄	> 0.25	> 0.25	> 0.25	> 0.25
Sucrose	> 1.0	0.28 ± 0.05	> 1.0	0.17 ± 0.013
Glucose	0.09 ± 0.01	0.017 ± 0.002	0.016 ± 0.002	0.008 ± 0.001
Fructose	> 1.5	0.65 ± 0.07	> 1.5	0.35 ± 0.038
Glycerol	> 0.25	0.17 ± 0.03	> 0.25	0.17 ± 0.01

All values are the result of at least three determinations ($\pm$ SEM) and values are in mol/L.

Table 3.11. Temperature effects on K_m values for maltose and glycogen for partially purified acidic α -glucosidase from control *R. sylvatica* and *R. pipiens*.

Animal	Maltose		Glycogen	
	5°C	20°C	5°C	20°C
<i>R. sylvatica</i>	2.39 ± 0.37	2.17 ± 0.22	1.98 ± 0.67	2.41 ± 0.22
<i>R. pipiens</i>	2.75 ± 0.14	$0.81 \pm 0.09^{*\$}$	1.16 ± 0.33	$0.61 \pm 0.16^{\$}$

Values are means $\pm$ SEM (n = 3-4) and are given in units of mM for maltose and mg/mL for oyster glycogen.

*- denotes a significant difference ($P < 0.05$) from the corresponding K_m value at 5°C.

§- denotes a significant difference ($P < 0.05$) from the corresponding K_m value for *R. sylvatica* at 20°C.

Figure 3.1. Effect of pH on the activity of α -glucosidase from *R. sylvatica* and *R. pipiens*. Activity was determined using optimal assay conditions (20°C) in the presence of 40 mM turanose (●) and the activity remaining once any activity detected in the presence of turanose was subtracted (■). Animals are: *R. sylvatica* (closed symbols) and *R. pipiens* (open symbols). Activities are relative to maximal enzyme activity from each species of frog.

Figure 3.2. Representative elution profile for DE-52 chromatography of *R. sylvatica* liver α -glucosidase. A linear gradient (0-1 M KCl in pH 7.0 phosphate buffer) was used to elute the enzyme. MU α G was used as substrate to determine the fluorescence units at pH 7.0 (●) and pH 4.0 (■); activity is expressed relative to the activity in the peak tube measured with the pH 4.0 assay.

Figure 3.3. Representative elution profile for *R. pipiens* liver α -glucosidase on DE-52 chromatography. The column was developed with a linear gradient of KCl (0-1 M) in phosphate buffer, pH 7.0 and the elution profile of α -glucosidase isozymes was assessed using MU α G as the substrate at pH 7.0 (●) and pH 4.0 (■). Activity expressed relative to the peak fraction measured at pH 7.0.

Figure 3.4. Isoelectrofocusing of liver α -glucosidase from control *R. sylvatica*.

Activities for both the neutral (●) and acidic (■) isozymes were measured using MU α G as the substrate; expressed relative to the peak fraction as measured with pH 4.0 assay. The broken line shows the measured pH gradient achieved.

Figure 3.5. Isoelectrofocusing profile of liver α -glucosidase from *R. pipiens*.

The neutral (●) and acidic (■) activities were measured with MU α G as the substrate; shown relative to peak tube measured with pH 4.0 assay. The measured pH gradient is shown as a broken line.

Figure 3.6. Effect of assay temperature on the K_m value for MU α G of neutral α -glucosidase from liver of control *R. sylvatica* (●) and *R. pipiens* (■). Data are the mean of 3-5 separate preparations and error bars denote SEM. a - Significantly different from the value at 5°C. $P < 0.05$; b - significantly different from the 20°C value, $P < 0.05$.

Figure 3.7. Effect of assay temperature on the K_m values for MU α G of acidic α -glucosidase from liver of control *R. sylvatica* (●) and *R. pipiens* (■). Data are the means of a minimum of 3 separate preparations where the SEM are indicated by error bars. a - Significantly different ($P < 0.05$) from either the 5°C or 20°C values.

Figure 3.8. Effect of incubation at different temperatures for 2, 4 or 6 hours on the activity of the neutral isozyme of α -glucosidase from *R. sylvatica* (●) and *R. pipiens* (■) liver. Data are the means of 3-5 determinations at each point with activity assayed using MU α G as the substrate: where not shown, error bars (SEM) are included within the dimensions of the symbol.

Figure 3.9. Effect of incubation at different temperatures for 2, 4 or 6 hours on the activity of the acidic isozyme of α -glucosidase from *R. sylvatica* (●) and *R. pipiens* (■) liver. All data points are from of 3-5 preparations with activity assayed using MU α G as the substrate; error bars denote SEM and may be incorporated into the symbol.

Figure 3.10. Thermal inactivation of liver α -glucosidase over time during incubation at 45°C for *R. sylvatica* (top) and *R. pipiens* (bottom). Neutral (●) and acidic (■) isozymes were measured under optimal assay conditions at pH 7.0 and 4.0, respectively, over 6 hours. Data points are the means of 3-5 determinations (error bars denote SEM). a - Significantly different ($P < 0.05$) from the activity at 5 minutes.

Discussion

Crude liver extracts exhibited both neutral and acidic α -glucosidase activities as seen in Figure 3.1. As there was an overlap of the two isozymes' activity profiles at the local maxima (pH 4.0 and 7.0), turanose, a specific inhibitor of the acidic isozyme, was utilized to separate the two activities. The acidic activity was determined by calculating the difference in activity in the absence versus presence of turanose whereas the neutral activity was determined in the presence of turanose allowing for the accurate separation of the two pH dependent α -glucosidase activities. The existence of multiple pH dependent isozymes of α -glucosidase in frogs is consistent with the earlier report of Fournier *et al.* (1992) on *Rana pipiens*.

Neutral and acidic α -glucosidases from *R. pipiens* liver could be well separated by DE-52 Sephadex column chromatography using a KCl gradient (Figure 3.3). However, due to the broad peak of the acidic isozyme, the same was not true for the *R. sylvatica* enzymes (Figure 3.2). The neutral isozyme from both species exhibited greater interaction with the column material than did the acidic form which would indicate that there is a structural difference between the two pH dependent α -glucosidases found in frog liver.

To find a method that adequately separated the two isozymes in both species and to further investigate the physical differences between the isozymes, liver extracts were subjected to isoelectrofocusing. This technique produced a good separation of the enzymes (by the choice of appropriate peak fractions)(Figures 3.4 and 3.5) and was used to provide partially pure enzyme for subsequent analyses of the two forms. The neutral isoforms from the two species showed similar pI values (6.0-6.2) whereas the pI values of the acidic enzymes differed widely (5.4 in *R. sylvatica* versus 6.1 in *R. pipiens*) (Table 3.2). The similar pI values of the neutral

isozymes from both species indicates that there is little difference in the charge of their surfaces but a difference of over half a pH unit for the acidic isoforms indicates these two have quite charge ratio on their surfaces and suggests that adaptive changes to protein structure may have been made to one or both enzymes. As will be further discussed below, this is consistent with the differences in kinetic properties (K_m , I_{50} for salts) seen in comparing the two acidic forms versus the neutral enzymes which were kinetically quite similar between the two species.

It is well known that freezing exposure of wood frogs stimulates an activation of liver glycogen phosphorylase (Storey, 1987a) that leads to a rapid build up of glucose as a cryoprotectant. Indeed, this is clearly seen from the present data (Table 3.5); overall, the amount of the active α form (total $\times$ % α) increased by 19-fold in liver of frozen frogs versus controls. To determine whether α -glucosidase activities were similarly activated in response to freezing in *R. sylvatica*, maximal activities of both the neutral and acidic enzymes were compared in control and frozen tissues (Table 3.3 and 3.4). Neutral α -glucosidase activity did not change in liver during freezing but rose significantly in heart, kidney and skeletal muscle. However, neutral α -glucosidase in gut from frozen *R. sylvatica* dropped to half of the value in controls. A unique α -glucosidase with activity at neutral pH has been known to be in the brush border of the intestine (Castilla *et al.*, 1980) and as the frog will not be ingesting food during the freezing bout, there is no need for the gut to produce digestive enzymes; hence, this may explain the drop in activity. However, an increase in activity in the heart and kidney would indicate that there is a need of glucose production within the internal organs for use as a cryoprotectant. Similarly, a retention of activity in the liver suggests that the ability of breaking down polysaccharides is important within the liver and for export to other organs.

Contrary to the neutral isozyme, activity of the acidic form increased significantly during freezing only within heart. Activity in liver, brain and muscle did not change during freezing but acidic α -glucosidase in gut and kidney was suppressed during freezing to very low levels. This indicates that the potential for endogenous glycogenolysis via this route is clearly strongly reduced in the frozen animal. When coupled with the opposite result for GP in these organs (both total GP and %a were increased significantly; Table 3.5) it suggests that GP is the primary enzyme responsible for any endogenous glycogenolysis occurring in these organs during freezing. The maintenance of acidic α -glucosidase in liver during freezing indicates that the enzyme probably retains a role in glycogenolysis during freezing although compared with the major activation of GP during freezing, it is obvious that the percentage contribution by α -glucosidase to the net output of glucose by the liver probably drops during freezing. As the animal came out of the frozen state, the need for glycogen breakdown was no longer a priority and the liver acidic α -glucosidase activity dropped. In fact, the production of glycogen during the recovery period following a freezing bout is initiated (Russell and Storey, 1995).

Species differences were evident for the neutral and acidic α -glucosidase activities for the various tissues surveyed. The activity of the acidic α -glucosidase from *R. pipiens* was lower in all tissues except heart, as compared to control *R. sylvatica*, which is consistent with a greater capacity for glycogenolysis in the wood frog. Similarly, the GP activities are overall higher in *R. sylvatica* tissues than in the tissues of the freeze intolerant leopard frog. Enzymatic activity in liver of *R. sylvatica* versus *R. pipiens* was 3-fold higher for the neutral α -glucosidase, 3-fold higher for the acidic α -glucosidase and 4-fold higher for GP. As stated above, even though the α -glucosidase activity does not appear to contribute greatly to the net cryoprotectant output, it undoubtedly plays some role as all glycogenolytic enzymes

in the freeze tolerant frog liver are present in higher activities than their counterparts in the freeze intolerant species. A similar pattern of acidic isozyme and GP activity in the other tissues (except for gut GP and heart acidic α -glucosidase) was seen when comparing the two species such that the glycogenolytic enzymes are always higher in *R. sylvatica*.

The same finding was not observed for the neutral isozyme. Neutral activity from heart, muscle and liver was consistently lower in *R. pipiens* than in *R. sylvatica* control tissues but kidney had a higher activity in *R. pipiens* and the activity of the neutral form from brain and gut were not significantly different between the two frog species. Thus, there is a relative activity difference between the different isozymes from the two frogs studied where the activity was neither consistently higher nor lower.

Similar to the activity of the crude neutral and acidic α -glucosidase in liver towards MU α G was their activity towards maltose and glycogen. In both species, the acidic isozyme showed a greater activity with these substrates than did the neutral form. However, the activity of the neutral isozyme towards MU α G from *R. pipiens* was lower than that of the wood frog, but with maltose as substrate this was inverted. The activity of the neutral *R. pipiens* α -glucosidase with glycogen was surprising as previous studies have found that glycogen not degraded by the neutral isozyme (Mehrani and Storey, 1993) although it is thought to be active concurrently with α -amylase to produce glucose from cytosolic glycogen (Smith *et al.*, 1968). Hence, there is the possibility that there was α -amylase present in the extract as well as endogenous GP which may have contributed to this activity. The finding that the activity of the acid and neutral isoform from *R. sylvatica* showed greater activity towards glycogen than maltose is in contrast to the finding by Calder *et al.* (1989) who found that the rate of hydrolysis was inversely related to the molecular

size of the substrate for rat liver, skeletal muscle and human placenta acid α -glucosidase.

Following IEF, the activities of the different isozymes were again examined (Table 3.9) and similar trends were noted for the acidic isoform as for the acidic crude extracts; i.e. there was greater activity with glycogen as the substrate in both species than if maltose was used. The partially purified neutral α -glucosidase from *R. sylvatica* did not exhibit activity towards either maltose or glycogen but the *R. pipiens* enzyme showed neutral activity with maltose ($7,722 \pm 1,469$ U/mg protein). The absence of activity towards glycogen by this partially purified extract could be due to the absence of α -amylase which has been known to aid in the degradation of glycogen by cytosolic α -glucosidase (Smith *et al.*, 1968).

Previous studies have shown that the concentration of glucose increases by as much as 100-fold upon the initiation of freezing and is supported by an increase in GP α (Storey, 1985a). Brain and heart were the only tissues which did not exhibit an increase in GP % α (Table 3.5) and in fact this parameter decreased significantly in the heart during freezing. Indeed, in heart the ratio of GP α to neutral α -glucosidase and GP α to acidic α -glucosidase under control conditions were found to be 2.5 and 7.5, respectively, yet during freezing, these ratios changed to 0.46 and 0.75, respectively. This would indicate that α -glucosidase is the important enzyme for the degradation of glycogen in heart following the onset of extracellular ice formation. As the blood starts to freeze and becomes more viscous, more glucose is required for the beating of the heart muscle and for heart cryoprotection and an increase in α -glucosidase in this tissue is reasonable.

Not only was the active GP increased upon freezing in the kidney, gut and liver, but so was an increase in the total activity of GP evident. This could imply two variables: an increase in the total amount of enzyme present; and/or a change in structure of the enzyme which results in an increase in total activity. An increase in

the percent a coupled with an increase in total activity of GP results in a greater capability in the degradation of glycogen. Similarly, one would expect the reverse during the recovery from freezing when glucose is cleared and the glycogen stores are regenerated and indeed this was observed in all three of these tissues as a decrease in total activity of GP as well as a decrease in % a .

It would appear that the percent of GP a present within some tissues is comparable between species under control conditions. Although there was a lower total GP between the control *R. sylvatica* and *R. pipiens* heart, kidney and brain, there is no significant difference in the % a within these tissues. However, in muscle there was a lower total GP as seen in heart, kidney and brain but the % a was higher. This change in total and percent active GP resulted in similar amount of active GP within these species. Liver values for total and % a GP were significantly lower in *R. pipiens* than those found in control *R. sylvatica*. This implies that there is a greater emphasis on liver glucose production via GP in *R. sylvatica* than in *R. pipiens*.

Not only is the activity of GP and α -glucosidase important, but also the activation energy in determining the relative importance of these enzymes in the breakdown of glycogen. All Arrhenius plots were linear and the activation energies were determined (Tables 3.7 and 3.8). In control *R. sylvatica* the activation energies of neutral, acidic α -glucosidase, GP a and GP b were not significantly different. However, E_a from *R. pipiens* neutral α -glucosidase was significantly lower than the activation energy of GP a but greater than that found for GP b which was also found to be true for the acidic α -glucosidase as compared to the two forms of GP. As the isozymes of α -glucosidase had a lower E_a than GP a in *R. pipiens* there is an energy saving in utilizing α -glucosidase rather than GP a in degrading glycogen.

To function effectively in an animal that experiences wide variation in its body temperature (such as wood frogs), an enzyme would be expected to retain its active site integrity over a wide range of temperatures. Figures 3.6 and 3.7 show the change in α -glucosidase K_m values for MU α G over a range of temperatures. The neutral α -glucosidase from *R. sylvatica* showed a very little change in substrate affinity over the temperature range studied (Figure 3.6). Over the natural temperature range experienced by the frogs (5-20°C), there is no significant change in affinity for either of the isozymes (Figures 3.6 and 3.7). Therefore, although the wood frog is subjected to vast changes in temperatures over the duration of a freeze/thaw cycle, there is no indication on the basis of substrate affinity that the structure of the active sites of the neutral and acidic α -glucosidase are altered. Hence, the degradation of polysaccharides by these isozymes are not be hampered by active site modification.

The neutral α -glucosidase from *R. pipiens* showed a decrease in affinity for MU α G at 12°C and 20°C whereas the acidic form did not exhibit this behaviour. At 5°C, there was an increase in affinity for MU α G as indicated by a lower K_m value than at either 12°C or 20°C. The freeze intolerant frog's active site integrity was not conserved as its freeze tolerant counterpart implying that there is a definite difference in the structure of the neutral isoform from *R. sylvatica* and *R. pipiens*.

Unlike the lack of temperature effect on the K_m for MU α G of acidic α -glucosidase from *R. pipiens*, the K_m for maltose at 5°C increased by 3-fold compared with the value at 20°C (Table 3.11). This implies that there is an alteration in the active site at low temperature which affects the binding of this substrate although not the other two. No change in substrate affinity was noted between 5 and 20°C assay temperatures for either maltose or glycogen substrate with *R. sylvatica* acidic α -glucosidase which also agrees with the results for these assay temperatures with MU α G as the substrate (Figure 3.7). Hence, it appears that

the active site of *R. sylvatica* acidic α -glucosidase is relatively insensitive to temperature change, particularly within the temperature range that would be considered physiological.

K_m is one of the variables which can give an inference into how enzyme function is affected by altering temperature. Another parameter than can be evaluated is the effect of temperature on enzyme maximal activity which indicates the thermal stability of an enzyme and is another property that can be altered in designing an enzyme to suit the natural temperature range experienced by an animal. As temperature increases there are greater hydrophobic interactions yet weaker hydrophilic ones and a reduction in activity could indicate a change in flexibility within the enzyme. When thermal denaturation was assessed for α -glucosidase the acidic enzyme from both species proved to be highly stable (Figure 3.9). The enzyme from both species was stable at 45°C at all incubation times as was the *R. sylvatica* enzyme at 55°C. Only the *R. pipiens* enzyme showed thermal decay and this occurred only after 6 h at 55°C. By contrast, the neutral enzymes from both species showed considerably less thermal stability. Neutral enzymes were fully stable over the range from 5°C to 27°C, as also was the *R. sylvatica* enzyme at 37°C (Figure 3.8). However, the neutral *R. pipiens* α -glucosidase began to lose activity with incubations at 37°C or above and the *R. sylvatica* showed thermal decay at 45°C and above. Hence, the neutral enzyme from both species showed greater thermal lability than did the acidic isozyme although between species, the *R. sylvatica* enzyme was slightly less labile. Differences in the thermal lability of the neutral isozymes were further explored with a detailed time course of incubation at 45°C (Figure 3.10). This confirmed a slightly greater stability of the *R. sylvatica* neutral enzyme for the half-time for thermal inactivation was 30.5 minutes for the enzyme from this species versus 21.5 minutes for the *R. pipiens* enzyme. Overall, these results indicate that the *R. sylvatica* enzyme has slightly

greater structural stability than does the *R. pipiens* protein and this may be adaptive in allowing protein function over a wider range of natural temperatures (including subzero temperatures) *in vivo*.

As *R. sylvatica* is known to survive freezing by using glucose as a cryoprotectant, it is expected that enzymes important in the degradation of glycogen to glucose would not be strongly inhibited by increasing glucose concentrations as compared to a non-freezing species. Although both neutral and acidic isozymes of α -glucosidase were found to be inhibited by glucose (Table 3.10), the *R. sylvatica* isozymes were significantly less affected by high glucose. Hence, I_{50} values for glucose were 5.6-fold higher for the neutral and 2-fold higher for the acidic enzyme from *R. sylvatica* compared with *R. pipiens*. Thus, the *R. sylvatica* enzymes are clearly better designed to continue glycogenolysis as product (glucose) levels rise.

During freezing, water is eliminated from cells and the local ionic strength increases such that salt effects on enzyme activity may also be of importance in regulating their function with respect to freeze/thaw cycles. Not surprisingly, then, the *R. sylvatica* α -glucosidase isozymes were less susceptible to inhibition by high salt than were the *R. pipiens* enzymes. For example, the I_{50} NaCl of *R. sylvatica* acidic α -glucosidase was 6-fold higher and the I_{50} KCl 2-fold higher than the corresponding values for the acidic enzyme from *R. pipiens*. Hence, the *R. sylvatica* enzymes are better designed to retain activity under the rising ionic strength conditions that occur as intracellular water is withdrawn into extracellular ice masses.

Species differences between the freeze tolerant and intolerant frogs indicates that there was a greater need of structural stability in the α -glucosidase from *R. sylvatica* than from *R. pipiens* as indicated by the denaturation variations of the neutral isozymes as well as the K_m values determined over the same temperature range. As well, the greater tolerance of the *R. sylvatica* α -glucosidases in the

presence of high concentrations of salts and carbohydrates indicates that these isoforms were better designed to sustain activity during periods of freezing when solute concentration increase.

Chapter 4

General Discussion

This study was initiated to examine the possible role of α -glucosidase in two animals which exhibit unique glycogen metabolism for overwintering. Both *S. lateralis* and *R. sylvatica* must survive very low temperatures for extended periods of time with endogenous supplies of glycogen being limited. The glucosidic pathway for the degradation of glycogen via α -glucosidase was examined and the isozymes were determined to possess similar properties in the two species at low temperatures. The acidic isoform was more inert to denaturation at high temperatures and this retention of structure was evident in the substrate affinity measurements over a wide temperature range where there was no change in K_m values. However, the neutral α -glucosidase was more susceptible to temperature effects as seen by the strong thermal denaturation at 45°C and above, as well as the increases in K_m values at low temperatures.

Studies have shown that α -glucosidase exists in both neutral and acidic isozymes and in some animals there may be multiple forms of one or both (Mehrani and Storey, 1993). Both the mammals and the frogs studied here showed acidic and neutral activities with pH optima of 4.0 and 7.0, respectively. However, in the rat and *S. lateralis* there were two distinct forms with neutral activity although only one was evident in the frogs. The acidic α -glucosidase from the mammals and amphibians were both inhibited by the disaccharide turanose whereas the activities of the neutral isozymes were not altered.

As there are two pathways for the degradation of glycogen to glucose, it was important to examine the phosphorolytic and glucosidic pathways for their contribution to carbon flux. In liver, there was greater GPa activity than α -glucosidase at low and high temperatures indicating a greater contribution to glucose output by the phosphorolytic pathway than the glucosidic pathway. In all

tissues investigated in the *S. lateralis* and *R. sylvatica*, only the α -glucosidase activity of the *R. sylvatica* heart indicated an inversion of this carbon flux. It appears that during the early stages of ice formation, when the heart is pumping more viscous blood, there is a greater contribution to glucose production from the glucosidic pathway than from the phosphorolytic pathway.

The Q_{10} of most chemical processes is 2-3 which also applies to the metabolic rate of animals (Nedergaard and Cannon, 1990) and active suppression or activation of an enzyme can be indicated by changes in Q_{10} values under different conditions. In *R. sylvatica*, the Q_{10} of the neutral α -glucosidase did not change for the control, frozen and recovered liver as indicated by the ratio of activity at 5°C versus 20°C being 1:4. However, the Q_{10} for the acidic isoform changed from 2 to 1.4 and finally to 2.6 in the control, frozen and recovered liver, respectively. This implies that although there is little contribution from acidic α -glucosidase to the total cryoprotectant concentration there still existed a relative activation of the acidic form during freezing.

In the golden-mantled ground squirrel the opposite trend was observed where the Q_{10} values of the control liver neutral and acidic enzymes was 1.9 and 1.8, respectively, compared with values for the enzymes from the hibernating animal which were 3.2 and 2.4, respectively. Therefore, there appears to be an active suppression of enzyme activity in liver of hibernating *S. lateralis* such that the degradation of glycogen would be reduced further than what would be expected based on the theoretical Q_{10} for the chemical hydrolysis of the glucosidic bond. Studies have shown that the Q_{10} values for respiration by some of the smallest hibernators are about 4 (Geiser, 1988). The observation that liver α -glucosidase from hibernating *S. lateralis* had Q_{10} values greater than the control values is

consistent with this finding. The suppression of this enzymatic activity could be one part of a general suppression of many cellular enzymes and processes that together extend the potential survival time of the animal while dormant.

Acidic isoforms utilized glycogen and maltose as substrates yet the neutral α -glucosidases possessed only limited use of glycogen and maltose. Therefore, the role of the acidic form for the degradation of di- and poly-saccharides in the lysosomes seems reasonable for the mammals and amphibians studied here as it has been consistently shown in other species. The exact role of the neutral form is still largely unknown although various proposed functions include the degradation of oligosaccharides, the transfer of carbohydrate moieties and the formation of glycoproteins (Castilla *et al.*, 1980; Murat *et al.*, 1978). As limited usage of the neutral isozymes towards glycogen and maltose was observed, its exact role still needs to be elucidated.

As the role of the neutral α -glucosidase is unknown, future studies should include the purification and activity of the neutral isozyme(s) towards a variety of physiologically available substrates may help to elucidate its role in the cell. In rat liver, the activity of the acidic α -glucosidase was inversely related to the molecular size of the substrate and it was determined that α -glucosidase favoured high molecular weight glycogen (Calder and Geddes, 1989). Therefore, studies involving the use of a variety of substrates could aid in determining the possible role of the neutral α -glucosidase within cells through its activity towards a variety of substrates. As there was differing substrate affinity of the neutral α -glucosidase in both frogs and ground squirrels, structural studies could help to determine the binding sites of substrates and a possible mechanism may be derived.

As well, work on the suppression of α -glucosidase during hibernation and on the activation of α -glucosidase during freezing needs be completed. The trigger and mechanism for the regulation of the two isozymes in the cell is of importance not only to this enzyme, but to a variety of enzymes which also show similar effects in these animals.

This study focused on the role of both the acidic and neutral α -glucosidase in hibernation and freeze tolerance, however little work has been completed dealing with other overwintering conditions such as prolonged underwater hibernation causing anoxia (the absence of oxygen). Since the present study has shown definite changes in the Q_{10} values of α -glucosidase within the animals in this study as well as denaturation and substrate affinity changes with temperature, the role of α -glucosidase isozymes should also be investigated in other species that also endure environmental stresses that necessitate altered glucose metabolism in order to determine the role of these enzymes to net glucose production.

Finally, α -glucosidase is but one enzyme which degrades polysaccharides to produce glucose. Future work should be completed on other glycosidases which may aid in the overall degradation of carbohydrates. α -Amylase is one such enzyme which cleaves $\alpha(1 \rightarrow 4)$ glucosidic bonds in the glycogen chain into smaller fragments and finally into maltose, isomaltose and glucose.

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