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The intertidal marine environment is home to a variety of plants and animals, including many members of the phylum Mollusca, such as the gastropod snail, Littorina littorea and the bivalve mussel, Modiolus demissus demissus. Due to the changing water levels of daily tides, animals inhabiting this zone are exposed to changes in environmental conditions such as large fluctuations in oxygen availability, salinity and temperature. At low tides, these gill breathing animals are out of the water and exposed to the air. While some marine species have a limited capacity for O₂ uptake from air, most depend on their ability to tolerate anoxic conditions for long periods of time, in order to survive in this habitat. At high latitudes, intertidal species may also be exposed at low tide to subzero air temperatures in the winter. Species who are exposed to subzero temperatures must also develop an effective method for dealing with the hazards associated with the frozen state, by either developing mechanisms to endure freezing of body fluids (freeze tolerance) or mechanisms to prevent freezing even at temperatures well below the freezing point of body fluids (freeze avoidance). Adaptation to the stresses faced by the molluscs inhabiting the intertidal zone occur at the behavioral, physiological and biochemical levels and it is the last of these which is of primary interest in this thesis.

Biochemical adaptations that have been observed in animals with high anoxia tolerance include the following: (1) High levels of fermentable fuels (carbohydrates such as glycogen) are maintained. (2) Buffering capacity of body fluids is enhanced, or there are changes in the types of fermentation end products produced, in order to minimize metabolic acidosis. (3) Alternative pathways of fermentative ATP production are used to increase anaerobic ATP yield. (4) Metabolic rate during anoxia is strongly reduced compared with the aerobic resting rate. While the first three are important, none of them are so closely linked to survival of harsh environmental conditions as is

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**REGULATION OF CARBOHYDRATE METABOLISM IN MARINE
MOLLUSCS:
EFFECT OF FREEZING AND ANOXIA**

by

Elizabeth Lucie Russell, B.Sc. (Hons) SSP

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

Chair, Department of Chemistry

Thesis Supervisor

Carleton University
1993

ABSTRACT

The snail Littorina littorea and mussel Modiolus demissus demissus are marine invertebrates that live in the intertidal zone and as a result of their habitat are exposed to varying environmental conditions such as periods of anoxia and freezing. The regulation of 5 key carbohydrate metabolism enzymes glycogen phosphorylase (GP), glycogen synthetase (GS), phosphofructokinase (PFK), pyruvate kinase (PK) and pyruvate dehydrogenase (PDH) during anoxia and freezing in the snail and mussel were investigated in this thesis. Changes in the activities of GS, GP, PK, and PDH, associated with changes in the phosphorylation state of the enzymes, were found. In addition, the activities of the enzymes GS and GP were controlled by degradation and in some tissues, synthesis of the enzymes. The phosphorylation state of PFK remained unchanged in both animals after exposure to periods of freezing and anoxia. Though the responses of GP and GS were tissue specific, GS, GP, PK and PDH showed a general decrease in activity in keeping with metabolic depression.

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LIST OF ABBREVIATIONS

AABS - p-(p-aminophenylazo)benzene sulphonic acid

AAT - Arylamine acetyltransferase

ATP, ADP, AMP - Adenosinetri-,di-, and monophosphate

cAMP - cyclic 3'5'AMP

CoASH - Coenzyme A

DHAP - Dihydroxyacetone phosphate

EDTA - ethylenediamine tetraacetate

EGTA - ethyleneglycol bis(β -aminoethyl ether) tetraacetate

F1,6P₂ - Fructose 1,6-bisphosphate

F2,6P₂ - Fructose 2,6-biphosphate

F6P - Fructose 6-phosphate

G1P - Glucose-1-phosphate

G3P - Glycerol-3-phosphate

G3PDH - Glycerol-3-phosphate dehydrogenase

G6P - Glucose-6-phosphate

G6PDH - Glucose-6-phosphate dehyrdrogenase

GAP - Glyceraldehyde-3-phosphate

GP - Glycogen phosphorylase

GP_a - Glycogen phosphorylase a

GS - Glycogen Synthetase

GS_I - Glycogen Synthetase I

I₅₀ - concentration of inhibitor that inhibits the maximal activity of an enzyme by 50 %

K_a - concentration of activator that produces half maximal enzyme activity

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

LDH - Lactate dehydrogenase

NAD⁺, NADH - oxidized and reduced forms of nicotinamide adenine dinucleotide
NADP⁺, NADPH - oxidized and reduced forms of nicotinamide adenine dinucleotide phosphate
PDH - Pyruvate dehydrogenase
PEP - Phosphoenolpyruvate
PFK - Phosphofructokinase
PGM - Phosphoglucomutase
P_i - Inorganic phosphate
PK - Pyruvate kinase
PMSF - phenylmethylsulphonyl fluoride
TPI - Triose phosphate isomerase
UDPG - uridine diphosphate glucose
UTP, UDP - uridine tri-, and diphosphate
V_{max} - maximal enzyme velocity

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INTRODUCTION

The intertidal marine environment is home to a variety of plants and animals, including many members of the phylum Mollusca, such as the gastropod snail, Littorina littorea and the bivalve mussel, Modiolus demissus demissus. Due to the changing water levels of daily tides, animals inhabiting this zone are exposed to changes in environmental conditions such as large fluctuations in oxygen availability, salinity and temperature. At low tides, these gill breathing animals are out of the water and exposed to the air. While some marine species have a limited capacity for O₂ uptake from air, most depend on their ability to tolerate anoxic conditions for long periods of time, in order to survive in this habitat. At high latitudes, intertidal species may also be exposed at low tide to subzero air temperatures in the winter. Species who are exposed to subzero temperatures must also develop an effective method for dealing with the hazards associated with the frozen state, by either developing mechanisms to endure freezing of body fluids (freeze tolerance) or mechanisms to prevent freezing even at temperatures well below the freezing point of body fluids (freeze avoidance). Adaptation to the stresses faced by the molluscs inhabiting the intertidal zone occur at the behavioral, physiological and biochemical levels and it is the last of these which is of primary interest in this thesis.

Biochemical adaptations that have been observed in animals with high anoxia tolerance include the following: (1) High levels of fermentable fuels (carbohydrates such as glycogen) are maintained. (2) Buffering capacity of body fluids is enhanced, or there are changes in the types of fermentation end products produced, in order to minimize metabolic acidosis. (3) Alternative pathways of fermentative ATP production are used to increase anaerobic ATP yield. (4) Metabolic rate during anoxia is strongly reduced compared with the aerobic resting rate. While the first three are important, none of them are so closely linked to survival of harsh environmental conditions as is

metabolic depression. In fact, the different lengths of time that varying species are able² to survive anoxia, can be correlated to the species ability to suppress metabolic activity (Storey, 1992).

Many animals are able to lower their metabolic rate well below their standard resting rate and enter a hypometabolic or dormant state. This facultative metabolic depression is used as a means of extending the animal's survival time during periods of harsh environmental conditions. The strategy of metabolic depression is highly developed in marine molluscs who daily face varying temperatures, water and oxygen availability, as a result of the tidal cycle. During periods of oxygen depletion, for example, marine invertebrates can depress their metabolic rate to only 5-10 % of their normal resting state (Famme et al., 1981; Schick et al., 1983).

What, then, is metabolic depression? To achieve metabolic rate depression, the physical and metabolic activities of the animals are slowed down or stopped. For example, heart and breathing rates are slowed down. Movement, feeding and digestion stops, and brain activity slows down. When faced with harsh conditions, animals usually try and remove themselves from the environment, by withdrawing into their shells, burying into the ground or surrounding themselves with cocoons. Whichever method is chosen, the end result enables the animal to be less affected by the changes occurring outside of their protective surroundings. Also, in situations where the animal is in danger of desiccation, a habitat is chosen where water loss would be minimized. Furthermore, many nonessential (largely biosynthetic) metabolic pathways are shut down, and essential (energy producing) pathways are slowed down.

These adjustments reduce both the amount of energy used and the amount produced. When the animal is placed in an environment where it is in danger of depleting its endogenous energy stores, hypometabolism enables the animal to greatly extend its potential survival time so that it can await the return of favourable conditions, in which fuel and water can be replenished.

An example of the ability of hypometabolism to extend an animal's survival time has been determined for the anoxia tolerant bivalve, Mytilus edulis. Hochacka and Somero (1984) calculated that M. edulis has sufficient reserves to survive anaerobically for a period of only 3 days at its normal resting metabolic rate. However, because *M. edulis* depresses its metabolic rate nearly 20-fold under anaerobic conditions, its glycogen reserves could then theoretically be extended to sustain the animal for sixty days of continuous anoxia (ie. if no other factors limiting).

Many functions in the cell require energy, and this is primarily derived from the hydrolysis of anhydride bound phosphate groups of ATP. Since animals' tissues have only a small pool of ATP (usually only 1-10mM), a constant supply of ATP is required. The necessary ATP is generated as a result of the catabolism of nutrient molecules. The main fuel substrates available to the cell are carbohydrates (glycogen, glucose, and other sugars), lipids (triglycerides, diglycerides and ketone bodies) and amino acids. Carbohydrates are the primary fuel source in molluscs under anoxic conditions (deZwaan, 1977).

While there are many ATP-utilizing reactions that occur in the cell, there are few pathways that produce the energy- rich ATP molecule. One pathway that does produce ATP, even under anoxic conditions, is glycolysis. Thus, to achieve a coordinated metabolic depression in anoxia, a key target must be glycolysis, the primary ATP-producing pathway in anoxia. It is for this reason that studies of control mechanisms of glycolytic activity have been the major focus of investigations into the molecular mechanisms of metabolic rate depression in facultative anaerobes.

Glycolysis is a sequence of enzyme-catalyzed reactions that catalyzes the conversion of glucose, a major source of energy for nearly all organisms, into pyruvate. The name glycolysis is derived from Greek words *glykos* meaning sweet and *lysis* meaning splitting. As is shown in Scheme 1 below, the conversion of one

molecule of glucose to 2 molecules of pyruvate results in the net production of 2 molecules of the energy rich compound ATP.

Scheme 1. The net reaction of glycolysis.

The regeneration of NAD^+ which accompanies the conversion of pyruvate into lactate, enables glycolysis to continue under anaerobic conditions. When the carbohydrate glycogen is the substrate, an additional molecule of ATP is produced and the pathway is known as glycogenolysis. Figure 1.1 shows all of the reactions involved in glycogenolysis.

The environmental stresses that induce hypometabolism in different species vary in the length of time they last and how often they occur. For example, inhabitants of the intertidal zone face fluctuations in oxygen availability, salinity and temperature twice a day. Insects that overwinter as larvae enclosed by cocoons are exposed to varying temperatures within even one day. Hibernation is not a continuous state and hibernating mammals wake up from their dormant states for short periods of time resuming their non-hibernating metabolic rate. Due to the ever changing environmental conditions an animal faces, it is necessary that the route to an animal's hypometabolic state be swift and readily reversible. This requirement seems to rule out an elaborate genetic rearrangement as the mechanism of control of metabolic rate. In addition, a great deal of co-ordination is necessary to ensure that the various pathways are slowed down or stopped in a balanced manner.

Three mechanisms of controlling glycolytic rate have been identified to date. They are as follows: (1) changes in the concentration of allosteric modulators of key control enzymes, (2) changes in the subcellular location of enzymes via reversible binding to macromolecular structures, and (3) postranslational modification of regulatory enzymes and functional proteins via protein phosphorylation and dephosphorylation.

One of the most potent regulatory modulators found in animal cells is F2,6P₂, a product of a reaction catalyzed by 6-phosphofructo-2-kinase (PFK-II). Under anoxic conditions, inactivation of PFK-II (via protein phosphorylation), results in a decrease in the amount of F2,6P₂ produced. Since F2,6P₂ is a potent activator of 6-phosphofructo-1-kinase (PFK) the rate limiting enzyme of glycolysis, as well as an inhibitor of PFK-II, decreases in its levels would dramatically reduce its regulatory role. Therefore, in some instances, metabolic rate depression could be partially a result of changes in the levels of modulators leading to a decrease in enzyme activation or an increase in enzyme inhibition.

Another method of controlling metabolic rate may involve the binding of enzymes to each other or to other subcellular structures, since such associations can alter their catalytic activity. For example, such complexes could cause a change in the kinetic properties of an enzyme as a result of conformational changes to the enzyme upon binding. The formation of multienzyme complexes could also place the catalytic sites of sequential enzymes into close proximity with each other, thus speeding up the rate of metabolic flux through a pathway. Indeed, initial studies focusing on skeletal muscle found that conditions that led to an increase in glycolytic rate also caused an increase in the proportion of certain glycolytic enzymes bound to particulate matter (Clarke et al., 1984; Brooks and Storey, 1988). Oppositely, under conditions (eg. anoxia) that reduce metabolic rate, enzyme binding has been shown to decrease. For example, studies on muscle from the marine whelk, Busycotypus canaliculatum, found

that the percentages of 8 glycolytic enzymes bound to particulate fractions decreased significantly during anoxia (Plaxton and Storey, 1986). The reverse effect was seen during aerobic recovery. These results indicate that an important facet of glycolytic control may lie in the reversible association of enzymes with the subcellular particulate fraction.

Another mechanism for enzyme control is post-translational modification via the addition or removal of covalently bound phosphate from proteins. Dephosphorylation and phosphorylation of regulatory enzymes is one of the quickest and most powerful ways of controlling the activity of enzymes and thus the rate of a pathway. A single extracellular signal, transmitted through one or more receptors, changes the level of intracellular second messenger molecules. These in turn activate specific protein kinases, which then phosphorylate a variety of enzymes and proteins to alter the cellular processes in which they are involved. A well known example involves an enzyme that controls glycogen catabolism, glycogen phosphorylase (GP) (Figure 1.1). The glycogen phosphorylase cascade is shown in Figure 1.2. A signal, such as a stimulatory hormone like glucagon or epinephrine, results in the activation of adenylate cyclase, which produces cAMP, which in turn activates cAMP-dependent protein kinase. The activated protein kinase activates phosphorylase $\bar{b}$ kinase which in turn phosphorylates the inactive phosphorylase $\bar{b}$ to convert it to the active form, phosphorylase $\bar{a}$, which then mediates glycogen breakdown. In marine molluscs, changes in the amount of active GP have been observed during periods of anaerobiosis (Storey, 1987, 1988).

Whereas glycogen phosphorylase catalyzes the removal of glucosyl residues from glycogen chains, glycogen synthetase (GS), as its name suggests, adds glucosyl units to growing glycogen chains (Figure 1.1). GP and GS are oppositely regulated such that when one enzyme is active the other is inactive, thus preventing opposing reactions from occurring simultaneously which would waste large amounts of energy.

GS activity is also controlled through reversible phosphorylation of the enzyme, but whereas the phosphorylated form of GP is the active form of the enzyme, it is the dephosphorylated form of GS which is active. Studies of marine invertebrates have found that the percentage of GS in the active form was greatest in the summer, corresponding to times of active feeding and storage of high levels of glycogen, and lowest in the winter months when animals are primarily catabolizing the stored glycogen (Gabbott et al, 1979, 1986).

Covalent modification via reversible phosphorylation has also been found to be a mechanism of control of the enzyme phosphofructokinase (PFK) (Figure 1.1). PFK is one of key enzymes of glycolysis, catalyzing the non-equilibrium conversion of F6P to F1,6P₂. Significant differences in the kinetic properties of PFK from the land snail *Otala lactea* were found between control, and anoxic and estivating snails (Whittam and Storey, 1991). Extracts from control snails that were incubated under in vitro conditions which promoted phosphorylation, were found to mimic the changes seen in the natural transition from aroused to estivating. In addition, extracts of estivated snails that were incubated under conditions promoting phosphorylation, showed the same properties as the unincubated extracts from estivating snails. The authors concluded that control of PFK via phosphorylation was key in controlling glycolytic rate and an important part of overall metabolic depression.

One of the first reports of phosphorylation/ dephosphorylation control of a glycolytic enzyme in marine invertebrates as a response to environmental anoxia involved the enzyme pyruvate kinase (PK) from the adductor muscle of the bivalve mollusc *M. edulis* (Seibenaller, 1979). Since then there have been numerous reports of similar control of PK in other marine molluscs (Gaitanaki et al., 1990; Hakim et al, 1985; Holwerda et al., 1989, Plaxton and Storey, 1984a, 1984b; de Vooy et al., 1986). In general, it was found that during periods of anoxia, phosphorylation of the enzyme lead to a less active enzyme. Common features found among the less active

forms of PK included a reduced maximal activity, a greatly reduced affinity for the substrate PEP and a greater sensitivity to inhibition by L-alanine. The increased sensitivity of the phosphorylated form of PK to inhibition by L-alanine is particularly interesting due to the fact that L-alanine is often an end product of the early stages of anaerobic metabolism in marine invertebrates. Therefore in addition to anoxia induced inactivation of PK through phosphorylation, the anoxic, phosphorylated enzyme form is also highly sensitive to potent allosteric inhibition by an amino acid that accumulates rapidly over the early hours of anoxia exposure. This depression of PK activity allows for the diversion of glycolytic carbon into PEP carboxykinase and hence into reactions that lead to the production of succinate or propionate with the associated synthesis of ATP from substrate-level phosphorylation that increase the net yield of ATP in anoxia.

Another enzyme found to be controlled via covalent modification is pyruvate dehydrogenase (PDH) (Figure 1.1). While PDH is not part of the glycolytic pathway itself, it acts as a bridge from glycolysis to the citric acid cycle. PDH is located in the inner mitochondrial membrane and catalyzes the conversion of pyruvate, CoA and NAD^+ into acetyl-CoA, NADH, and CO_2 . PDH from mammalian systems is a complex of mitochondrial enzymes, the three major enzymes being pyruvate decarboxylase, dihydrolipoyl acetyltransferase, and dihydrolipoyl dehydrogenase. The pyruvate decarboxylase component of the enzyme is regulated by reversible phosphorylation, the dephosphorylated form of the enzyme being the active form. While little is known about PDH from marine invertebrates, a study was performed investigating the possible mechanisms of control of PDH from the foot muscle and hepatopancreas of the land snail, Otala lactea (Brooks and Storey, 1992). During estivation in this species, a decrease in the amount of active enzyme was observed, with a return to control values when the animals were aroused. The authors concluded that covalent modification of PDH was the major mechanism of control used to decrease the overall PDH activity during estivation.

Two animals belonging to the phylum Mollusca, the gastropod snail, L. littorea and the bivalve mussel, M. demissus, were used in this study. Both animals are able to survive long periods of anoxia as well as endure subzero temperatures. Figure 3 shows the features of a generalized mollusc, and the more specific features of a gastropod and a bivalve. In recent studies, it was found that the gastropod L. littorea is able to survive at least 6 days under anoxic conditions at 5°C and at temperatures as low as -8°C, with 100 % recovery (unpublished results). The bivalve M. demissus is able to survive at least 72 hours of anoxia at 5°C, and at temperatures as low as -6°C, with a 90 % recovery rate (unpublished results). Both animals are classed as freeze tolerant species, since when they are exposed to subzero temperatures, they are able to endure the freezing of their body fluids (Aarset, 1982).

As discussed earlier, reversible phosphorylation of glycolytic enzymes has been found to play an important role in metabolic rate depression during periods of anoxia. There have been few studies on the effect of freezing on the behaviour of glycolytic enzymes. However, since extracellular freezing cuts off the circulation of O₂ to tissues, it could reasonably be expected that metabolic depression and changes in the phosphorylation state of the enzymes would also be an adaptation for natural freezing survival. This study investigated the effect of different periods of anoxia and freezing on metabolism in tissues of L. littorea and M. demissus focusing primarily on the role of phosphorylation in the regulation of 5 key enzymes involved in carbohydrate metabolism, GS, GP, PFK, PK and PDH. Enzymes from both L. littorea and M. demissus were studied to determine if there were differences either in the molecular control mechanisms of metabolic rate depression in the different animals, or in the response of the animals to the different stresses. This study discusses the relevance of enzyme covalent modifications to the regulation of the hypometabolic state and the similarities between the modifications induced by anoxia and freezing.

Figure 1.1 The reactions involved in glycogenolysis.

Enzymes are: 1a) glycogen phosphorylase*, 1b) glycogen synthetase*, 2) phosphoglucomutase, 3) glucokinase and hexokinase, 4) phosphoglucoisomerase, 5) phosphofructokinase* 6) fructose-1,6-bisphosphate aldolase, 7) triose phosphate isomerase, 8) phosphoglyceraldehyde dehydrogenase, 9) phosphoglycerate kinase, 10) phosphoglyceromutase, 11) enolase, 12) pyruvate kinase*, 13) lactate dehydrogenase 14) pyruvate dehydrogenase complex*.

* Enzymes investigated in this thesis.

Figure 1.2 Glycogen Phosphorylase Cascade

Activation of adenylate cyclase leads to a sequence of reactions resulting in the activation of glycogen phosphorylase and subsequent glycogen breakdown.

Figure 1.3 Features of a generalized mollusc and the gastropoda and bivalvia species (Sherman and Sherman, 1970).

Extracts of the foot muscle and hepatopancreas tissues of the gastropod L. littorea and of the adductor, gill (ctenidium), hepatopancreas and mantle tissues of the bivalve M. demissus were used in this study.

BIVALVIA

GASTROPODA

METHODS AND MATERIALS

CHEMICALS AND ANIMALS

All biochemicals and coupling enzymes were obtained from Sigma Chemical Co. and Boehringer-Mannheim Corp. Distilled, deionized water was used throughout for the preparation of biochemical solutions. All ATP, ADP and citrate stock solutions contained added $MgCl_2$ in 1:1 molar amounts. Marine periwinkles, Littorina littorea, were obtained from a local supplier. Marine mussels, Modiolus demissus der issus, were obtained from Wood's Hole Marine Lab, Maine, USA. Animals were acclimated in aerated artificial sea water at 5°C for at least 3 weeks prior to experimentation.

PREPARATION OF EXPERIMENTAL ANIMALS

This study investigated 2 environmental stresses, freezing and anoxia. Control animals were taken directly from the 5°C seawater.

Freezing - Animals were removed from the 5°C seawater and placed in air in an incubator at -6.0°C for M. demissus and at -8.0°C for L. littorea. YSI telethermometers, wired to a Kipp and Zonen BD 111 chart recorder, were attached to the animals before they were placed in the incubators. Cooling rates were approximately 1.0 - 2.0°C/min. Nucleation was observed as an exotherm after the animals had reached their supercooling points. L. littorea were frozen for periods of 12 or 24 hours, and M. demissus were frozen for periods 2 or 12 hours. Survival rates were 100 % for both animals at both time points.

Anoxia - A small amount of seawater was placed in a jar to prevent dehydration. Nitrogen gas (100%) was then bubbled through the water for at least 15 minutes to displace any oxygen present. Animals were removed from the aerated 5°C seawater and placed in the anoxic jar. The jar was then flushed with nitrogen gas for a further 20 minutes, after which the lid was sealed and the jar was returned to the 5°C incubator. L. littorea were held in this state of anoxia for 12 or 24 hours, and M.

demissus for 2 and 12 hours. Survival rates were 100% for both animals at both time points.

TISSUE PREPARATION

To dissect the animals, the shell was broken open and the respective tissues were rapidly removed. The tissues were immediately frozen in liquid nitrogen and stored at -70°C until further use. Enzyme extracts were prepared by homogenizing frozen tissue samples with a Ultra-Turrax homogenizer or a Polytron PT10 sonicating homogenizer using a weight to volume ratio ranging from 1:2 to 1:10 depending on the tissue. The homogenization buffer used for pyruvate kinase (PK), phosphofructokinase (PFK), glycogen phosphorylase (GP), and glycogen synthetase (GS) enzyme extracts contained 100 mM NaF, 10 mM EDTA, 10 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 15 mM 2-mercaptoethanol in 20 mM imidazole-HCl (pH 7.2).

Homogenates used in the GP assay were centrifuged for 2 minutes at 5°C in a Brinkman 5412 refrigerated microcentrifuge. The supernatant was removed and used for enzyme analysis.

For analysis of GS, the supernatant was removed from homogenates that had been left to settle on ice for a minimum of 30 minutes. However, L. littorea foot and M. demissus adductor muscle were centrifuged in a Brinkman 5412 refrigerated microcentrifuge for 2 minutes at 5°C .

The homogenates used for PK and PFK assays were centrifuged for 20 minutes at 25,000 g in a Sorvall RC-5B refrigerated centrifuge at 5°C . Supernatants were removed and then passed through 5 ml columns of G-25 Sephadex equilibrated in the homogenization buffer. Columns were spun in a desktop centrifuge at top speed for 1 minute. This procedure removed low molecular weight metabolites that might interfere with the reactions and also determination of the kinetic properties. The filtrate was then removed, stored on ice, and used for analysis of PK and PFK.

Tissues used for the pyruvate dehydrogenase (PDH) assays were homogenized in ice-cold 50 mM potassium phosphate buffer (pH 7.8) containing 2 mM 2-mercaptoethanol, 1 mM EDTA, 0.1 mM PMSF and 0.1% (w/v) Triton X-100. The homogenates were then placed at -20°C for 2 hours. The freezing of the homogenates aided in the break up of the mitochondrial membrane and increased the yield of the mitochondrial enzyme PDH. The samples were then thawed gently, and stored on ice.

ENZYME ASSAYS

The activity of many enzymes can be monitored by linking the formation of their products to coupled reactions whose products are more easily detected.

Glycogen Phosphorylase: The activity of GP was monitored by coupling the formation of the product glucose-1-phosphate (G1P) to the 2 coupling enzymes, phosphoglucomutase (PGM) and glucose-6-phosphate dehydrogenase (G6PDH) and recording the increase NADPH concentration (Scheme 1).

Scheme 1. GLYCOGEN PHOSPHORYLASE ANALYSIS THROUGH PGM and G6PDH.

Assay conditions for GP_a were 50 mM potassium phosphate buffer (pH 7.0) containing 0.25 mM EDTA, 10 mM MgCl₂, 0.4 mM NADP, 10 μM glucose-1,6-bisphosphate, 2 mg/ml glycogen (previously dialyzed), and 0.7 U/ml PGM and 0.2 U/ml G6PDH. For measurements of total phosphorylase, assays were conducted in the presence of 2 mM AMP.

Glycogen Synthetase: The enzyme GS adds another glucose unit to the glycogen chain resulting in the conversion of UDPG to UDP. The product UDP can be linked to the PK coupled enzyme assay. While PK prefers to use ADP in the conversion of PEP to pyruvate, it is also able to use UDP in its place (Scheme 2)

Scheme 2. GLYCOGEN SYNTHETASE ANALYSIS THROUGH PK AND LDH

GS activity was assayed in the presence of 20 mM imidazole-HCl buffer containing 1 mM PEP, 5 mM MgCl₂, 20 mM KCl, 0.15 mM NADH, 2 mg/ml glycogen (previously dialyzed), 1 U/ml PK, 1 U/ml LDH, and 5 mM UDPG. For measurements of total synthetase activity, 5 mM G6P was included in the assay mixture.

Phosphofructokinase: PFK activity was determined by monitoring the production of F1,6P₂ and linking this, through aldolase and triose phosphate isomerase (TPI), to NADH oxidation by glycerol-3-phosphate dehydrogenase (G3PDH) as seen in Scheme 3.

Scheme 3. PFK ANALYSIS THROUGH ALDOLASE, TPI, AND G3PDH

Assay conditions for maximal L. littorea PFK activity during isozyme studies were 20 mM imidazole-HCl (pH 7.2) containing 5 mM MgCl₂, 50 mM KCl, 0.15 mM NADH, 1 mM MgATP, 1 mM F6P and 0.2 U/ml TPI, 2.4 U/ml G3PDH, 0.4 U/ml aldolase. During the determination of the kinetic properties of L. littorea PFK, the above assay conditions were used with varying substrate concentrations of MgATP and F6P as described in Results. Assay conditions used in the M. demissus PFK isozyme studies were 20 mM imidazole-HCl (pH 7.2) containing 5 mM MgCl₂, 20 mM KCl, 0.15 mM NADH, 1 mM F6P, 0.5 mM MgATP and 0.2 u/ml triosephosphate isomerase, 2.4 U/ml glycerol-3-phosphate dehydrogenase, 0.4 U/ml aldolase.

Pyruvate Kinase: The activity of PK was monitored by coupling the formation of its product pyruvate to the coupling enzyme LDH. The progress of the reaction was followed by measuring the decrease in NADH absorbance at 340 nm, as shown in Scheme 4.

Scheme 4. PK ANALYSIS THROUGH LDH

PK activity was assayed in the presence of 20 mM imidazole-HCl (pH 7.2), 5 mM MgCl₂, 20 mM KCl, 0.15 mM NADH and 1 IU LDH, with varying amounts of PEP and ADP-Mg as described in Results.

All of the above enzyme assays were conducted by monitoring changes in concentration of NADH or NADPH at 340 nm using a Gilford 240 recording spectrophotometer and Kipp and Zonen BD 111 chart recorder. The assays were

initiated by addition of the enzyme preparation and performed at $21 \pm 1^\circ\text{C}$, with a final cuvette volume of 1 ml.

Pyruvate Dehydrogenase: The activity of the mitochondrial enzyme PDH was also assayed using a coupled enzyme reaction, coupling the product acetyl-CoA to the enzyme arylamine acetyltransferase (AAT). While PDH does produce NADH, (Scheme 5, reaction 1), the LDH present in a crude enzyme extract quickly causes its reoxidation (Scheme 5, reaction 2), thereby preventing measurement of PDH activity by monitoring NADH production. Instead the production of acetyl-CoA by PDH is coupled to the decolourization of the dye p-(p-aminophenylazo)benzene sulphonic acid (AABS) at 460 nm as shown in Scheme 5, reaction 3.

Scheme 5. PDH ANALYSIS THROUGH LDH AND AAT

PDH activity was determined immediately after the homogenates were thawed to ice cold temperature, under the following conditions, 50 mM Tris-HCl (pH 7.8), 1 mM MgCl_2 , 0.5 mM EDTA, 0.2 % (w/v) Triton X-100, 2.5 mM NAD^+ , 0.2 mM thiamine pyrophosphate, 0.2 mM Coenzyme A, 0.1 mM AABS, 0.05 IU AAT. The reaction was initiated by the addition of an appropriate volume of homogenate and allowed to run for approximately 5 minutes to establish a background rate. PDH activity was then measured after the addition of 10 mM pyruvate. This activity represents the amount of enzyme in the α form. In order to measure the total PDH activity in the sample, it was necessary to incubate the samples in the presence of MgCl_2 and CaCl_2 at concentrations, temperatures and time periods as described in

Results, to convert the inactive PDH to active PDH. Total PDH activity was defined as the activity in the sample after the incubation period and was determined under the same conditions as the α form of the enzyme.

COUPLING ENZYMES

Commercial coupling enzymes are stored in the presence of ammonium sulfate. Since in the case of PFK, this added stabilizer actually activates PFK, the ammonium sulfate had to be removed. This was achieved by dialyzing the coupling enzymes, aldolase, TPI and G3PDH, against 500 volumes of dialysis buffer (20 mM imidazole-HCl, pH 7.2, and 10 mM 2-mercaptoethanol) overnight at 4°C with 2 changes of buffer. Alternatively, the ammonium sulfate was removed by gel filtration (x2) of the enzyme stock solution through small columns (5ml) of G-25 Sephadex.

The coupling enzyme AAT used in PDH analysis was partially purified from chicken liver using polyethylene glycol fractionation and hydroxylapatite chromatography as outlined by Brooks and Storey (personal communication).

STABILITY TESTS

In order to enhance the stability of PFK during the *L. littorea* PFK isozyme studies, PFK was incubated with different stabilizing solutions, and then its activity was monitored over an 11 day period. Frozen tissue samples were homogenized 1:5 (w/v) in 20 mM imidazole buffer containing 30 mM NaF, 3 mM EDTA, 3 mM EGTA, 5 mM 2-mercaptoethanol, and 10 % (w/v) glycerol using an Ultra-Turrex tissue homogenizer. The homogenates were then centrifuged for 20 min at 25,000 g in a Sorvall RC-5B refrigerated centrifuge at 5°C. The supernatant was passed through 5 ml columns of G-25 Sephadex equilibrated in the homogenization buffer. Columns were spun in a desktop centrifuge at top speed for 1 minute. The filtrate was then removed, and placed in one of eleven different incubation solutions whose final concentrations were as follows: 40% (w/v) glycerol, 1 M sucrose, 1 mM F6P, 5 mM F6P, 0.5 mM AMP, 50 mM phosphate buffer, 100 mM phosphate buffer, 1 mM ATP,

10 mM MgSO₄, 40% glycerol + 1 mM F6P, or 1 mM F6P + 50 mM phosphate buffer. The samples were then incubated at room temperature and their activity determined every second day for 11 days.

ISOZYME STUDIES

To determine if there were different forms (phosphorylated and unphosphorylated) of the enzymes PK and PFK in L. littorea tissues, homogenates of both foot and hepatopancreas were passed through ion exchange columns of DE-52 Sephadex. Fractions were collected and assayed for the presence of multiple enzyme peaks. For PK analysis, foot and hepatopancreas tissues were homogenized 1:5 (w/v) in a 20 mM imidazole-HCl buffer (pH 7.2) containing 3 mM EDTA, 3 mM EGTA, 0.1 PMSF, 5 mM 2-mercaptoethanol, 30 mM NaF and 10% w/v glycerol using an Ultra-Turrax tissue homogenizer. Homogenates were centrifuged for 20 min at 25,000 g in a Sorvall RC-5B refrigerated centrifuge at 5°C. The resulting supernatant was passed through a 5 ml column of fine G-25 Sephadex equilibrated in the homogenization buffer. The 5 ml columns were then spun in a desk top centrifuge at top speed for 1 minute. The filtrate was placed on a DE-52 Sephadex column (6 cm X 1.5 cm) equilibrated in the homogenization buffer, and washed twice with several column volumes of homogenization buffer. Enzymes were then eluted with a linear salt gradient of 0-250 mM KCl in 40 ml homogenization buffer for foot and hepatopancreas. The resulting 1 ml fractions were assayed for PK activity. In addition for control foot samples, PK was eluted with a linear salt gradient of 0-125 mM KCl for better separation of the peaks, and the fractions containing the 2 different peaks were then pooled separately and their kinetic properties determined.

The same procedure was followed during L. littorea PFK analysis except that the buffer used contained in addition 5 mM F6P. The enzyme was applied to a DE-52 Sephadex column equilibrated in the buffer + 5 mM F6P and washed with several column volumes of same buffer. The enzyme was eluted with a linear KCl gradient, 0-

150 mM or 0-400 mM (in 40 ml) for foot and hepatopancreas respectively. One ml fractions were collected and assayed for PFK activity.

The same procedure was followed during M. demissus PFK analysis except that a 10 mM P_i buffer (pH 8.0) containing 2 mM EDTA, 2 mM EGTA, 50 mM NaF, 15 mM 2-mercaptoethanol, 1 mM F6P and 10 % (w/v) glycerol was used. The extracts were placed on DE-52 Shephadex columns and washed with several volumes of buffer. PFK was eluted with a linear 0-200 mM KCl gradient (40 ml) for all tissues. One ml fractions were collected and assayed for PFK activity.

L. littorea tissue homogenates were also incubated under different conditions in an attempt to phosphorylate or dephosphorylate PFK and PK in vitro. Tissue samples were homogenized 1:5 (w/v) in 20 mM imidazole-HCl (pH 7.2) containing 15 mM, 2-mercaptoethanol, 10 % (w/v) glycerol and 0.1 mM PMSF using an Ultra-Turrax homogenizer. The samples were centrifuged and passed through Sephadex G25 columns as described above. The homogenate was then divided into three aliquots. The first aliquot was used as a control and the homogenate was incubated in 20 mM imidazole-HCl buffer (pH 7.0) containing 30 mM NaF, 3 mM EDTA, 3 mM EGTA, 5 mM 2-mercaptoethanol. The second incubation solution contained a final concentration of 30 mM NaF, 1 mM cyclic 3'5'AMP, 10 mM $MgCl_2$ and 10 mM ATP in 20 mM imidazole-HCl buffer (pH 7.0). The third aliquot of enzyme was incubated in 20 mM imidazole-HCl buffer (pH 7.0) containing 10 mM $MgCl_2$.

The same procedure was followed when PFK was analyzed except that each solution also contained 5 mM F6P. The samples were incubated overnight at 4°C and then placed on ion exchange DE-52 Sephadex columns and eluted as described above.

PROTEIN DETERMINATION

Protein concentration was determined by the Bio-Rad protein assay kit based on the Coomassie Blue G-250 dye-binding method with Bovine Gamma Globulin as the standard (Bradford, 1976).

KINETICS AND STATISTICS

The substrate affinity constant, K_m , was defined as the concentration of substrate at which the enzyme reaction proceeds at 50% of its maximal velocity. K_a was defined as the concentration of activator at which the effector produces 50% of its maximal activity. I_{50} was defined as the concentration of inhibitor required to reduce enzyme activity to one half its value in the absence of inhibitor.

The substrate affinity constant K_m was determined by fitting the data to the Hill equation using a non-linear least square regression program provided by Dr. S.P.J. Brooks (1992). A modified Hill equation that introduced a V_0 term (rate at zero activator concentration) was used for determining K_a values. The inhibitor constant values (I_{50}) were obtained from plots of rate vs [inhibitor].

Data was analyzed using analysis of variance (Model I) followed by testing of differences between groups using a two tailed Dunnett's test, unless otherwise indicated.

RESULTS

GLYCOGEN PHOSPHORYLASE

Table 3.1 shows the percentage of GP in the active *a* form and the total activities of GP in two tissues of the gastropod mollusc *L. littorea* after 12 and 24 hours of freezing or anoxia. The activity of phosphorylase *a* (IU/g wet weight) in each tissue during anoxia and freezing is plotted in Figure 3.1 and 3.2; these illustrate the combined effects of changes in percentage of the *a* form, and in total phosphorylase activity, on the amount of active enzyme in each tissue.

The response of GP to freezing and anoxia was tissue specific in the snail and differed between the two stresses. Figure 3.1 shows that during anoxia the amount of active enzyme decreased by nearly one half in foot muscle but remained unchanged in hepatopancreas. The effect observed in foot muscle was due to a decrease in the amount of total phosphorylase (*a* + *b*) expressed in the tissue. The amount of active GP in foot muscle also decreased during freezing, with a significant difference found after 24 hours. Again, this result was due to a decrease in the amount of total phosphorylase. The response of hepatopancreas GP to freezing was the opposite to that seen in foot muscle, with a significant increase in phosphorylase activity. This effect was due to a rise in the total amount of phosphorylase (*a* + *b*) expressed in the tissue.

Table 3.2 shows the comparable effects of freezing and anoxia on GP in the bivalve mollusc *M. demissus*. Total activities of GP and the percentage of the active *a* form are shown for four tissues after 2 and 12 hours of freezing and anoxia. The activity of phosphorylase *a* (IU/g wet weight) in each tissue during anoxia and freezing is plotted in Figures 3.3 and 3.4, respectively.

The response of GP in the mussel was also tissue specific and differed between the two stresses. As Figure 3.3 shows, the amount of active enzyme in adductor muscle increased during anoxia. This activation of GP was due to an increase in both

the total amount of phosphorylase ($\alpha + \beta$) and the percentage of GS in the active α form. Phosphorylase activity in gill increased 6-fold after 12 hours of anoxia due to a large increase in the total amount of phosphorylase expressed in the tissue. Both hepatopancreas and mantle showed no significant change in phosphorylase activity in response to the anoxic conditions.

Figure 3.4 shows that there was no change in phosphorylase α content in adductor muscle after either 2 and 12 hours of freezing. In gill, however, phosphorylase α activity increased 3-fold after 2 hours frozen but returned to control values by 12 hours. This increase in enzyme activity was due to a rise in the total amount of phosphorylase expressed in the tissue. In both hepatopancreas and mantle tissues, the amount of active enzyme remained unchanged after 2 hours of freezing exposure but decreased significantly by 3-fold in hepatopancreas and 2-fold in mantle after 12 hours frozen. In hepatopancreas this effect was due to a decrease in both the percentage of the α form and the total phosphorylase content, whereas in mantle, a decrease in only the total amount of phosphorylase ($\alpha + \beta$) was seen.

GLYCOGEN SYNTHETASE

Table 3.3 shows the percentage of GS in the active I form and the total activities of GS ($I + D$) in two tissues of the snail *L. littorea*. The response of GS in the snail to freezing and anoxia was similar between the two tissues. Both foot muscle and hepatopancreas showed significant increases in the amount of active I form after freezing and anoxia. After 24 hours of freezing, the percentage of active synthetase rose from $10.5 \pm 1.3\%$ to $28.7 \pm 3.1\%$ in foot, and from $26.7 \pm 1.7\%$ to $47.2 \pm 2.8\%$ in hepatopancreas. Corresponding to the increase in percentage of active synthetase, was a decrease in the total amount of GS ($I + D$) expressed. While there were significant changes in both the total amount of GS expressed, and the percentage

of GS in the active **I** form, there was no significant change in the amount of active enzyme (IU/g wet weight) in response to freezing and anoxia.

Table 3.4 shows the comparable effects of freezing and anoxia on GS in the mussel *M. demissus*. Total activities of GS (**I** + **D**) and the percentage of the active **I** form are shown for four tissues after 2 and 12 hours of freezing and anoxia. Adductor showed no change in any of the parameters during freezing and anoxia. In gill, the percentage of GS in the active **I** form increased in response to 12 hours of freezing. At 12 hours anoxia, mantle showed a decrease in the percent **I**, and an increase in the total amount of GS expressed. While mantle and gill showed changes in the percentage of GS in the active form and/ or changes in the total amount of enzyme in response to freezing and anoxia, once again there was no significant change in the amount of active GS (IU/g wet weight). The only tissue to show a decrease in the amount of active GP was hepatopancreas, after 12 hours of freezing, largely due to a decrease in the total enzyme activity.

PHOSPHOFRUCTOKINASE

PFK Stability Tests

The effect of different stabilizing solutions on control *L. littorea* foot and hepatopancreas PFK activity are shown in Figures 3.5 and 3.6, respectively. As can be seen in Figure 3.6, the only solution that stabilized hepatopancreas PFK to any extent was that containing a final concentration of 5 mM F6P. Foot muscle PFK was stabilized by AMP, in addition to F6P (Figure 3.5). Other additions, such as P_i , did not improve F6P stabilization. Since the only solution that stabilized hepatopancreas PFK to any extent was that containing F6P, 5 mM F6P was added, as described in Methods and Materials, to stabilize the enzyme during the PFK isozyme studies that followed.

Isozyme studies

PFK from the foot muscle and hepatopancreas of L. littorea was placed on the ion exchange column, DE-52 Sephadex, to determine if there were isozymes present. PFK activity from control, frozen (24 h) and anoxic (24 h) foot muscle was found in one peak eluted at about 50 mM KCl (Figure 3.7). Hepatopancreas PFK from control, anoxic (24 hrs) and frozen (24 hrs) tissues was also eluted as a single peak at about 90 mM KCl (Figure 3.8).

Extracts from adductor, gill, hepatopancreas and mantle tissues of M. demissus were placed on DE-52 Sephadex anion exchange columns to determine if PFK isozymes were present. Adductor, gill, hepatopancreas and mantle PFK activity was found as a single peak under control, anoxic (12 h) and frozen (12 h) conditions (Figure 3.9-3.12). The enzyme was eluted at 90 mM KCl in adductor and gill, and 80 mM KCl in hepatopancreas and mantle.

Tissue homogenates from control L. littorea were also incubated under different conditions in an attempt to change the phosphorylation state of the enzyme. The first incubation solution containing 30 mM NaF, 3 mM EDTA, 3 mM EGTA, 5 mM 2-mercaptoethanol, 5 mM F6P in 20 mM imidazole-HCl (pH 7.0) acted as a control. The second incubation solution contained 1 mM cyclic 3'5'AMP, 10 mM ATP, 10 mM MgCl₂, 5 mM F6P in 20 mM imidazole-HCl (pH 7.0) and promoted phosphorylation. The third incubation contained 10 mM MgCl₂ in 20 mM imidazole (pH 7.0) and promoted the dephosphorylation of the enzyme. After incubation, extracts were analyzed by ion exchange chromatography. Ion exchange chromatography of the incubated foot and hepatopancreas extracts on DE-52 Sephadex resulted in a single peak, similar to the unincubated extracts. Figure 3.13 and 3.14 show chromatography of the L. littorea foot muscle and hepatopancreas PFK after in vitro phosphorylation and dephosphorylation incubations on DE-52 Sephadex.

Kinetic Properties

A summary of the kinetic properties of PFK of foot muscle and hepatopancreas of control L. littorea is listed in Table 3.5. PFK from foot and hepatopancreas showed different kinetic properties. PFK from foot muscle showed a higher affinity for the substrate F6P (K_m 0.22 ± 0.03) than hepatopancreas PFK (K_m 0.44 ± 0.01). In addition, the foot PFK showed a greater sensitivity to the activators, AMP, KCl, aspartate, and F26P₂, and decreased sensitivity to the inhibitors MgATP, MgCitrate and PEP.

PYRUVATE KINASE

Isozyme Studies

A typical separation of the forms of PK found in the foot muscle of control, frozen and anoxic animals after ion exchange chromatography on DE-52 Sephadex is shown in Figure 3.15. An extract of foot muscle from control L. littorea was placed on a DE-52 Sephadex anion exchange column in order to determine if isozymes of PK were present. Enzymatic activity was found in 2 peaks, as shown in Figure 3.15. Peak I was eluted at 30 mM KCl and peak II was eluted at 90 mM KCl. After ion exchange chromatography on DE-52 Sephadex, PK from foot muscle of frozen (24 h) and anoxic (24 h) L. littorea was found as a single peak, which corresponded to peak I of the control tissue.

A typical separation of the forms of PK found in the hepatopancreas of control, frozen and anoxic animals after ion exchange chromatography on DE-52 Sephadex is shown in Figure 3.16. As with the enzyme from the foot muscle, PK from the hepatopancreas of control snails could also be separated into 2 peaks by chromatography on DE-52 Sephadex. After 24 hours of freezing, the two forms of the enzyme were still present, however after 24 hours of anoxia, only one peak,

corresponding to peak I of the control animal, was observed. Peak I was eluted at a concentration of KCl 50 mM, and peak II at 110 mM KCl.

L. littorea foot and hepatopaneas extracts were subjected to in vitro incubations, as described in Methods and Materials, to promote either phosphorylation or dephosphorylation of the enzyme. Figure 3.17 and 3.18 show the chromatography of the in vitro phosphorylation and dephosphorylation incubations of L. littorea foot muscle and hepatopaneas on DE-52 Sephadex. Ion exchange chromatography on DE-52 Sephadex of the incubated control foot and hepatopaneas homogenates resulted in 2 peaks, similar to the unincubated homogenates. In both tissues, the in vitro phosphorylation of the enzyme resulted in a single peak whose elution concentration corresponded to that of peak II of control animals. In vitro dephosphorylation of hepatopaneas PK resulted in a single peak which corresponded to that of peak I of the control incubation. After in vitro dephosphorylation of foot muscle PK, enzyme activity was still found in 2 peaks, but the ratio of peak I to peak II was greater than in the control animal. In order to try and obtain a more complete dephosphorylation of the foot muscle PK, in vitro dephosphorylation incubation of the foot muscle was carried out at room temperature. However, the room temperature incubation gave the same results as the incubation carried out at 4°C.

Kinetic Properties

In order to obtain a better separation of the 2 peaks of PK from the foot muscle of control animals, the enzyme was eluted with a linear salt gradient of 0-125 mM KCl, and the fractions containing the 2 different peaks were then pooled separately and their kinetic properties determined. A summary of the kinetic properties of the two peaks of foot muscle PK of control L. littorea is shown in Table 3.6. Significant differences between the kinetic properties of the two peaks were found. Peak II PK showed a greater affinity for both substrates, PEP and ADP than did peak I. In addition, peak II PK was much less sensitive to inhibition by KCl and L-alanine than peak II. The

difference in L-alanine sensitivity was particularly large: peak I PK showed an I_{50} of 0.38 mM whereas peak II PK showed no inhibition even at L-alanine levels as high as 40 mM.

Effect of anoxia and freezing on PK activity from different tissues of M. demissus.

Another method for determining the effect of environmental stress on an enzyme involves measuring the rate of the enzyme at low and saturating concentrations of substrate. During periods of stress, the V_{max} of an enzyme remains constant, however there is often an increase in the apparent K_m for the substrate. Thus the activity ratio of an enzyme is a good indication of whether stress leads to a change in the enzyme's substrate affinity. The ratio of enzyme activities at low and saturating substrate concentrations for PK from the tissues of M. demissus during freezing and anoxia are shown in Table 3.7. The ratio of PK activity at low and saturating conditions decreased dramatically in M. demissus hepatopancreas, adductor, and gill tissues after 2 and 12 hours of anoxia and freezing. However, there was little or no change in mantle tissue under the same conditions. In hepatopancreas, the activity ratio decreased from 0.18 to 0.05 after 12 hours of anoxia and to 0.11 after 12 hours of freezing. Adductor muscle showed a 3-fold decrease in activity ratio during freezing and anoxia. Mantle tissue did show a decrease in activity after 12 hours of anoxia, however this is probably due to the observed increase in the maximal activity of the enzyme from 0.22 ± 0.02 to 0.31 ± 0.03 IU/g wet weight. In gill tissue, there was a significant decrease during freezing and almost a 4-fold decrease during anoxia in the PK activity ratio.

PYRUVATE DEHYDROGENASE

In order to obtain the optimal conditions for conversion of PDH into its active form, tissue homogenates were incubated under different conditions and the enzyme activity determined over a period of time. Figures 3.19 and 3.20 shows the amount of

active PDH in the foot and hepatopancreas tissues of L. littorea respectively, as a function of time under different in vitro incubation conditions. Figures 3.21 - 3.24, show the results of the incubation time courses for the M. demissus tissues, adductor, gill, hepatopancreas, and mantle. As a result of these studies, the indicated incubation conditions were used in the % active PDH determinations. While all of the incubation conditions did result in a slight conversion of PDH into its active form, different conditions resulted in a greater conversion into the active form or an increased time period of stability. For example, all four conditions used in the incubation of L. littorea foot muscle resulted in roughly the same amount of conversion (Figure 3.19), but the extract at 4°C was stable over a longer period of time. In general, the foot muscle of L. littorea and the adductor muscle of M. demissus required only 10 mM MgCl₂ compared to the 20 mM MgCl₂ required by the other tissues.

Figure 3.25 shows the effect of freezing and anoxia on the % of PDH in the active form in L. littorea foot and hepatopancreas. In both cases, the stresses lead to significant decreases in the amount of active PDH. In foot muscle, PDH α decreased from 73 % for control animals to 61 % for frozen animals and 59% for anoxic animals. In hepatopancreas, PDH activity decreased from 70% in control animals to 49% after 24 hours of freezing, and to 55% after 24 hours of anoxia. Total PDH activity remained constant during freezing and anoxia at 0.149 ± 0.008 IU/g wet weight in foot muscle, and 0.066 ± 0.006 IU/g wet weight in hepatopancreas.

PDH α activity also decreased significantly in the four tissues of M. demissus during anoxia (Figure 3.26) and freezing (Figure 3.27). Decreases in PDH α were most dramatic in the gill and mantle. In gill, the amount of active PDH decreased from 80% in the control animals to 57% in anoxic animals (24 hours). The amount of active PDH in mantle decreased from 84% in control animals to 57% in animals frozen for 24 hours.

Total PDH activity in M. demissus remained constant during freezing and anoxia at 0.082 ± 0.003 IU/g wet weight in adductor, 0.057 ± 0.003 IU/g wet weight in gill, 0.168 ± 0.011 IU/g wet weight in hepatopancreas, and 0.091 ± 0.005 IU/g wet weight in mantle.

Table 3.1 Total glycogen phosphorylase (a + b) activities and the percentage of the active a form in tissues of *L. littorea* during freezing and anoxia.

	Phosphorylase a%	
	Foot	Hepatopancreas
Control	34.6 ± 2.3	36.8 ± 2.7
Anoxic 12 h	$42.1 \pm 2.3^*$	$48.4 \pm 3.6^*$
Anoxic 24 h	28.1 ± 1.4	32.2 ± 4.0
Frozen 12 h	$55.7 \pm 1.5^{**}$	41.6 ± 2.8
Frozen 24 h	39.5 ± 2.1	41.7 ± 1.1

	Total phosphorylase	
	Foot	Hepatopancreas
Control	2.09 ± 0.15	0.26 ± 0.015
Anoxic 12 h	$1.06 \pm 0.07^{**}$	0.24 ± 0.036
Anoxic 24 h	$1.57 \pm 0.13^*$	0.22 ± 0.008
Frozen 12 h	$1.16 \pm 0.13^{**}$	0.29 ± 0.039
Frozen 24 h	$1.17 \pm 0.18^{**}$	$0.41 \pm 0.025^{**}$

Note: Activities are IU/g wet weight, means $\pm$ SEM, n=4-6.

* Values are significantly different from corresponding control values, $P < 0.05$.

** $P < 0.01$.

Figure 3.1 Effect of anoxia on the activity of phosphorylase a in tissues of the snail L. littorea.

Activities are IU/g wet weight, means $\pm$ SEM, n=4-6.

* Values are significantly different from corresponding control value ; $P < 0.05$.

Figure 3.2 Effect of freezing on the activity of phosphorylase a in tissues of the snail L. littorea.

Activities IU/g wet weight, means $\pm$ SEM, n=4-6.

* Values are significantly different from corresponding control values, $P < 0.05$; ** $P < 0.01$.

Table 3.2 Total glycogen phosphorylase (a + b) activities and percentage of the active a form in tissues of *M. demissus* during freezing and anoxia.

	Phosphorylase a (%)			
	Adductor	Gill	Hepatopancreas	Mantle
Control	32.4 ± 0.4	40.3 ± 2.3	35.9 ± 1.0	38.0 ± 0.7
Anoxic 2 h	48.9 ± 1.2 ^{**}	27.8 ± 2.0 ^{**}	32.1 ± 3.4	N.D.
Anoxic 12 h	38.3 ± 1.2 [*]	50.1 ± 1.1 ^{**}	35.4 ± 5.8	35.8 ± 2.7
Frozen 2 h	36.9 ± 1.6 [*]	45.7 ± 4.1	37.1 ± 2.0	50.6 ± 3.4 ^{**}
Frozen 12 h	34.1 ± 0.8	38.0 ± 3.7	25.2 ± 1.7 [*]	35.6 ± 1.1

	Total Phosphorylase (IU/g wet wt.)			
	Adductor	Gill	Hepatopancreas	Mantle
Control	0.28 ± 0.02	0.007 ± 0.0003	0.39 ± .07	0.32 ± 0.04
Anoxic 2 h	0.50 ± 0.02 ^{**}	0.036 ± 0.003 ^{**}	0.54 ± .05	N.D.
Anoxic 12 h	0.42 ± 0.04 ^{**}	0.038 ± 0.002 ^{**}	0.56 ± .03	0.29 ± 0.02
Frozen 2 h	0.24 ± 0.02	0.017 ± 0.001 ^{**}	0.45 ± .04	0.19 ± 0.03 ^{**}
Frozen 12 h	0.27 ± 0.03	0.009 ± 0.001	0.18 ± .01 ^{**}	0.14 ± 0.01 ^{**}

Note: Activities are IU/g.wet weight, means ± SEM, n=4-6. N.D. - not determined.

* Values are significantly different from corresponding control values, P < 0.05.

** P < 0.01.

Figure 3.3 Effect of anoxia on the activity of phosphorylase a in tissues of the mussel M. demissus.

N.D. - not determined.

Activities are IU/g wet weight, means $\pm$ SEM, n=4-6.

* Values are significantly different from corresponding control values, $P < 0.05$; ** $P < 0.01$.

Figure 3.4. Effect of freezing on the activity of phosphorylase α in tissues of the mussel M. demissus.

Activities are IU/g wet weight, means $\pm$ SEM, n=4-6.

* Values are significantly different from corresponding control values, $P < 0.05$; ** $P < 0.01$.

Table 3.3 Total glycogen synthetase (I + D) activities and the percentage of the active I form in tissues of *L. littorea* during freezing and anoxia.

	Synthetase I%	
	Foot	Hepatopancreas
Control	10.5 $\pm$ 1.3	26.7 $\pm$ 1.7
Anoxic 24 h	19.4 $\pm$ 2.2*	45.4 $\pm$ 7.5*
Frozen 24 h	28.7 $\pm$ 3.1**	47.2 $\pm$ 2.8*

	Total Synthetase (IU/gww)	
	Foot	Hepatopancreas
Control	1.01 $\pm$ 0.11	0.19 $\pm$ 0.01
Anoxic 24 h	0.38 $\pm$ 0.03**	0.17 $\pm$ 0.02
Frozen 24 h	0.53 $\pm$ 0.08**	0.13 $\pm$ 0.01*

Note: Activities are IU/g wet weight, means $\pm$ SEM, n=4-6.

* Values are significantly different from corresponding control values, $P < 0.05$.

** $P < 0.01$.

Table 3.4 Total glycogen synthetase (I + D) activities and percentage of the active I form in tissues of *M. demissus* during freezing and anoxia.

	Synthetase I (%)			
	Adductor	Gill	Hepatopancreas	Mantle
Control	37.5 $\pm$ 1.8	33.0 $\pm$ 3.9	18.0 $\pm$ 0.5	27.8 $\pm$ 2.6
Anoxic 12 h	40.8 $\pm$ 4.1	40.6 $\pm$ 2.5	23.7 $\pm$ 2.3	19.5 $\pm$ 1.4*
Frozen 12 h	41.3 $\pm$ 1.9	59.4 $\pm$ 2.6	37.5 $\pm$ 7.2**	21.4 $\pm$ 0.9
	Total Synthetase (IU/g wet wt.)			
	Adductor	Gill	Hepatopancreas	Mantle
Control	0.22 $\pm$ 0.02	0.18 $\pm$ 0.03	0.44 $\pm$.01	0.34 $\pm$ 0.04
Anoxic 12 h	0.26 $\pm$ 0.04	0.15 $\pm$ 0.02	0.35 $\pm$.02*	0.47 $\pm$ 0.06
Frozen 12 h	0.23 $\pm$ 0.02	0.12 $\pm$ 0.01	0.09 $\pm$.01**	0.28 $\pm$ 0.01

Note: Activities are IU/g.wet weight, means $\pm$ SEM, n=4-6.

* Values are significantly different from corresponding control values, P < 0.05.

** P < 0.01.

Figure 3.5 Stability tests of PFK from L. littorea foot muscle.

PFK from control foot muscle was incubated at $21 \pm 1^{\circ}\text{C}$ over a period of 11 days in solutions whose final concentrations were as follows:

A Control (closed triangles), 10 mM MgCl (open triangles), 1 M sucrose (closed circles), 40% w/v glycerol (open circles).

B 1 mM MgATP (closed triangles), 1 mM F6P & 50 mM P_i (open triangles), 50 mM P_i (closed circles), 0.5 mM AMP (open circles).

C 40% w/v glycerol & 1 mM F6P (closed triangles), 100 mM P_i (open triangles), 5 mM F6P (closed circles), 1 mM F6P (open circles).

Data are expressed relative to starting activity, in the absence of additions, at time zero.

Figure 3.6 Stability tests of PFK from L. littorea hepatopaneas.

PFK from control hepatopaneas was incubated at $21 \pm 1^{\circ}\text{C}$ over a period of 9 days in solutions whose final concentrations were as follows:

A Control (closed triangles), 10 mM MgCl (open triangles), 1 M sucrose (closed circles), 40% w/v glycerol (open circles).

B 1 mM MgATP (closed triangles), 1 mM F6P & 50 mM P_i (open triangles), 50 mM P_i (closed circles), 0.5 mM AMP (open circles).

C 40% w/v glycerol & 1 mM F6P (closed triangles), 100 mM P_i (open triangles), 5 mM F6P (closed circles), 1 mM F6P (open circles).

Data are expressed relative to starting activity, in the absence of additions, at time zero.

Figure 3.7 Chromatography of PFK from the foot muscle of L. littorea on DE-52 Sephadex.

Foot muscle PFK from control (circles), frozen 24 hours (squares) and anoxic 24 hours (triangles) snails was eluted using a linear gradient of KCl, 0-150 mM (dashed line), in a 20 mM imidazole-HCl buffer (pH 7.2) containing 3 mM EDTA, 3 mM EGTA, 5 mM 2-mercaptoethanol, 30 mM NaF, 5 mM F6P and 10 % (w/v) glycerol.

Figure 3.8 Chromatography of PFK from hepatopancreas of L. littorea on DE-52 Sephadex.

Hepatopancreas PFK from control (circles), frozen 24 hours (squares) and anoxic 24 hours (triangles) snails was eluted using a linear gradient of KCl, 0-400 mM (dashed line), in a 20 mM imidazole-HCl buffer (pH 7.2) containing 3 mM EDTA, 3 mM EGTA, 5 mM 2-mercaptoethanol, 30 mM NaF, 5 mM F6P and 10 % (w/v) glycerol.

Figure 3.9 Chromatography of PFK from adductor muscle of M. demissus on DE-52 Sephadex.

Adductor muscle PFK from control (circles), frozen 24 hours (squares) and anoxic 24 hours (triangles) mussels was eluted using a linear gradient of KCl, 0-200 mM (dashed line), in a 10 mM P_i buffer (pH 8.0) containing 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 15 mM 2-mercaptoethanol, 1 mM F6P, and 10 % glycerol.

Figure 3.10 Chromatography of PFK from gill of M. demissus on DE-52 Sephadex.

Gill PFK from control (circles), frozen 24 hours (squares) and anoxic 24 hours (triangles) mussels was eluted using a linear gradient of KCl, 0-200 mM (dashed line), in a 10 mM P_i buffer (pH 8.0) containing 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 15 mM 2-mercaptoethanol, 1 mM F6P, and 10 % glycerol.

Figure 3.11 Chromatography of PFK from hepatopancreas of M. demissus on DE-52 Sephadex.

Hepatopancreas PFK from control (circles), frozen 24 hours (squares) and anoxic 24 hours (triangles) mussels was eluted using a linear gradient of KCl, 0-200 mM (dashed line), in a 10 mM P_i buffer (pH 8.0) containing 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 15 mM 2-mercaptoethanol, 1 mM F6P, and 10 % glycerol.

Figure 3.12 Chromatography of PFK from mantle of M. demissus on DE-52 Sephadex.

Mantle PFK from control (circles), frozen 24 hours (squares) and anoxic 24 hours (triangles) mussels was eluted using a linear gradient of KCl, 0-200 mM (dashed line), in a 10 mM P_i buffer (pH 8.0) containing 50 mM NaF, 2 mM EDTA, 2 mM EGTA, 15 mM 2-mercaptoethanol, 1 mM F6P, and 10 % glycerol.

Figure 3.13 Chromatography of PFK from foot muscle of L. littorea after in vitro phosphorylation or dephosphorylation incubations.

All solutions were incubated overnight at 4°C. After incubation, homogenates were loaded onto a DE-52 Sephadex column, and PFK was eluted with a 0-150 mM KCl gradient (dashed line).

Incubation conditions were:

- A. Control** (circles) - 20 mM imidazole-HCl, 30 mM NaF, 3 mM EDTA, 3 mM EGTA, 5 mM F6P and 5 mM 2-mercaptoethanol (pH 7.0).
- B. In vitro phosphorylation** (triangles) - 20 mM imidazole-HCl, 1 mM cyclic 3'5'AMP, 10 mM ATP, 10 mM MgCl₂, 30 mM NaF, and 5 mM F6P (pH 7.0).
- C. In vitro dephosphorylation** (squares) - 20 mM imidazole-HCl, 10 mM MgCl₂ and 5 mM F6P (pH 7.0).

Figure 3.14 Chromatography of PFK from hepatopancreas of L. littorea after in vitro phosphorylation or dephosphorylation incubations.

All solutions were incubated overnight at 4°C. After incubation, homogenates were loaded onto a DE-52 column, and PFK was eluted with 0-400 mM KCl gradient (dashed line).

Incubation conditions were:

- A. Control** (circles) - 20 mM imidazole-HCl, 30 mM NaF, 3 mM EDTA, 3 mM EGTA, 5 mM F6P and 5 mM 2-mercaptoethanol (pH 7.0).
- B. In vitro phosphorylation** (triangles) - 20 mM imidazole-HCL, 1 mM cyclic 3'5'AMP, 10 mM ATP, 10 mM MgCl₂, 30 mM NaF, and 5 mM F6P (pH 7.0).
- C. In vitro dephosphorylation** (squares) - 20 mM imidazole-HCl, 10 mM MgCl₂ and 5 mM F6P (pH 7.0).

Table 3.5 Kinetic properties of PFK from foot muscle and hepatopancreas of control L. littorea.

	Foot Muscle ^a	Hepatopancreas ^b
K_m F6P, mM	0.22 $\pm$ 0.03	0.44 $\pm$ 0.01
K_a AMP, mM	0.016 $\pm$ 0.002	0.045 $\pm$ 0.001
K_a F2,6P ₂ , μ M	1.7 $\pm$ 0.2	8.0 $\pm$ 0.6
K_a KCl, mM	11 $\pm$ 0.7	17 $\pm$ 0.5
K_a Aspartate, mM	14 $\pm$ 1.2	16 $\pm$ 1.6
I_{50} MgATP, mM	0.30 $\pm$ 0.03	0.51 $\pm$ 0.06
I_{50} MgCitrate, mM	1.6 $\pm$ 0.14	4.9 $\pm$ 0.19
I_{50} PEP, mM	0.90 $\pm$ 0.10	1.34 $\pm$ 0.08

Results are means $\pm$ SEM for determinations on enzyme preparations from n=4 different animals.

^a - K_m (F6P) values were determined at 0.1 mM MgATP. I_{50} and K_a values were determined at 0.2 mM F6P and 0.1 mM MgATP.

^b - K_m (F6P) values were determined at 0.1 mM MgATP. I_{50} and K_a values were determined at 0.4 mM F6P and 0.1 mM MgATP.

Figure 3.15 Chromatography of PK from the foot muscle of L. littorea on DE-52 Sephadex.

Foot muscle PK from control (circles), frozen 24 hours (squares) and anoxic 24 hours (triangles) snails was eluted using a linear gradient of KCl, 0-250 mM (dashed line), in a 20 mM imidazole-HCl buffer (pH 7.2) containing 3 mM EDTA, 3 mM EGTA, 5 mM 2-mercaptoethanol, 30 mM NaF and 10 % (w/v) glycerol.

Figure 3.16 Chromatography of PK from hepatopancreas of L. littorea on DE-52 Sephadex.

Hepatopancreas PF from control (circles), frozen 24 hours (squares) and anoxic 24 hours (triangles) snails was eluted using a linear gradient of KCl, 0-250 mM (dashed line), in a 20 mM imidazole-HCl buffer (pH 7.2) containing 3 mM EDTA, 3 mM EGTA, 5 mM 2-mercaptoethanol, 30 mM NaF and 10 % (w/v) glycerol.

Figure 3.17 Chromatography of PK from foot muscle of L. littorea after in vitro phosphorylation or dephosphorylation incubations.

All solutions were incubated overnight at 4°C. After incubation, homogenates were loaded onto a DE-52 Sephadex column, and PK was eluted with a 0-250 mM KCl gradient (dashed line). Incubation conditions were:

A. Control (circles) - 20 mM imidazole-HCl, 30 mM NaF, 3 mM EDTA, 3 mM EGTA, and 5 mM 2-mercaptoethanol (pH 7.0).

B. In vitro phosphorylation (triangles) - 20 mM imidazole-HCl, 1 mM cyclic 3'5'AMP, 10 mM ATP, 10 mM MgCl₂, and 30 mM NaF (pH 7.0).

C. In vitro dephosphorylation (squares) - 20 mM imidazole-HCl, and 10 mM MgCl₂ (pH 7.0).

Figure 3.18 Chromatography of PK from hepatopancreas of *L. littorea* after in vitro phosphorylation and dephosphorylation incubations.

All solutions were incubated overnight at 4°C. After incubation, homogenates were loaded onto a DE-52 column, and PK was eluted with a 0-250 mM KCl gradient (dashed line). Incubation conditions were:

- A. Control** (circles) - 20 mM imidazole-HCl, 30 mM NaF, 3 mM EDTA, 3 mM EGTA, and 5 mM 2-mercaptoethanol (pH 7.0).
- B. In vitro phosphorylation** (triangles) - 20 mM imidazole-HCl, 1 mM cyclic 3'S'AMP, 10 mM ATP, 10 mM MgCl₂, and 30 mM NaF, (pH 7.0).
- C. In vitro dephosphorylation** (squares) - 20 mM imidazole-HCl, and 10 mM MgCl₂ (pH 7.0).

Table 3.6 Kinetic properties of two forms of PK from foot muscle of control L. littorea.

	Peak I	Peak II
K_m PEP, mM	0.21 ± 0.03	$0.10 \pm 0.02^*$
K_m ADP, mM	0.20 ± 0.01	$0.15 \pm 0.02^*$
I_{50} L-alanine, mM	0.38 ± 0.02	$> > 40$
I_{50} KCl, mM	208 ± 0.40	$356 \pm 25^*$
I_{50} MgCitrate, mM	2.79 ± 0.40	3.82 ± 0.34
I_{50} MgATP, mM	2.78 ± 0.45	4.04 ± 0.55
I_{50} NH_4Cl , mM	244 ± 50	231 ± 37
I_{50} $(NH_4)_2SO_4$, mM	138 ± 26	112 ± 22

Note: Results are means $\pm$ SEM for determinations on enzyme preparations from $n=4$ different animals.

K_m PEP values were determined at 2 mM ADP. K_m ADP values were determined at 1 mM PEP. I_{50} values were determined at 1 mM ADP and 0.3 mM PEP.

* Values are significantly different from the corresponding values for Peak I enzyme according to the Student's t-test, $P < 0.05$.

Table 3.7 Total activities and ratio of enzyme activities at low and at saturating substrate concentrations for PK from the tissues of *M. demissus* during freezing and anoxia.

	Activity Ratio			
	Adductor ^a	Gill ^b	Hepatopancreas ^b	Mantle ^c
Control	0.120 $\pm$ 0.006	0.390 $\pm$ 0.080	0.180 $\pm$ 0.020	0.230 $\pm$ 0.020
Anoxic 2 h	0.040 $\pm$ 0.006**	0.080 $\pm$ 0.010**	0.080 $\pm$ 0.010**	N.D.
Anoxic 12 h	0.050 $\pm$ 0.008**	0.110 $\pm$ 0.040**	0.050 $\pm$ 0.004**	0.160 $\pm$ 0.010*
Frozen 2 h	0.040 $\pm$ 0.008**	0.100 $\pm$ 0.020**	0.070 $\pm$ 0.010**	0.200 $\pm$ 0.020
Frozen 12 h	0.040 $\pm$ 0.006**	0.210 $\pm$ 0.030*	0.110 $\pm$ 0.020*	0.220 $\pm$ 0.010

Note: Values are the mean $\pm$ SEM of 3-4 determinations

^a Enzyme activity ratio was determined at 0.50 mM and 5.0 mM PEP.

^b Enzyme activity ratio was determined at 0.75 mM and 7.5 mM PEP.

^c Enzyme activity ratio was determined at 0.40 mM and 2.0 mM PEP.

* Values are significantly different from corresponding controls, $P < 0.05$, ** $P < 0.01$.

Figure 3.19 Time course for the in vitro incubation of L. littorea foot extracts for the conversion of PDH into the active a form.

Samples were incubated under 4 different conditions; 1) 10 mM MgCl₂ and 1 mM CaCl₂ at 21 $\pm$ 1°C (open circles), 2) 20 mM MgCl₂ and 1 mM CaCl₂ at 21 $\pm$ 1°C (closed circles), 3) 10 mM MgCl₂ and 5 mM CaCl₂ at 21 $\pm$ 1°C (open triangles), 4) 10 mM MgCl₂ and 1 mM CaCl₂ on ice (closed triangles).

Samples were incubated with 10 mM MgCl₂ and 1 mM CaCl₂ on ice (condition 4) for 40 minutes for % active PDH determinations.

Figure 3.20 Time course for the in vitro incubation of L. littorea hepatopancreas extracts for the conversion of PDH into the active a form.

Samples were incubated under 4 different conditions; 1) 10 mM MgCl₂ and 1 mM CaCl₂ at 21 $\pm$ 1°C (open circles), 2) 20 mM MgCl₂ and 1 mM CaCl₂ at 21 $\pm$ 1°C (closed circles), 3) 10 mM MgCl₂ and 5 mM CaCl₂ at 21 $\pm$ 1°C (open triangles), 4) 10 mM MgCl₂ and 1 mM CaCl₂ on ice (closed triangles).

Samples were incubated with 20 mM MgCl₂ and 1 mM CaCl₂ at 21 $\pm$ 1°C (condition 2) for 40 minutes for % active PDH determinations.

Figure 3.21 Time course for the in vitro incubation of M. demissus adductor extracts for the conversion of PDH into the active a form.

Samples were incubated under 4 different conditions; 1) 10 mM MgCl₂ and 1 mM CaCl₂ at 21 $\pm$ 1°C (open circles), 2) 20 mM MgCl₂ and 1 mM CaCl₂ at 21 $\pm$ 1°C (closed circles), 3) 10 mM MgCl₂ and 5 mM CaCl₂ at 21 $\pm$ 1°C (open triangles), 4) 10 mM MgCl₂ and 1 mM CaCl₂ on ice (closed triangles).

Samples were incubated with 10 mM MgCl₂ and 1 mM CaCl₂ at 21 $\pm$ 1°C (condition 1) for 40 minutes for % active PDH determinations.

Figure 3.22 Time course for the in vitro incubation of *M. demissus* gill extracts for the conversion of PDH into the active a form.

Samples were incubated under 4 different conditions; 1) 10 mM MgCl₂ and 1 mM CaCl₂ at 21 ± 1°C (open circles), 2) 20 mM MgCl₂ and 1 mM CaCl₂ at 21 ± 1°C (closed circles), 3) 10 mM MgCl₂ and 5 mM CaCl₂ at 21 ± 1°C (open triangles), 4) 10 mM MgCl₂ and 1 mM CaCl₂ on ice (closed triangles).

Samples were incubated with 20 mM MgCl₂ and 1 mM CaCl₂ at 21 ± 1°C (condition 2) for 20 minutes for % active PDH determinations.

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NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

Figure 3.23 Time course for the in vitro incubation of M. demissus hepatopancreas extracts for the conversion of PDH into the active α form.

Samples were incubated under 4 different conditions; 1) 10 mM MgCl_2 and 1 mM CaCl_2 at $21 \pm 1^\circ\text{C}$ (open circles), 2) 20 mM MgCl_2 and 1 mM CaCl_2 at $21 \pm 1^\circ\text{C}$ (closed circles), 3) 10 mM MgCl_2 and 5 mM CaCl_2 at $21 \pm 1^\circ\text{C}$ (open triangles), 4) 10 mM MgCl_2 and 1 mM CaCl_2 on ice (closed triangles).

Samples were incubated with 20 mM MgCl_2 and 1 mM CaCl_2 at $21 \pm 1^\circ\text{C}$ (condition 2) for 40 minutes for % active PDH determinations.

Figure 3.24 Time course for the in vitro incubation of *M. demissus* mantle extracts for the conversion of PDH into the active α form.

Samples were incubated under 4 different conditions; 1) 10 mM MgCl_2 and 1 mM CaCl_2 at $21 \pm 1^\circ\text{C}$ (open circles), 2) 20 mM MgCl_2 and 1 mM CaCl_2 at $21 \pm 1^\circ\text{C}$ (closed circles), 3) 10 mM MgCl_2 and 5 mM CaCl_2 at $21 \pm 1^\circ\text{C}$ (open triangles), 4) 10 mM MgCl_2 and 1 mM CaCl_2 on ice (closed triangles).

Samples were incubated with 20 mM MgCl_2 and 1 mM CaCl_2 at $21 \pm 1^\circ\text{C}$ (condition 2) for 40 minutes for % active PDH determinations.

Figure 3.25 Percentage of pyruvate dehydrogenase in the active a form in the tissues of L. littorea after 24 hours of anoxia (coarse fill) and 24 hours of freezing (solid fill).

Values are means $\pm$ SEM, n=4.

* values are significantly different from corresponding control values, $P < 0.05$; ** $P < 0.01$.

Figure 3.26 Percentage of PDH in the active α form in the tissues of M. demissus after 2 hours (coarse fill) and 12 hours (solid fill) of anoxia.

N.D. - not determined.

Values are means $\pm$ SEM, n=4.

* values are significantly different from corresponding control values, $P < 0.05$; ** $P < 0.01$.

Figure 3.27 Percentage of PDH in the active a form in the tissues of M. demissus after 2 hours (coarse fill) and 12 hours (solid fill) of freezing.

Values are means $\pm$ SEM, n=4.

* values are significantly different from corresponding control values, $P < 0.05$; ** $P < 0.01$.

DISCUSSION

Marine invertebrates that live along the coastal shores are exposed to varying environmental conditions. Due to the changing water levels of the daily tides, animals inhabiting the intertidal zone are exposed to changes such as large fluctuations in oxygen availability, salinity and temperature. Though these animals often belong to different phyla and face a variety of stresses, common adaptations have been found among them.

Among those biological adaptations found in animals with high anoxia tolerance are the following: (1) Maintenance of high levels of fermentable fuels (carbohydrates such as glycogen) (2) Enhancement of the buffering capacity of body fluids or changes in the types of fermentation end products produced, in order to minimize metabolic acidosis (3) Use of alternative pathways of fermentative ATP production to increase anaerobic ATP yield. (4) Reduction of metabolic rate during anoxia compared to that of the aerobic resting rate. While the first three adaptations are important, none of them are so closely linked to survival of harsh environmental conditions as is metabolic rate depression.

While it has been assumed that metabolic rate depression also occurs in animals in the frozen state, it has not yet been experimentally proven. However, since extracellular freezing cuts off the circulation of O_2 to tissues and hence creates an anoxic state, it could reasonably be expected that metabolic depression would also be an adaptation for natural freezing survival.

It has become apparent that the extent to which the above adaptations are put into play are often tissue specific. The tissue specific variations enable the animal to accommodate the specific functions of each tissue and allow the animal to reorganize itself for stress survival in a balanced manner. As an example, tissue specific differences of metabolic rate during anoxia (as determined by end product

accumulation) have been found in the clam, Mercenaria mercenaria (Korycan and Storey, 1983).

One mechanism closely associated with metabolic rate depression involves the reversible phosphorylation of regulatory enzymes. Post-translational modification, via the addition or removal of covalently bound phosphate from proteins, is one of the quickest and most powerful ways of controlling an enzyme's activity, and thus the flux through a pathway. The phosphorylation/ dephosphorylation process can act as an "on/off" switch by converting inactive enzymes into their active forms and visa versa. This type of regulation has been well documented for many different types of animals and is a well understood method of control for the enzymes GP, GS, and PDH (Woodford et al, 1992). Enzymatic control, via reversible phosphorylation, is a powerful method of control during periods of stress because of the speed with which it can be executed. Furthermore, the fact that it is easily reversible makes it a very useful tool when the animal is facing changing environmental conditions. Though phosphorylation of an enzyme does require ATP, the cost of phosphorylating an enzyme is cheap, energetically speaking, compared with alternative modes of changing enzyme activity such as enzyme synthesis. Researchers have estimated that the energy cost of phosphorylating enzymes takes up only about 5% of the total energy budget (P. Cohen, personal communication). In addition, the mechanism of reversible phosphorylation gives the animal control over more than one enzyme at a time. A prime example of this involves the glycogen synthesis and degradation enzymes, GS and GP. Phosphorylation of GP results in its activation, while at the same time phosphorylation of GS results in its inactivation. Such regulation prevents opposing reactions from occurring simultaneously which would waste large amounts of valuable energy.

Whereas the research undertaken for this thesis set out to study the role of reversible phosphorylation in the regulation of carbohydrate metabolism during anoxia

and freezing in the tissues of L. littorea and M. demissus, the studies also revealed two other regulatory mechanisms that play important roles. These are degradation and synthesis of enzymes. The degradation of enzyme results from the action of proteases, of which there are many in the cell, and requires little or no energy input. Degradation of an enzyme is a relatively cost efficient way of decreasing the amount of an enzyme and as a result the net enzyme activity. However, synthesis is not nearly so easy as degradation especially during periods of stress. Not only does synthesis of proteins involve many more steps than degradation, it is also energetically costly, requiring large amounts of ATP. Synthesis of proteins may be the last resort of an animal faced with a long period of stress but requiring an increase in enzyme activity. While changes in enzyme activity were found to be due to conversion between inactive and active forms via reversible phosphorylation, in some cases degradation and synthesis of enzymes was also observed. It was found in this study that the methods used by the animals differed not only between enzymes but also between stresses and even species and tissues themselves.

GLYCOGEN PHOSPHORYLASE

A prime example of the different mechanisms that the two species used to control enzymatic activity was found in the response of GP to anoxia and freezing. During stressful conditions that involve a lack of oxygen, the carbohydrate glycogen is the main source of fuel, with little or no contribution from other fuel sources such as lipids and amino acids. Since the enzyme GP catalyzes the first step in glycogen breakdown, control of GP results in direct control of the cell's fuel source. Three factors are thought to control the response of GP to stress (i) stress induced depression of metabolic rate (ii) anoxic ATP requirements of individual tissues and (iii) in some cases, a switch to anoxic glycogen fermentation from aerobic fermentation of an alternative fuel. The importance of each factor in each tissue determines the amount of active enzyme during periods of stress. Specific differences in the amount of active

glycogen phosphorylase during periods of anoxia were found in the marine mollusc B. canaliculatum (Storey, 1988). Similarly, research reported in this thesis shows that the phosphorylase response of L. littorea and M. demissus to environmental stress was tissue specific as well as stress related.

During freezing and anoxia, the foot muscle of L. littorea showed a decrease in the amount of active phosphorylase (Figures 3.1, 3.2). There are two mechanisms that account for the observed decrease. For both anoxia and freezing, the decrease in active enzyme was in part due to a decrease in the total amount of enzyme (Table 3.1). The decrease in amount of total enzyme could be due to: a) degradation of GP by proteolysis and/ or b) dissociation of the subunits that make up GP. Interestingly, Ebberink and Salimans (1982) have reported that GP_b purified from the posterior adductor muscle of the sea mussel M. edulis consists of 2 forms, probably a monomer and a dimer which are in equilibrium. The other mechanism, which was seen at both 12 hours frozen and 12 hours anoxic in the foot, involves conversion of inactive GP into the active form. However, the overall result of these two mechanisms was a decrease of nearly one half in the amount of active phosphorylase in frozen or anoxic. The decrease in active phosphorylase would result in a decrease in glycogen catabolism, in keeping with the principles of metabolic depression.

However, the mechanism found in the foot muscle, which involved a decrease in the total amount of GP via degradation of the enzyme or dissociation of the enzyme into subunits, was not present in the hepatopancreas of L. littorea. Conversion of inactive GP into the active form, occurred to a small extent in hepatopancreas as seen at 12 hours anoxic (Table 3.2). In addition, in hepatopancreas at 24 hours frozen, there was almost a 2 fold increase in the amount of phosphorylase activity as a result of an increase in the total amount of GP (Figure 3.2). The activation of GP in hepatopancreas at 24 hours frozen seems contrary to what would be expected during a period of environmental stress. However, the products of the reaction catalyzed by GP

or subsequent products, may be those needed by the animal to protect itself from the hazards associated with the frozen state. Indeed, such activation of GP is often seen in insects in response to cold temperatures for the purpose of cryoprotectant synthesis (Bahjou et al., 1987; Hayakawa, 1985).

In L. littorea hepatopancreas the changes in GP due to anoxia and freezing were generally of a much smaller magnitude than those seen in foot. This result, in addition to the fact that hepatopancreas used a different combination of mechanisms to control GP activity, indicates that regulation of GP was tissue specific.

Unlike the gastropod L. littorea, many bivalves may carry out anaerobic metabolism even in the presence of aerobic conditions according to the findings of Broth and Magnum (1978). Many bivalves lack respiratory pigments in their blood, and as a result their blood is a poor supplier of oxygen. Since most of the oxygen, 85% in the case of M. demissus, is absorbed directly by the tissues via their exposed surfaces, cells in the interior of these tissues and tissues in the interior of the animal, are unable to utilize oxygen. Since some tissues of M. demissus are normally hypoxic even under control conditions, with the exception of gill which is the O₂ exchange organ of this animal, anoxia might not be a great change to some of these animal's tissues. This respiration physiology may be key to understanding the tissue specific response seen during freezing and anoxia.

The response of GP in M. demissus, was tissue specific, as well as stress related (Figures 3.3, 3.4; Table 3.2). Under anoxic conditions, the mantle and hepatopancreas showed no significant change in GP content. This may be due to the fact that these tissues are under hypoxic stress even under control conditions. The response of adductor and gill differed since both showed an increase in phosphorylase activity during anoxia. The increase seen in adductor may be due to an increase in the demand for energy brought about by shell closure. In gill the increase could be explained by a

switch to anaerobic metabolism of glycogen from aerobic metabolism of an alternative fuel.

In M. demissus, increases in GP activity in response to anoxia were primarily the result of an increase in the total amount of enzyme. The mechanism of converting b to a played a minor role.

Freezing is a different stress from anoxia, since it carries the additional stress of cellular dehydration, and M. demissus responds uniquely. The decrease in active GP found in hepatopancreas and mantle during freezing, probably due to the additional stresses associated with freezing on top of the anoxic conditions, are in keeping with a general depression of metabolic rate. Gill again showed an activation of GP during freezing, while adductor showed no significant change in GP activity.

The regulatory mechanisms of GP seen during freezing in M. demissus involve interconversion between the active and inactive enzyme forms, and a decrease in the total amount of enzyme, as can be seen in hepatopancreas and mantle tissues. In addition, similar to the response seen in anoxia, activation of gill is due an increase in the total amount of enzyme.

The molecular mechanism in these animals that controls changes in amount of active GP seems to be somewhat unusual when compared to previously studied animals. In L. littorea and M. demissus, nearly all change in GP activity was derived from changes in the total amount of phosphorylase expressed in each tissue, with interconversion between the inactive and active forms playing a minor role. In contrast, mammalian systems change the percentage of the active form a, with the total phosphorylase content remaining constant (Cohen, 1980). However, the molecular mechanism seen in these animals has also been recently observed in other marine invertebrates (Storey, 1988) and in lower vertebrates such as frogs (Storey and Storey, 1988). While it is becoming obvious that control of GP differs between animals, other unusual properties of GP from marine molluscs have been observed. These include the

presence of a third form of GP in Pecten maximus, which requires AMP at a concentration 20-fold higher than phosphorylase b for activation, and unusual AMP activation kinetics for phosphorylase b from M. edulis adductor muscle (Ebberink and Salimans, 1982; Vazquez-Baanate and Rosell-Perez, 1979).

The response of GP to freezing and anoxia varied between tissue and animal. These results indicate that GP is indeed an important control point in the adaptation of both animals to freezing and anoxia, involving not just phosphorylation/dephosphorylation control but degradation and synthesis of the enzyme as well.

GLYCOGEN SYNTHETASE

Similar to GP, GS of L. littorea also seemed to be controlled by 2 different mechanisms. One mechanism involves a decrease in the total amount of GS, possibly through degradation of the enzyme or dissociation of the subunits. The other mechanism involves conversion of inactive GS into its active form. Unlike GP, both mechanisms played a role in both tissues during anoxia and freezing. In both hepatopancreas and foot of L. littorea, during anoxia and freezing, there is an increase in the percentage of GS in the active form, at the same time as there is a decrease in total amount of enzyme (Table 3.3). Despite these changes, an unusual result occurred: the amount of active GS remains unchanged.

These same mechanisms were in play in most tissues of M. demissus. However, like GP, the response of GS to freezing and anoxia was tissue specific (Table 3.4). While mantle and gill showed changes in the percentage of GS in the active form and/ or changes in the total amount of enzyme in response to freezing and anoxia, once again there was no significant change in the amount of active GS. Adductor showed no change in any of the parameters during freezing and anoxia. The only tissue to show a decrease in the amount of active GS was hepatopancreas, after 12 hours of freezing, largely due to a decrease in the total enzyme activity.

In keeping with the metabolic depression introduced by the animal when faced with environmental stresses, there would be a decrease in the demand for glycogen, and as a result one would predict a decrease in GS activity. Perhaps early on in the stress there is no need to control GS activity since, while the animal does not have the energy nor the need for increased glycogen synthesis, it does not wish to decrease its fuel source yet.

While there have been no studies that correlate the effect of freezing and anoxia to GS activity, seasonal changes in activity associated with feeding and/or gametogenesis have been observed (Gabbott et al, 1979; Gabbott and Whittle, 1986). The mechanism of control seen in L. littorea and M. demissus which involved a decrease in the total amount of enzyme activity, has also been observed in the mussel M. edulis (Cook and Gabbott, 1978; Gabbott and Whittle, 1986). The decreased I activity seen in M. edulis during the winter months was due to a decrease in the amount of total enzyme as well as conversion of the enzyme into its inactive D form.

An interesting aside which highlights the interest associated with GS, is that many phosphoproteins, including GS, have been found to contain multiple phosphorylation sites, and these sites are often located in clusters. Studies of GS isolated from rabbit liver have discovered that the observed multiple phosphorylation occurs in a hierarchical fashion and actually results from the action of 2 or more kinases (Roach, 1990). In addition, while phosphorylation of GS generally leads to decreased activity, it was found that different phosphorylations had different effects. Indeed, phosphorylation at some sites resulted in little or no inactivation of the enzyme. While the rationale for multisite, hierarchical phosphorylation is not yet clear, such control allows for a more intricate and integrated regulation mechanism than enzymes controlled via a single level of modification.

PHOSPHOFRUCTOKINASE

PFK is one of the key regulatory enzymes of glycolysis due to the fact that the reaction it catalyzes is the first unidirectional reaction in the pathway. Control of PFK has already been found to involve reversible aggregation of the enzyme with subcellular particles, allosteric regulation by a variety of metabolites, polymerization of the enzyme itself, as well as covalent modification through reversible phosphorylation of the enzyme (Choate et al, 1985; Hofer, 1985; Soling and Brand, 1981; Somero et al, 1991). Control of PFK through reversible phosphorylation has already been found to occur in marine molluscs and is thought to be the method of control in other cases where kinetic differences are observed between PFK isolated from control and anoxic tissues (Whitwam and Storey, 1991; Plaxton and Storey 1986).

Ion exchange chromatography on a DE-52 Shephadex column revealed that PFK from the foot muscle and hepatopancreas of control L. littorea existed in only one form (Figure 3.7, 3.8). Subsequent chromatography indicated that PFK from anoxic and frozen snails also existed in one form and was eluted at the same salt concentration as PFK of control animals. Although only one form of PFK seemed to exist in control, frozen and anoxic snails, in vitro phosphorylation and dephosphorylation incubations of control extracts were carried out in order to determine if reversible phosphorylation of the enzyme could play a role in control of PFK. DE-52 ion exchange chromatography of the enzyme after incubation resulted in one peak under all three incubation conditions; control, phosphorylation and dephosphorylation (Figures 3.13, 3.14). Furthermore, PFK was eluted at the same salt concentration in all three cases. There are two possible explanations for these observations. The first is that the components of the incubation solutions were not those necessary to promote a change in the phosphorylation state of the enzyme or phosphorylation/ dephosphorylation of PFK is not the control mechanism used by this animal.

In addition to different salt elution concentrations, PFK from foot and hepatopancreas showed different kinetic properties probably suited to tissue-specific enzyme functions (Table 3.5). PFK from foot muscle showed a higher affinity for the substrate F6P (K_m 0.22 ± 0.03) than hepatopancreas PFK (K_m 0.44 ± 0.01). In addition, the foot PFK showed a greater sensitivity to the activators, AMP, KCl, and F26P₂. PFK from *L. littorea* differs from the classical mammalian model because it is inhibited by the PK substrate PEP. The difference in the kinetic properties of PFK from the two tissues, in addition to the different salt elution concentrations from the anion exchange column, indicate that PFK is present as two different isozymes in the two tissues. The presence of the two isozymes probably reflects the different functional properties of these tissues. For example, the kinetic properties of the foot muscle may result in a quicker activation of PFK, compared to hepatopancreas PFK, which may be necessary for either locomotion or short burst work.

Ion exchange chromatography on DE-52 Sephadex revealed that PFK in the adductor muscle, gill, hepatopancreas and mantle of *M. demissus* existed as only one form in control animals (Figures 3.9-3.12). PFK activity was also observed in a single peak after ion exchange chromatography of tissue extracts from frozen and anoxic animals. The elution salt concentration of PFK from the different tissues was quite close, indicating that the enzymes from the different tissues were similar.

The chromatographic results of the snail matched those of the mussel, both revealing only one form of PFK present under control and stressed conditions in all tissues studied. These results, in addition to the unsuccessful in vitro phosphorylation and dephosphorylation incubations of control *L. littorea* tissues, indicate that covalent modification of PFK does not play a role in controlling PFK activity in these animals.

Another possible mechanism of PFK control that has already been found to exist in marine molluscs is allosteric regulation by the powerful activator F26P₂ (Storey, 1985, 1988). While other activators certainly have important regulatory roles to play,

no other activator has been found to have such an effect on PFK as F2,6P₂. The activator F2,6P₂ is a product of a reaction catalyzed by 6-phosphofructo-2-kinase (PFK-II). During anoxic conditions, inactivation of PFK-II (via phosphorylation), results in a decrease in the amount of F2,6P₂ produced. Since F2,6P₂ is a potent activator of PFK, decreases in its levels would dramatically reduce its regulatory role, and hence lead to reduced PFK activity. In whelk organs, it has been found that PFK-II activity drops 2 to 4 fold in the early hours of anoxia (Storey, 1988). This leads to a rapid drop in F2,6P₂ levels in all organs during anoxia, ranging from a 4-fold decrease in kidney to a 224-fold drop in ventricle. The F2,6P₂ response to anoxia is not restricted to marine molluscs but also occurs in mammalian organs during hypoxia and anoxia (Hue and Rider, 1987). Further studies of the effect of anoxia and freezing on the levels of F2,6P₂ may shed more light on the mechanism of control of PFK in L. littorea and M. demissus.

PYRUVATE KINASE

Ion exchange chromatography on DE-52 Sephadex revealed that PK in foot muscle and hepatopancreas of control L. littorea, existed in two forms (designated peak I and peak II) which show different sensitivities to KCl concentration (Figures 3.15, 3.16). Peak I of foot and hepatopancreas was eluted at 30 and 50 mM KCl, respectively; peak II at 90 and 110 mM KCl, respectively. Chromatographic separation of the two forms of PK has also been reported for numerous marine animals such as the crab Carcinus maenas (Giles et al, 1976), the mussels M. edulis (Howerda et al, 1983) and M. galloprovincialis (DeVooy and Holwerda, 1986) and the limpet Patella caerulea (Michaelidis et al, 1988; Gaitanaki et al, 1990).

The results of kinetic studies of the two forms of PK found in the foot muscle showed that they had very different kinetic properties (Table 3.6). Peak II showed a greater affinity for both the substrates, ADP and PEP (K_m PEP 0.10 ± 0.02 , K_m ADP 0.15 ± 0.02) than peak I (K_m PEP 0.21 ± 0.03 , K_m ADP 0.20 ± 0.01). In

addition peak II was less sensitive to inhibition by KCl. However, the most distinguishing feature between the two forms of the enzyme is the high sensitivity of peak I to inhibition by L-alanine in comparison to the lack of inhibition of peak II by L-alanine even at concentrations as high as 40 mM. The large sensitivity to L-alanine is very interesting due to the fact that its levels have been found to rise in the snail during anoxia (Storey et al., 1982). Due to the differences in kinetic properties, peak II can be labelled the more active enzyme.

After ion exchange chromatography, PK from the foot muscle of frozen (24 hrs) and anoxic (24 hrs) snails was found as a single peak, whose salt elution concentrations corresponded to that of peak I of the control tissue. After 24 hours of freezing, 2 forms of PK were still present in the hepatopancreas of L. littorea), while after 24 hrs of anoxia, only one peak, corresponding to peak I of the control animal was observed. The results of the chromatography indicates that during most periods of stress, such as anoxia and freezing, PK of foot muscle and hepatopancreas was converted into the less active (peak I) form of the enzyme. These results are consistent with metabolic rate depression in the animal.

To determine if this conversion was due to reversible phosphorylation, as has been found in other species (Seinbenaller. 1979; Whitam and Storey, 1990a. 1990b), control tissue extracts were incubated under conditions which promoted phosphorylation and dephosphorylation of the enzyme. Subsequent chromatography revealed that peak I represented the low phosphate or unphosphorylated form and peak II represented the phosphorylated form of the PK molecule (Figures 3.17, 3.18). These results corresponded with the prediction that a more negatively charged (phosphorylated) molecule would be eluted from the anion exchange column, DE-52 Sephadex at a higher salt concentration.

It therefore appears that PK from foot muscle of L. littorea is regulated by several modulators as well as interconversion between 2 forms of the enzyme, the

dephosphorylated inactive form and the phosphorylated active form. Hepatopancreas PK also showed the same mechanism of control via reversible phosphorylation.

A quick method for assessing the effect of environmental stress on an enzyme involves measuring the rate of the enzyme at low vs saturating concentrations of substrate; this screens for stable changes in K_m which could be caused by reversible phosphorylation. The effect of stressful environments on PK has been found to be quite consistent with common differences in kinetic properties between the control and stressed enzyme. The most common change after exposure to stress is an increase in the apparent K_m for the substrate PEP. While the kinetic properties have been found to change, V_{max} is not influenced by the interconversion of the enzyme. After exposure to anoxic and subzero temperatures, M. demissus showed significant changes in the ratio of PK activity at low and saturating conditions (Table 3.7). During anoxia and freezing, the PK activity at the low PEP concentrations decreased significantly in the adductor, gill and hepatopancreas but remained constant at the saturating PEP concentrations. As a result the ratio of enzyme activities dropped significantly during both freezing and anoxia in adductor, gill, and hepatopancreas with a similar trend present in mantle at only 12 hours anoxic. The decrease in PK activity is similar to that seen in L. littorea and corresponds to that expected during metabolic rate depression.

Freezing and anoxia resulted in decreased PK activity in both L. littorea and M. demissus. It is probable that a decrease in PK activity, for example via dephosphorylation of the enzyme as in L. littorea, would result in a shift from the conversion of PEP into pyruvate to the conversion of PEP into oxaloacetate. Increases in the end product succinate seen in L. littorea (Storey et al, 1982) are indicative of this shift at the PEP branchpoint.

PYRUVATE DEHYDROGENASE

Under aerobic conditions, pyruvate enters the mitochondrial matrix where it is converted to acetyl-coenzyme A. The acetyl group of acetyl-CoA then enters the citric acid cycle. The citric acid cycle is the final oxidative pathway in the catabolism of carbohydrates, fatty acids and amino acids and is an important source of intermediates to biosynthetic pathways. Since the citric acid cycle is the central pathway of aerobic metabolism, it would seem an obvious target for depression during periods of anaerobic stress. In addition, enzymes providing substrates for the cycle would also be an ideal targets for inactivation. Such an enzyme is PDH, which links glycolysis to the citric acid cycle. The PDH complex catalyzes the irreversible oxidative decarboxylation of pyruvate and formation of acetyl-CoA.

It has been found in mammalian systems, and has been suggested for invertebrate systems, that PDH activity is controlled by reversible phosphorylation of the enzyme, the dephosphorylated form of the enzyme being the active form (Woodford et al., 1992; Brooks and Storey, 1991). The amount of inactive and active form is controlled via the effects of inhibitors and activators on PDH kinase, which leads to phosphorylation of the enzyme, and PDH phosphatase, which leads to dephosphorylation and activation of the enzyme. The inhibitors of PDH kinase include the substrates of the PDH reaction; CoA, pyruvate, and its activators include the products of the PDH reaction; CO₂, acetyl-CoA, in addition to Ca²⁺. Activation of PDH phosphatase, which would lead to activation of PDH due to dephosphorylation, results from the presence of Mg²⁺ and Ca²⁺ (Williamson and Cooper, 1980).

Whereas, the optimal conditions for incubation were tissue specific, the response of PDH to freezing and anoxia was not (Figures 3.19-3.27). Both the foot and hepatopancreas tissues of L. littorea experienced a decrease in the amount of active PDH during periods of stress. The same trend was seen in the tissues of M. demissus,

again under both stresses. The observed decreases are due solely to a decrease in percentage of the enzyme in the active form, since the total amount of PDH remained constant. The decrease in amount of active enzyme corresponds to the general picture of metabolic depression. However, due to the fact that PDH links glycolysis to an aerobic pathway, a more drastic decrease in enzyme activity would have been expected. However decreases in PDH activity of similar magnitudes were seen during estivation of the land snail *Q. lactea* (Brooks and Storey, 1991). Control of the depression of the citric acid cycle may lie in other directions, ie. decrease in substrate levels due to inactivation of PK or even within the cycle itself. Lack of O₂ itself plus inhibition of TCA cycle enzymes due to a build up of their products would halt the cycle as well. In addition, since the flux of the pathway seems to be down regulated at the PK locus, a greater decrease in PDH activity may be redundant.

In both *L. littorea* and *M. demissus* during freezing and anoxia, the activity of the enzymes GP, GS, PK, and PDH were controlled by reversible phosphorylation. PFK however, was not controlled by reversible phosphorylation, as a result studies investigating its control should look in other directions. In addition, the activity of the enzymes GP and GS was controlled by degradation and in some tissues, synthesis of the enzymes. Interestingly enough, although both animals are from different classes of the Mollusca which have been evolutionarily distinct for thousands of years, both *L. littorea* and *M. demissus* showed the same control points along the glycogenolytic pathway, with changes in activity seen at the GP, GS, PK and PDH loci.

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